RESPONSES OF TEA PLANTS TO CLIMATE CHANGE: FROM MOLECULES TO ECOSYSTEMS

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RESPONSES OF TEA PLANTS TO CLIMATE CHANGE: FROM MOLECULES TO ECOSYSTEMS

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EDITORIAL
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Editorial: Responses of Tea Plants to Climate Change: From Molecules to Ecosystems

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Editorial on the Research Topic

Responses of Tea Plants to Climate Change: From Molecules to Ecosystems

We find ourselves at a time when climate change is impacting almost every facet of society, including the sustainability of agricultural and food systems. The science is clear that climate change is happening and its impact will worsen without efforts to reverse the trend with more sustainable and eco-friendly approaches (IPCC, 2014). As scholars, practitioners, and citizens of this planet, we must collectively act to understand the risks posed by climate change and then to apply this knowledge for climate mitigation and adaptation. Science plays a key role in understanding climate effects on earth's systems toward co-designing evidence-based solutions. This Research Topic seeks to advance our understanding of the effects of climate change on the multiple dimensions of crops through the lens of the tea plant.

The tea plant, *Camellia sinensis* (L.) Kuntze is an ideal model perennial plant for examining climate effects on crops as it is the most widely consumed drink globally after water with notable cultural, dietary, and health values. Tea is cultivated on five continents in diverse types of agricultural systems and supports livelihoods and contributes to regional economies (Han et al., 2018). The consumption of tea is linked to its flavor and health attributes which are based on distinct secondary metabolite biochemical profiles comprised of polyphenols, amino acids, alkaloids, terpenoids, and other volatile compounds. Tea secondary metabolites are key determinants of quality that are sensitive to climate variability; this variability has notable implications throughout the tea system from farmer management to consumer preferences and tea sector livelihoods (Ahmed et al., 2014).

Articles in this Research Topic provide evidence on the effects of climate change on tea plants based on physiological, molecular, and biochemical responses, and ecosystem interactions and industry more broadly. Some articles in this Research Topic provide a survey of literature on existing evidence on the effects of global change on tea plants, whilst others propose new methodologies for understanding their impact or unveil novel findings on the topic. Collectively, the articles in this collection advance our understanding of the effects of various environmental stressors linked to climate change including water stress, salt stress, temperature fluctuations, solar radiation, herbivory, and pathogens. The evidence compiled in this collection demonstrates that tea plants react with different defense mechanisms including physiological, molecular, and biochemical responses to environmental stressors linked to climate change.

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A unique emphasis of this Research Topic is the focus on the effects of climate on tea quality as determined by secondary metabolite biochemical profiles. While extensive research has focused on the effects of multiple stresses on crop yields, this collection also advances our understanding of their impact on crop quality. Below we provide a general overview of the collection of articles in this Research Topic, starting with the literature reviews and then the primary research articles. For the primary research articles, we first present articles focused on plant performance, and then those focused on the biochemical responses that influence tea quality.

The three literature reviews in this Research Topic highlight what we know about climate effects on tea and elucidate current gaps in research. With an emphasis on the plant performance aspects of tea production, Muoki et al.'s review compiles evidence on the varied responses of tea plants to climate change with a focus on the Kenyan tea industry. They synthesize strategies for conventional and molecular breeding and selection toward informing climate resilient tea cultivation. With a focus on the biochemical responses of tea plants, Ahmed et al.'s systematic review synthesizes the effects of environmental factors on tea quality from 86 articles, carried out in tea producing regions globally. This systematic review demonstrates that shifts in water stress, seasonality, geography, light factors, altitude, herbivory and microbes, temperature, and soil factors can result in both increases and decreases of up to 50% in tea secondary metabolites. A key gap in the literature elucidated through this review is a lack of studies examining the effects of elevated CO_2 on tea quality. Ahammed et al. build on this finding through a review characterizing the physiological and metabolic responses of tea plants to elevated CO_2 including alterations in concentrations of polyphenols, free amino acids, catechins, theanine, and caffeine as well as associated gene expression.

Drought, low temperatures, pests and diseases, salt, and light are key factors that determine plant performance in existing and new areas of production (Han et al., 2018). At the morpho-anatomical level, the plant cuticle plays a major role in regulating water loss and drought tolerance, an attribute that is essential for climate adaptation in many tea-producing areas. Zhang Y. et al. propose a novel method that can be applied for measuring water loss through the leaf cuticle toward understanding drought tolerance and selecting drought tolerant tea varieties. The implementation of this method can potentially be applied to other plants to examine cuticular transpiration in the context of climate-change-promoted drought events.

Cold stress is a key environmental factor that adversely impacts plant growth and is a major factor limiting the expansion of tea cultivation (Chinnusamy et al., 2007). Low temperatures can result in the accumulation of reactive oxygen species in tea plants which in turn can promote the antioxidant capacity including the synthesis of flavonoids (Li et al., 2018). Glycosyltransferases (UGTs) are involved in the transport of flavonoids and render them more water soluble and less toxic (Song et al., 2018). Zhao et al. identify a UGT gene involved in the regulation of plant cold stress tolerance.

At the molecular level, microRNAs (miRNAs) are recognized as key modulators of gene expression in response to various environmental stressors (Khraiwesh et al., 2012). Jeyaraj et al. provide evidence on the role of miRNAs in tea plants in response to the plant pathogen *Colletotrichum gloeosporioides*. Findings from this study have implications for understanding pathogen susceptibility and resistance in tea plants. Wan et al. highlight how salt stress severely affects the growth and quality of tea and examine the molecular mechanisms involved in responses to salt stress. Specifically, they provide evidence on the roles of long non-coding RNAs (lncRNAs) in transcriptional regulation as ubiquitous regulators in response to salt stress in tea plants. Liu et al. examine how light is a key environmental regulator of the chlorophyll biosynthesis pathway in higher plants and provide evidence on the molecular mechanisms that regulate chlorophyll biosynthesis in response to light and hormones. At the ecosystem level, Zhang Q. et al. examine cultivars that are suitable for adaptation to a new tea producing areas in the United States by examining cold tolerance along with plant growth, morphology, and biochemical profiles. Together, these studies provide a model for selecting and developing crop cultivars that can be adaptable to new agricultural areas or changing conditions.

The biochemical plant responses linked to secondary metabolite synthesis are important to both plant resistance and plant quality. Five articles in this Research Topic focus on the shifts in various classes of secondary metabolites that influence tea flavor in response to multiple environmental stressors. Shamala et al. demonstrate how ultraviolet-B (UV-B) radiation stimulates the biosynthesis of phenolic compounds. Huang et al. shed insight on the biosynthesis of gallic acid, which is a precursor for polyphenol synthesis in tea plants. They provide experimental evidence from enzyme assays and kinetic analysis on favorable candidate genes for gallic acid biosynthesis in tea plants and connections of gallic acid biosynthesis to the shikimate pathway. Li et al. present a study examining the interplay of the secondary metabolite theanine, which contributes an umami taste to tea, and gene expression in diverse tea cultivars in the context of seasonal variability. They provide evidence that theanine fluctuations are both season- and developmental stage-dependent. Furthermore, they provide evidence that while theanine-biosynthesis genes are generally negatively correlated with theanine content, amino acid transporter genes are generally positively correlated with theanine accumulation.

Kfoury et al. provide evidence on the variation of secondary metabolites based on spatial and temporal factors. They demonstrate that elevation is a key determinant of crop quality based on the profiling of over 500 volatile secondary metabolites in one tea producing area of China, Yunnan Province, but not in another, Fujian Province. They show that seasonality and the annual variation associated with changing environmental conditions are determinants of crop quality in both locations. Scott et al. provide evidence on the relationship of increasing leafhopper density in tea agricultural systems to damaged leaf area and both volatile and non-volatile secondary metabolites through a manipulative experiment. Both Kfoury et al. and Scott et al. show that induced biochemical responses to environmental factors can be linear or non-linear. For example, Scott et al. show that some secondary

metabolites change linearly with herbivore pressure, and that others respond after reaching a specific threshold following herbivore pressure.

Collectively, the findings of the articles in this Research Topic have notable implications for the resilience of crops and agricultural systems in the context of global change. While the articles in this Research Topic focus on tea, we expect that they will provide methodologies and insights on examining the responses of other agriculturally- and economically-important plants to climate change. This Research Topic further highlights the critical role of plant sciences in understanding responses to global change and designing sustainable and resilient crop systems. To reduce the detrimental impact of climate change is not an easy task, however, if we do not take rapid actions, the world will be less habitable. Cooperation between researchers, practitioners, producers, and policy stakeholders is essential to co-design and implement evidence-based mitigation and adaptation strategies for tea and, more generally, for plant cultivation.

AUTHOR CONTRIBUTIONS

SA and WH wrote the draft of the editorial, with all co-authors jointly editing the final version.

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SYSTEMATIC REVIEW
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Environmental Factors Variably Impact Tea Secondary Metabolites in the Context of Climate Change

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Climate change is impacting food and beverage crops around the world with implications for environmental and human well-being. While numerous studies have examined climate change effects on crop yields, relatively few studies have examined effects on crop quality (concentrations of nutrients, minerals, and secondary metabolites). This review article employs a culturally relevant beverage crop, tea (*Camelia sinensis*), as a lens to examine environmental effects linked to climate change on the directionality of crop quality. Our systematic review identified 86 articles as relevant to the review question. Findings provide evidence that shifts in seasonality, water stress, geography, light factors, altitude, herbivory and microbes, temperature, and soil factors that are linked to climate change can result in both increases and decreases up to 50% in secondary metabolites. A gap was found regarding evidence on the direct effects of carbon dioxide on tea quality, highlighting a critical research area for future study. While this systematic review provides evidence that multiple environmental parameters are impacting tea quality, the directionality and magnitude of these impacts is not clear with contradictory evidence between studies likely due to confounding factors including variation in tea variety, cultivar, specific environmental and agricultural management conditions, and differences in research methods. The environmental factors with the most consistent evidence in this systematic review were seasonality and water stress with 14 out of 18 studies (78%) demonstrating a decrease in concentrations of phenolic compounds or their bioactivity with a seasonal shift from the spring and /or first tea harvest to other seasons and seven out of 10 studies (70%) showing an increase in levels of phenolic compounds or their bioactivity with drought stress. Herbivory and soil fertility were two of the variables that showed the greatest contradictory evidence on tea quality. Both herbivory and soil fertility are variables which farmers have the greatest control over, pointing to the importance

of agricultural management for climate mitigation and adaptation. The development of evidence-based management strategies and crop breeding programs for resilient cultivars are called for to mitigate climate impacts on crop quality and overall risk in agricultural and food systems.

Keywords: climate change, crop quality, secondary metabolites, food systems, agriculture

INTRODUCTION

Climate Change and Crop Quality

Impacts of climate change on agricultural systems threaten crops with notable implications for farmer livelihoods and human health (Odada et al., 2008; Pachauri et al., 2014; Campbell et al., 2016). Since the 1950s, agricultural systems have experienced gradual systematic changes in average climate conditions including unprecedented multi-decadal warming, increased inter-annual variability of the Earth's surface temperatures, changes in average precipitation, greater weather variability, and more extreme weather conditions (Pachauri et al., 2014). These climatic changes have decreased agricultural productivity and shifted the geographic range of many crops (Lobell and Asner, 2003; Porter and Semenov, 2005). Concurrently, productivity of some crops has increased with a rise in temperatures (Ewert et al., 2005). Increasing atmospheric carbon dioxide (CO_2) has decreases concentrations of micronutrients zinc (Zn) and iron (Fe) as well as protein for C_3 grains and legumes (Myers et al., 2014). While the literature provides substantial evidence on the impact of climate change on crop yields (Lobell and Asner, 2003; Hertel et al., 2010; Lobell et al., 2011), fewer studies have focused on the effects of climate change on crop quality (Porter and Semenov, 2005; Ahmed and Stepp, 2016).

Crop quality is a multi-dimensional parameter that refers to the nutritional, health, and sensory attributes of crops as measured by the presence, absence, and/or concentrations of phytonutrients, minerals, and primary and secondary metabolites (i.e., bioactive food components or phytochemicals) as well as associated bioactivity, shelf life, and organoleptic properties (i.e., color, visual appeal, aroma, taste, and texture) (Mattos et al., 2014; Ahmed and Stepp, 2016). Secondary metabolites are non-nutrient plant constituents that impact crop quality through an influence on flavor, appearance, stability (Tomás-Barberán and Espin, 2001) and health-promoting attributes including mitigating micronutrient deficiencies (Johns and Sthapit, 2004) and the risk of diet-related chronic disease including cancer, heart disease, and diabetes (Liu, 2013). Over 100,000 secondary metabolites have been identified in five chemical classes including phenolics, terpenes, alkaloids, and other nitrogen-containing compounds, phytosterols, and organosulfur compounds (Goldberg, 2003; Swift et al., 2004).

Plants have evolved secondary metabolites to protect themselves from various abiotic and biotic stressors (Fraenkel, 1959; Coley et al., 1985). Since the synthesis of secondary metabolites represents a metabolic cost, plants tend to produce these compounds in notable concentrations if they have the ecological cue to do so based on interactions between environmental (Lower and Orians, 2003; Björkman et al., 2011; Kowalsick et al., 2014), agricultural (Ahmed et al., 2013), genetic (van-Dam and Vrieling, 1994), and physiological factors (Coley et al., 1985; Glynn et al., 2007). Each crop has specific secondary metabolites that characterize crop quality while the presence of other secondary metabolites is viewed as deleterious for crop quality due to off-flavors or toxicity attributes. Climate change factors have resulted in a decrease of multiple secondary metabolites in a range of food and beverage crops including coffee (Villarreal et al., 2009), tea (Ahmed et al., 2014a,b; Kowalsick et al., 2014), grapes (Xu et al., 2011), brown rice (Britz et al., 2007), kale (Zietz et al., 2010), tomatoes (Kacjan-Maršič et al., 2010), and peanuts (Casini et al., 2003).

Tea as a Model System for Examining Climate Effects on Crop Quality

This review article employs tea (*Camelia sinensis*; Theaceae) to understand how climate change impacts crop quality of a culturally-relevant beverage plant. Tea, the botanical source of all white, green, oolong, black and pu-erh tea beverages, was selected as a study system because of its prevalence in diets globally, its production in over 50 countries on five continents (FAOSTAT, 2016), and because it is a plant that is cultivated for its quality based on secondary metabolites (Ahmed et al., 2010). Polyphenolic catechin, methylxanthine (i.e., caffeine), and volatile secondary metabolites, coupled with carbohydrates and amino acids, contribute to tea quality through influencing the flavor and appearance of tea (Drewnowski and Gomez-Carneros, 2000; Scharbert et al., 2004) as well as its health claims including anti-oxidative, anti-inflammatory, neuro-protective, cardio-protective, anti-cancer, anti-microbial, and anti-atherosclerotic activities (Trevisanato and Kim, 2000; Lin et al., 2003; Clement, 2009). Tea is further suitable as a study system to examine the effects of climate change because it is a woody perennial that experiences multiple decadal effects of climate change. Successful tea cultivation tea is dependent on climatic conditions including temperature, rainfall, humidity, and solar radiation (Wijeratne, 1996).

Marx et al. (2017) identified 14 articles highlighting that shifts in rainfall and solar radiation influence tea yields and quality with implications for farmer livelihoods. For example, Biggs et al. (2018) demonstrate that climate change is impacting farmer livelihoods across four major tea-growing regions of Assam, India. Boehm et al. (2016) show that a decrease in solar radiation in the previous growing season is associated with lower tea yields. However, the literature is missing a systematic review that synthesizes the evidence of environmental effects on tea quality and, the directionality of changes in quality parameters.

This study addresses the aforementioned knowledge gap through a systematic literature review that examines the following question: *What are effects of environmental variation related to climate change on tea quality?* Given the lack of studies examining long-term effects of climate change on tea quality, we synthesize studies that examine the effects of environmental factors that are shifting with climate change. This review can inform future research by identifying literature gaps and new research questions while serving as a model for literature reviews on climate change effects on crop quality.

SYSTEMATIC REVIEW METHODS

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; Moher et al., 2009) and the Guidelines for Systematic Review and Environmental Synthesis in Environmental Management (Collaboration for Environmental Evidence, 2013) were applied to design a systematic review protocol to collect evidence on the following closed-frame study question: *What are effects of environmental variation related to climate change on tea quality?* We used the PICO (Population, Intervention/Exposure, Comparator, Outcome) framework elements (Schardt et al., 2007) to structure our systematic review.

The biological population/study unit is tea (*Camellia sinensis* L.; Theaceae). The intervention/exposure that we examined is environmental variability linked to climate change, which includes variables including temperature, precipitation, elevation, seasonality, location, herbivory, soil, and solar radiation. As we found a lack of published studies directly examining climate effects on tea quality based on our preliminary review, we included environmental factors that vary with global change in systematic review search protocol. The comparator assessed in our review was variation in a specific environmental variable from a baseline evaluation or other comparison. The outcomes that we examined were impacts on the biochemical parameter of tea quality including secondary metabolite chemistry that consists of phenolic catechins, methylxanthines (including caffeine), amino acids (including theanine), and a range of volatile compounds.

A multidisciplinary team of subject experts and a review methodology expert collaboratively designed the systematic review protocol. Specifically, the multidisciplinary team of subject experts included scientists with expertise in the fields of plant biology, chemistry, soil science, herbivory, phytochemistry, biochemistry, agronomy, food science, tea science, and climate change. Establishment of the review protocol involved preliminary scoping of search terms to test the search strategy for indication of the volume of relevant literature. Search terms were identified and revised during an iterative process after examining relevant articles from the search and refining search terms to meet both evidence needs as well as feasibility for a systematic review. Following are the key components of the final search terms that included a combination of tea, environmental factor linked to climate change, and quality parameter: "tea OR camellia sinensis" AND "climate change OR global warming OR season OR solar radiation OR precipitation OR geographic area OR temperature OR soil OR wind OR annual bud burst OR carbon dioxide" AND "antioxidant OR caffeine OR phytochemical OR catechin OR theaflavin OR flavonol OR polyphenol OR secondary metabolite." **Supplementary Table 1** lists the final search terms used in this systematic review. The final search terms were tested with a set of known relevant articles.

A total of five publication databases were searched: PubMed, Web of Science, EBSCO GreenFILE, Scopus, and, Agricola. Databases were selected that are commonly used to search for articles in the areas of food systems, agriculture, climate change, secondary metabolite chemistry, and human health. Inclusion criteria were restricted to peer-review articles published in the English language from 2000 to 2015. The software program Covidence was used to manage articles identified within the publication databases and was further used for screening of articles. Following the search, the retrieved articles were screened by a review panel of three reviewers (KS, MH, and SA) to minimize reviewer bias in identifying articles for their relevance to the review question using *a-priori* inclusion criteria. Two reviewers from the review panel screened each article. Decision discrepancies (inclusion or exclusion) were discussed by the entire panel for resolution. The final set of articles was critically appraised for their design on focusing on environmental effects on tea quality.

The final set of articles in the study was read by a separate data extraction review panel (DK, DS, and AL). Appropriate data from each of these articles were extracted by one of three members of the data extraction panel and placed into a table highlighting specific study parameters including environmental factor, variety and cultivar of tea, quality indicator, method(s) of analysis, and outcomes. A second member on the data extraction panel reviewed extracted data in the table to verify that appropriate data was extracted.

The final search resulted in the retrieval of 9,750 articles including 1,754 duplicates for a total of 7,996 articles (**Supplementary Figure 1**). From this, 93 articles were identified by the review panel for inclusion in the review. These articles were relevant to the review question using our *a priori* inclusion criteria. Nine of these articles were removed from the search because it was not possible to retrieve the full text of these papers, resulting in a total of 84 articles that were included in the synthesis of our systematic review.

Each article included in the study was grouped on the basis of which environmental factor(s) were assessed in the study, and then placed in a corresponding table of the most prevalent environmental factors that emerged from the systematic review. Articles were placed in multiple tables if they examine multiple environmental variables. For each environmental factor table, we applied a coding framework to quantify the number of articles that demonstrated directional changes (increase, decrease, or no change) of specific tea quality parameters. Specifically, we quantified the following quality parameters: (1) increase, decrease, or no change in catechins and other phenolic compounds with an increase in [environmental factor, i.e., temperature], (2) increase, decrease, or no change in catechins and other phenolic compounds with a decrease in [environmental factor, i.e., temperature], (3) increase, decrease,

or no change in caffeine with an increase in [environmental factor, i.e., temperature], (4) increase, decrease, or no change in caffeine with a decrease in [environmental factor, i.e., temperature], (5) increase, decrease, or no change in terpenoids and other volatiles with an increase in [environmental factor, i.e., temperature], (6) increase, decrease, or no change in terpenoids and other volatiles with a decrease in [environmental factor, i.e., temperature], (7) increase, decrease, or no change in amino acids with an increase in [environmental factor, i.e., temperature] and, (8) increase, decrease, or no change in amino acids with a decrease in [environmental factor, i.e., temperature]. Unfortunately, as the studies used different measures of crop quality and variable experimental designs, we were unable to quantitatively compare between studies. Three reviewers (DS, DK, and SA) coded the outcomes for each article to ensure there were no discrepancies. The resulting data were summarized into a narrative synthesis and a quantitative synthesis.

Some environmental factors were merged together due to their relatedness and presentation in the literature. Geographic area was separated from altitude as it was presented separately in the literature. While the systematic review did not identify a study on carbon dioxide effects on tea quality, we summarize a recent study outside of the time frame as this is a critical abiotic stressor associated with climate change. Those studies that included multiple variables were analyzed individually for all relevant environmental factors.

RESULTS

Findings from the systematic review highlighted the following environmental factors as being prevalent in the literature as impacting tea quality:

(1) seasonality (including change of harvest season such as shifts between spring, summer, autumn, and winter harvest seasons or shifts between dry and wet seasons as well as bud burst; 18 studies; **Supplementary Table 2**);
(2) water stress (including precipitation; 10 studies; **Supplementary Table 3**);
(3) geography (8 studies; **Supplementary Table 4**);
(4) light factors (including solar radiation; 6 studies; **Supplementary Table 5**);
(5) altitude (5 studies; **Supplementary Table 6**);
(6) herbivory and microbes (5 studies; **Supplementary Table 7**);
(7) temperature (4 studies; **Supplementary Table 8**) and;
(8) soil and nutrient factors (27 studies; **Supplementary Table 9**).

The most prevalent crop quality parameters in the literature addressing the study question were total phenolic concentration, catechin content [including epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and catechin gallate (CG)], and antioxidant activity. Other prevalent quality parameters include caffeine, theaflavin, thearubigin, tannins, carotenoids, terpene alcohols (i.e., linalool and geraniol) and other aromatic volatiles, fatty acid composition, chlorophyll, glycoside precursors, proanthocyanidin, and lipoxygenase and glycosidase enzymatic activities. Overall, increases in the aforementioned secondary metabolites is associated with increased crop quality until a specific threshold for human flavor preference when a further increase is no longer considered desirable. Total color, brightness, and flavor of brewed tea were measured as organoleptic quality parameters linked to secondary metabolite concentrations. High-performance liquid chromatography (HPLC) and colorimetric spectrophotometry assays were the most prevalent methods for analyzing tea quality.

The reviewed studies were carried out in a range of tea producing and consuming countries (**Figure 1**). In addition, the studies included a wide range of different tea cultivars of the *assamica* and *sinensis* varieties of *Camellia sinensis* including AC-259, T-78, B-157, B-777, S-449, UPASI-9, UP-2, UP-3, UP-8, BSS-2, TV-23, TV-25, TV-26, ZC108, ZC302, LJ 43, Derepazari 7, Fener, Yulan, and Fudingdaba. However, many of studies did not specify the variety, cultivar, or landrace of the tea examined. The lack of information on the variety of tea as well as the broad range of cultivars examined prevented analysis of the response of tea plants to environmental variables on the basis of tea variety and cultivar.

Seasonality

One-fifth (18 of 86) of the studies reviewed assessed the effects of seasonality factors on tea quality including change of harvest season between spring, summer, autumn, and winter harvest seasons or shifts between dry and wet seasons as well as bud burst (**Figure 2**; **Supplementary Table 2**). The main seasonality factor examined was comparing the spring harvest to either the summer, monsoon, autumn, and winter, with a comparison between the spring and monsoon season being the most prevalent seasonal comparator. The spring tea harvest season is generally the first harvest season that follows winter dormancy in direct response to increased temperatures (Ahmed and Stepp, 2012). The main quality parameters measured in reviewed studies on seasonality were phenolic secondary metabolites (including individual catechin compounds) and / or antioxidant activity (14 studies), followed by volatile metabolites (4 studies), and caffeine (a methylxanthine compound; 4 studies).

Fourteen seasonality studies demonstrated that concentrations of individual catechins, phenolic secondary metabolites, and / or antioxidant activity decreased with a seasonal shift from the spring and/or first tea harvest to other seasons, particularly the monsoon season (Sud and Baru, 2000; Saikia and Mahanta, 2002; Akhlas, 2003; Venkatesan et al., 2007; Chen et al., 2010, 2014; Kottur et al., 2010; Ansari et al., 2011; Jayasekera et al., 2011, 2014; Xu et al., 2012; Ahmed et al., 2014b; Baptista et al., 2014; Topuz et al., 2014). Phenolic catechin compounds are generally associated with high tea quality; however, relatively high concentrations of particular phenolics are linked to a bitter or astringent taste that is generally less desired by consumers (Ahmed et al., 2014b). Two seasonal studies showed that while some phenolic secondary metabolites, and/or antioxidant activity decreased with a seasonal shift from the spring and / or first tea harvest to other seasons, other secondary metabolites and / or antioxidant activity increased. Ahmed et al. (2014b) demonstrated that concentrations of

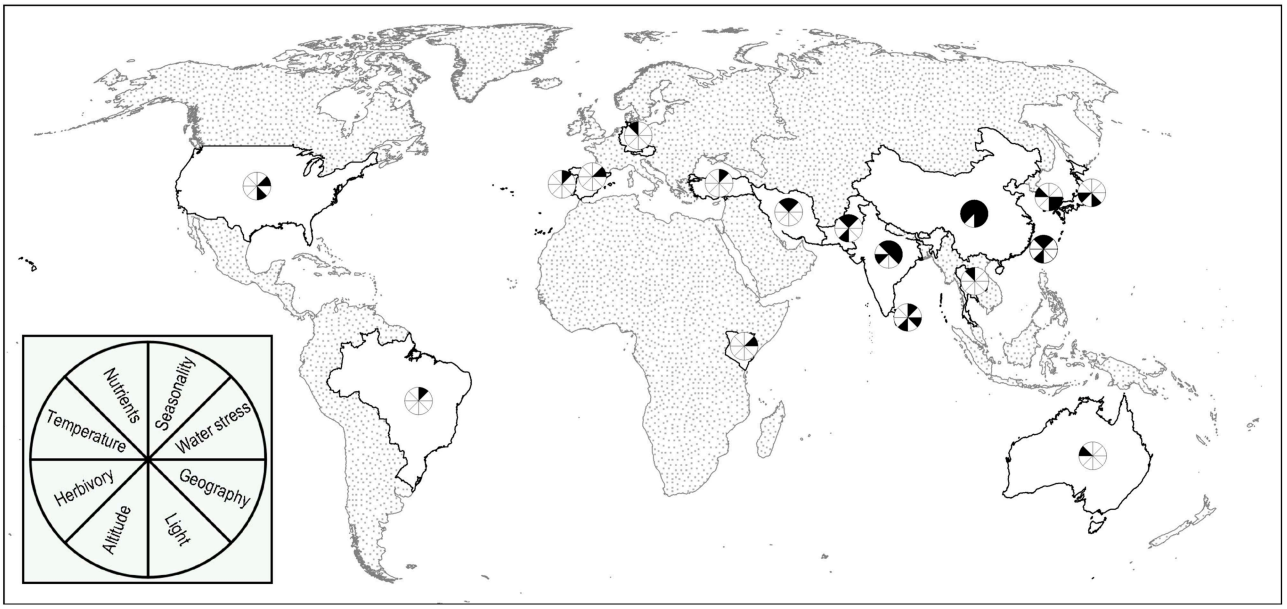

FIGURE 1 | Environmental factors assessed by location. Map depicting the range of tea producing and consuming countries where the studies identified in this systematic review were carried out along with the corresponding environmental factors assessed.

FIGURE 2 | Effects of seasonality on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

desirable phenolic catechins were up to 50% lower during the beginning of the monsoon season compared to the spring, while total phenolic concentrations and antioxidant activity increased.

The four seasonality studies measuring tea volatiles demonstrated that some volatile compounds increased with the seasonal transition from the spring and/or first tea harvest and that other compounds decreased (Saikia and Mahanta, 2002; Rawat and Gulati, 2008; Tontul et al., 2013; Kowalsick et al., 2014). Kowalsick et al. (2014) demonstrated that the number and concentration of over 300 volatile compounds from 18

chemical families either increased, decreased, or stayed the same with the onset of the monsoon from the spring season. This study showed that in general the spring season has a greater number and higher concentration of volatiles associated with high tea quality (Kowalsick et al., 2014). Three studies showed that caffeine concentrations decreased with a transition from the spring season to the summer monsoon season (Akhlas, 2003; Ansari et al., 2011; Ahmed et al., 2014b) and one study found caffeine to increase during the summer (Saito et al., 2007). One study provided evidence of an increase in amino acids with the transition from the spring to the summer monsoon (Saikia and Mahanta, 2002).

Six of the 14 seasonality studies provided evidence that while some quality parameters decreased with a shift in season, other measures of quality increased (Saikia and Mahanta, 2002; Venkatesan et al., 2007; Rawat and Gulati, 2008; Tontul et al., 2013; Ahmed et al., 2014b; Kowalsick et al., 2014). Rawat and Gulati (2008) found that total content of glycosidic bound flavor compounds was highest during early growth flush (April—mid June), declined during rains (mid June—September), and exhibited recovery during the backend flush (mid September—November). The authors further found an increase in the terpene index and a decrease in terpenoid/non-terpenoid ratio during the rains due to a marked decrease in terpenoid compounds (mainly aromatic geraniol). Jayasekera et al. (2011) found that variation of tea quality on the basis of season was further dependent on geographic location with some plantations having higher quality during the dry season and others having higher quality during the monsoon.

Water Stress

A total of 10 studies examined responses of tea plants to low water availability, including drought stress and soil moisture content (**Figure 3**; **Supplementary Table 3**). Five of these studies showed an increase in phenolic compounds and/or antioxidant activity with drought stress (Hernández et al., 2006; Upadhyaya et al., 2011, 2013; Ahmed et al., 2013; Bhattacharya et al., 2014) while two studies showed a decrease in these compounds (Cheruiyot et al., 2007, 2008) and two other studies showed both an increase and decrease in these compounds with drought stress (Chakraborty et al., 2002; Upadhyaya and Panda, 2004). Hernández et al. (2006) found that phenolics (including epicatechin and epigallocatechin gallate) accumulated in drought-stressed tea plants. Cheruiyot et al. (2007) found that declining soil water content over a 12-week period significantly reduced growth and total polyphenolic concentrations in tea shoots depending on cultivar type. Chakraborty et al. (2002) highlight that while phenolic secondary metabolites initially increase with drought stress, they decrease during extended drought stress.

One water stress study examined the effect of drought stress on levels of volatile compounds (Cao et al., 2007). Cao et al. (2007) showed that the number of aromatic constituents in fresh tea leaves was highest under a soil water content of 54% (relative to field capacity) and lowest under a soil relative water content of 100%. None of the water stress studies examined the effects of water stress on caffeine levels in tea.

Geography

While geography is not directly changing with climate change, areas that are suitable for agriculture are shifting with climate change. The eight studies examining the effects of geography on tea quality (**Figure 4**; **Supplementary Table 4**) focused on agroclimatic zones, location, and karst topography. Geography was found to notably impact catechins and other tea polyphenols in seven studies (Borse et al., 2002; Li et al., 2007; Bhuyan et al., 2009, 2013; Lee et al., 2015). Two studies showed that caffeine varied with geography (Borse et al., 2002; Lee et al., 2010), two studies showed that amino acids varied with geography (Lee et al., 2010, 2015), and no studies showed that volatiles varied with geography. One study found that location notably impacts sensory parameters of tea and that this directly corresponds to higher phenolic theaflavin and therubigin tea constituents (Bhuyan et al., 2009).

Light Factors

The six studies on the effects of light factors explored shading treatment, light intensity, and light quality (**Figure 5**; **Supplementary Table 5**). One of these studies found that polyphenols increased with an increase in light (Zhang et al., 2014) while one study found an inverse relationship of light and polyphenols (Zheng et al., 2008) and two studies showed that some polyphenols increase with an increase in light and others decrease (Ku et al., 2010; Song et al., 2012). Ku et al. (2010) found that shade cultured green tea (tencha) had lower levels of phenolic epigallocatechin and epicatechin compounds as well as higher levels of amino acids compared to unshaded green tea while having higher levels of gallocatechin. These difference in polyphenol profiles resulted in the shade cultured tea to have higher umami profile and less astringent taste than the unshaded tea.

Three light studies found that caffeine levels decrease with an increase in light (Song et al., 2012; Lee et al., 2013; Zhang et al., 2014) while one of these studies found that caffeine levels increased at specific amounts of light (Song et al., 2012). Song et al. (2012) demonstrated a non-linear relationship between shade levels (10% shade to 60% shade) and tea quality based on six secondary metabolites (L-theanine, caffeine, -epicatechin, -epicatechin gallate, -epigallocatechin gallate, and –epigallocatechin). Three of the light studies indicate that some terpenoids and other volatiles increase while others decrease with a change in light levels (Ku et al., 2010; Song et al., 2012; Zhang et al., 2014). Overall, increased light results in decreased concentrations of amino acids (Ku et al., 2010; Song et al., 2012; Deng et al., 2013; Lee et al., 2013; Zhang et al., 2014).

Altitude

Studies demonstrate that higher elevation is generally associated with higher tea quality as measured by higher levels of catechins and other polyphenols as well as caffeine (**Figure 6**; **Supplementary Table 6**). Four studies found that tea catechins and other polyphenols increased with elevation (associated with cooler temperatures and higher oxidative stress) (Akhlas, 2003; Abeywickrama et al., 2010, 2011; Ohno et al., 2011) and one study found lower catechin levels with higher elevation

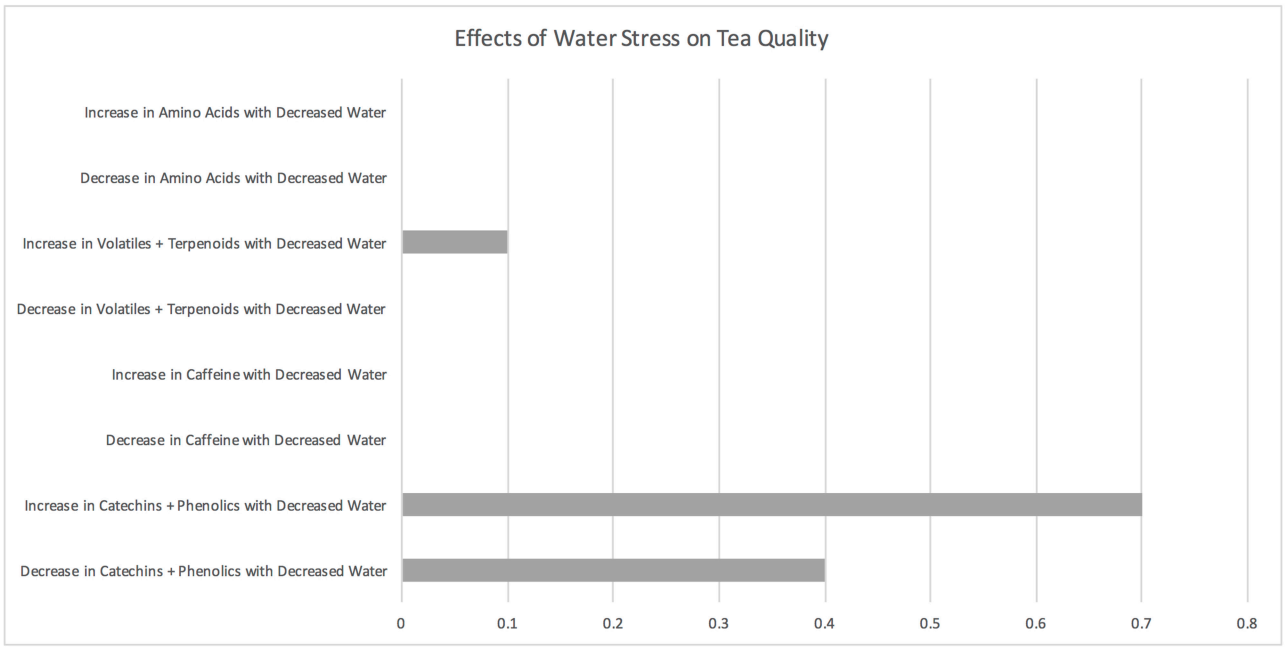

FIGURE 3 | Effects of water stress on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

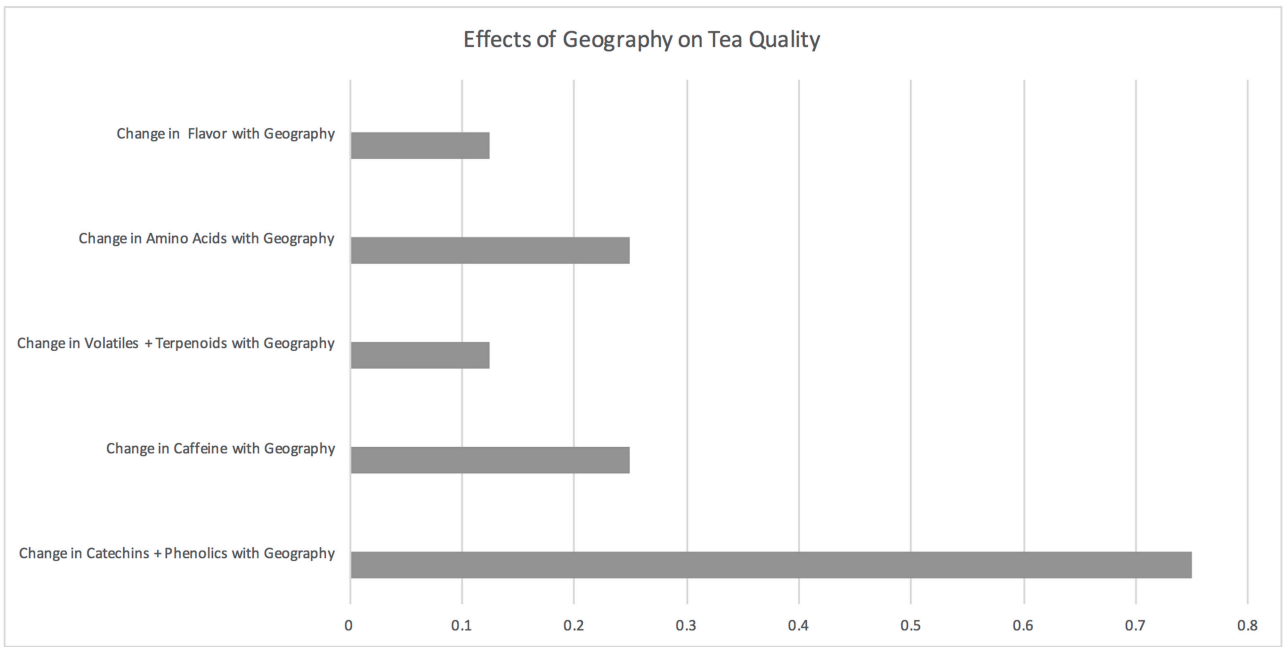

FIGURE 4 | Effects of geography on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

(Chen et al., 2014). Three studies found that caffeine was higher in tea cultivated at higher elevation as compared to lower elevation (Akhlas, 2003; Abeywickrama et al., 2010; Ohno et al., 2011) and one study found lower caffeine levels with higher elevation (Abeywickrama et al., 2011). One study demonstrated that amino acid levels increase with higher elevation compared to lower elevation (Ohno et al., 2011) while one study found that amino acids were higher for lower

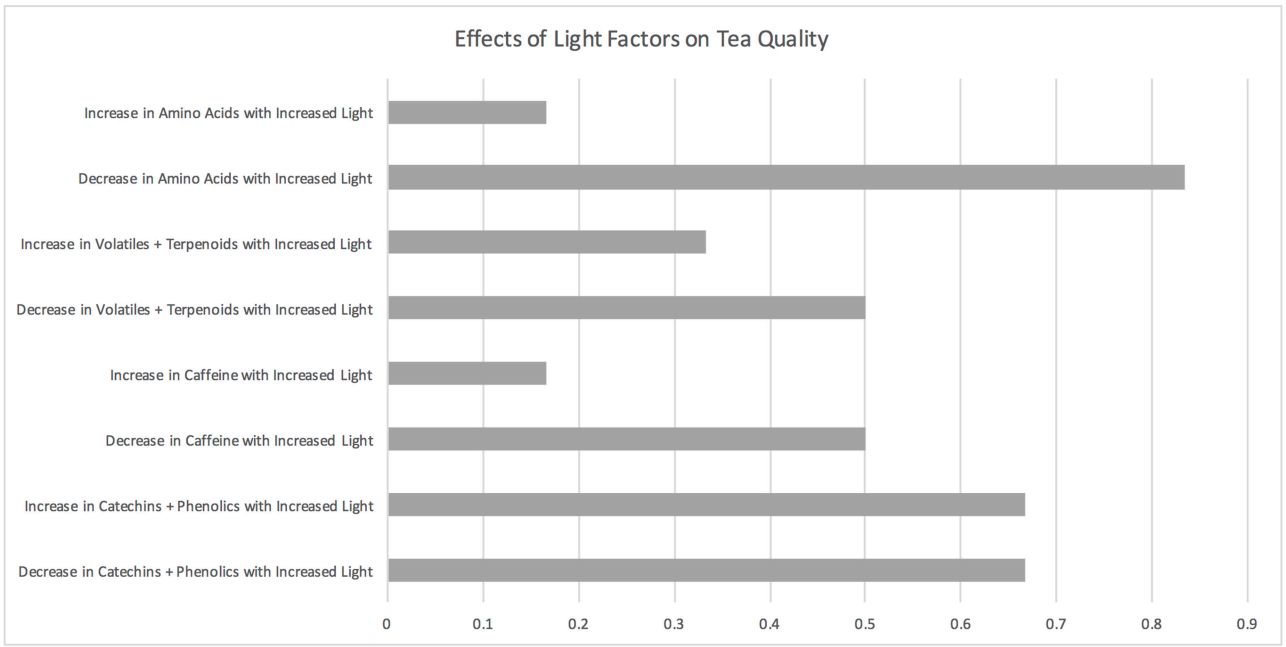

FIGURE 5 | Effects of light factors on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

elevation tea samples (Abeywickrama et al., 2011). One recent study (not included in this review) demonstrated an increase in favorable aromatic compounds that have sweet, floral, and honey-like notes with an increase in elevation while exhibiting a decrease in caffeine, epicatechin gallate, gallocatechin, and catechin (Kfoury et al., 2018).

Herbivory and Microbes

Three studies on herbivory and two studies on microbes examined the effects of various insects (tea aphids, tea mosquito bug, and Kanzawa spider mites) and microbes (*phomospsis* and *Arbusular mucro* fungi from natural and cultivated tea rhizospheres) (**Figure 7**; **Supplementary Table 7**). Herbivory resulted in either a decrease in catechins (1 study; Chakraborty and Chakraborty, 2005), increase in catechins (1 study; Dong et al., 2011), increases in volatiles (1 study; Dong et al., 2011), and increase in some volatiles and decrease in other volatiles (1 study; Han and Chen, 2002). Han and Chen (2002) found that tea aphid-damaged tea shoots showed the presence of volatile compounds benzaldehyde and E-2-hexenoic acid which favorably contribute to aroma. Intact tea shoots on the other hand had butanoic acid-3-hexenyl ester and 1-octanol. None of the herbivory studies demonstrated that caffeine and amino acid levels were impacted.

One microbe study focused on the pathogen *phomospsis* (Ponmurugan and Baby, 2007) and the other focused on the healthy soil fungi *Arbusular mucro* (Singh et al., 2010). The presence of the pathogen reduced polyphenol and amino acid levels in Ponmurugan and Baby (2007). Inoculating tea plants with arbuscular mycorrhizal fungi increased catechin and other polyphenol levels in tea as well as amino acids and caffeine (Singh et al., 2010).

Temperature

Three of the four temperature studies (**Figure 8**; **Supplementary Table 8**) found an inverse relationship between temperature and tea quality on the basis of catechins, phenolic secondary metabolites, and antioxidant compounds/activity (Lee et al., 2010; Wang et al., 2011; Wei et al., 2011) while one study showed that increased temperature resulted in increased catechin compounds (Yao et al., 2005). Wei et al. (2011) showed that higher catechin content was related to lower temperatures (same as higher elevation). Yao et al. (2005) showed that tea quality as determined by key catechin compounds [(-)-epigallocatechin gallate, (-)-epicatechin gallate, and catechin gallate] was higher during warm months and lower during cool months. Wang et al. (2011) showed that increased temperatures were associated with an increase in (-)-epigallocatechin, (-)-epicatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate while catechin (C) decreased. Only one temperature study examined caffeine levels in tea (Lee et al., 2010). Lee et al. (2010) showed that green tea samples grown at high temperature had lower levels of caffeine and catechin compounds compared to those at lower temperature.

Soil and Nutrient Factors

The 26 soil studies examined a range of soil nutrients including aluminum, zinc, iron, nitrogen, phosphorus, magnesium, selenium, manganese, potassium, and boron (Al, Zn, Fe, N,

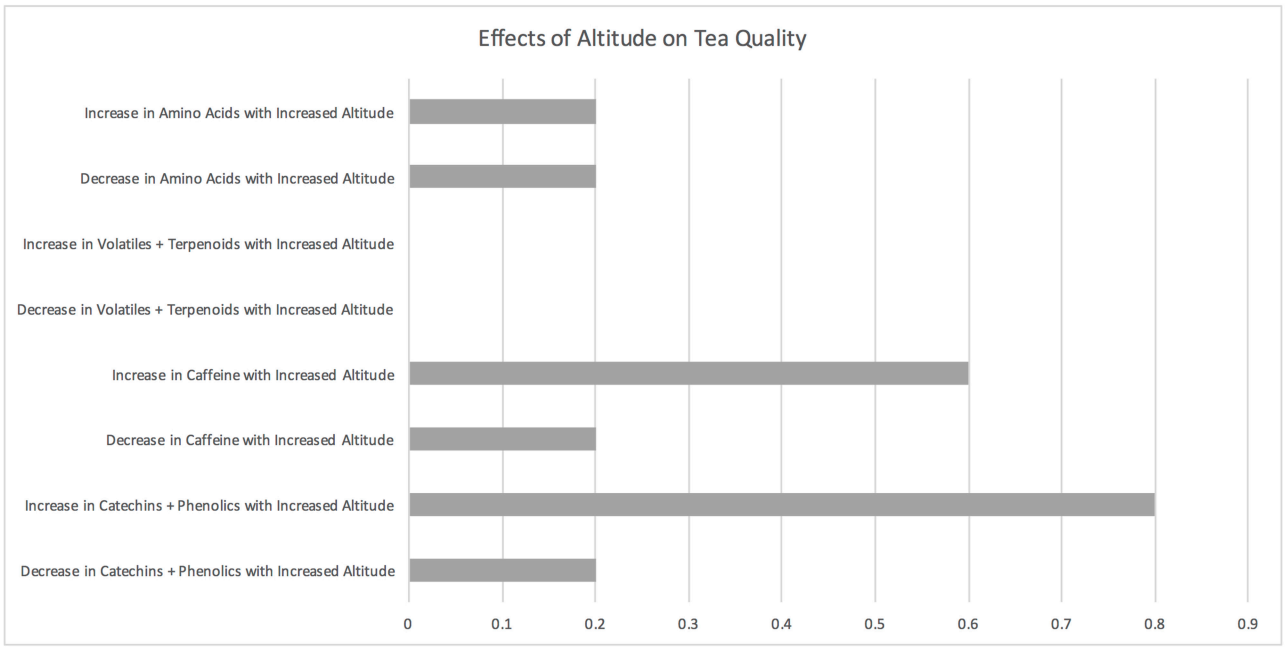

FIGURE 6 | Effects of altitude on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

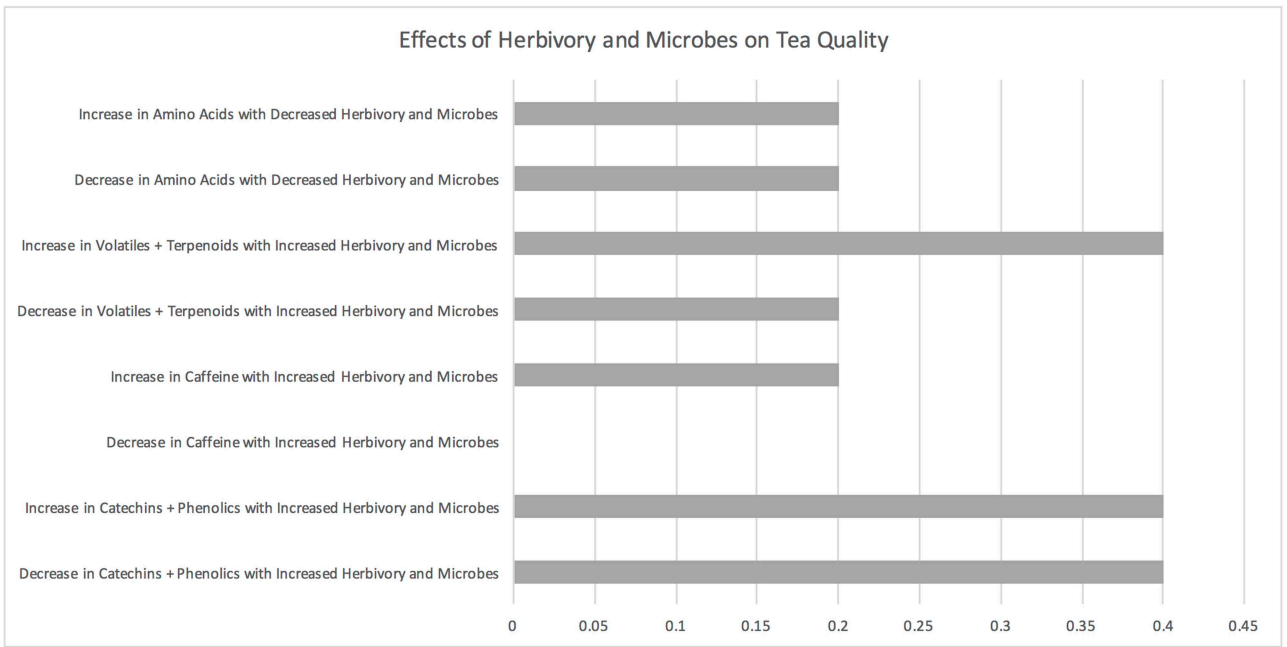

FIGURE 7 | Effects of herbivory on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

P, Mg, Se, Mn, K, and B, respectively) as well as pH levels (**Supplementary Table 9**). Broadly, these studies have two (sometimes overlapping) foci: (1) micro-nutrient fertility, and (2) macro-nutrient fertility, with several of the micro-nutrient studies assessing toxicity. Several of the reviewed soil studies assessed the impacts of both deficiency and sufficiency on quality parameters. These studies highlight how soil and nutrient factors notably impact catechin and other phenolic compounds (Hu

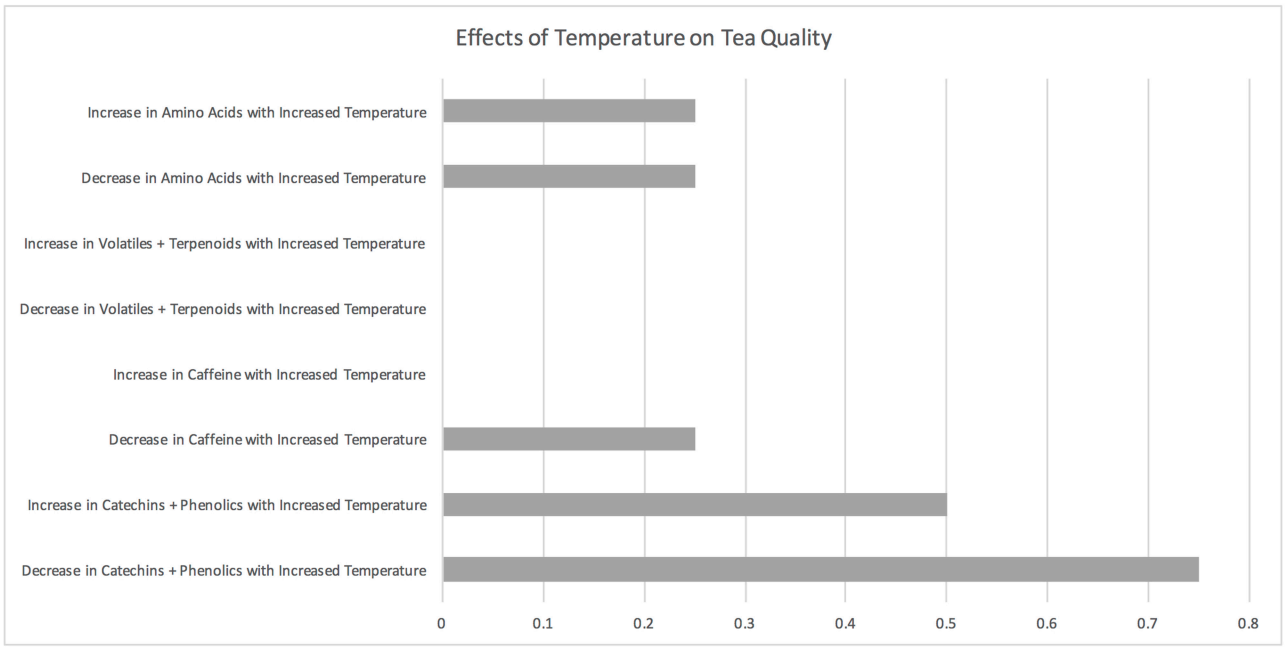

FIGURE 8 | Effects of temperature on tea quality. The horizontal axis depicts the percentage of studies and the vertical axis depicts directional changes in key quality parameters corresponding to climate change related environmental shifts.

et al., 2003; Ruan et al., 2007a; 2010, 2013; Chen et al., 2011; Jayaganesh et al., 2011; Duan et al., 2012; Lin et al., 2012; Sae-Lee et al., 2012; Yang et al., 2014), antioxidant defense enzymes (Hajiboland et al., 2013), caffeine (Yang et al., 2014; Sedaghathoor et al., 2019), amino acids (Hu et al., 2003; Venkatesan et al., 2004, 2005; Ruan et al., 2007a; 2010, 2013; Jayaganesh and Venkatesan, 2010; Jayaganesh et al., 2011; Lin et al., 2012; Yang et al., 2014), vitamins (Hu et al., 2003), and flavor (Venkatesan et al., 2006). Each of these is summarized below (**Supplementary Table 9**).

Although many crop species are inhibited by high levels of soluble aluminum (Al) in the soil, most often found at low soil pH (e.g., pH $<$ 5.0), we found multiple studies that demonstrated that Al stimulates tea growth (Mukhopadyay et al., 2012), phytochemical concentration associated with high-quality tea (Chen et al., 2011; Duan et al., 2012; Sae-Lee et al., 2012; Hajiboland et al., 2013), or both. Both Duan et al. (2012) and Sae-Lee et al. (2012)found that increasing Al resulted in higher polyphenol, caffeine, and amino acid content of tea leaves; Sae-Lee et al. (2012) also noted higher content of catechins with increasing Al, and also with increasing Se. Hajiboland et al. (2011) found that Al supplementation could compensate for B deficiency.

The stimulation of tea growth resulted in increased amino acid and vitamin C from application of Se (Hu et al., 2003). Deficiency and toxicity impacts from Zn (Mukhopadhyay et al., 2013) and Fe (Hemalatha and Venkatesan, 2011) were observed on growth and enzymatic activity in tea; Venkatesan et al. (2007) also observed that excess Mn resulted in lower amino acid and carotenoid content of tea leaves. Three papers evaluated the impacts of Mg fertilization on growth and composition of tea. The work of Ruan et al. (2012) focused on growth, which increased with Mg addition, but also Mg content and amino acid mobility in tea plants. Jayaganesh and Venkatesan, (2010) and Jayaganesh et al. (2011) also noted increased amino acid content with the addition of Mg, and Jayaganesh et al. (2011) observed that different forms of Mg had differential effects on catechin and carotenoid content of tea leaves. Increasing the supply of available N in soil was found to increased amino acid content of tea leaves (Venkatesan et al., 2005; Ruan et al., 2007b, 2010), although other studies (Han et al., 2008; Hamid et al., 2014) noted primarily increased growth with increasing N availability, rather than changes in plant composition.

Carbon Dioxide

While this review did not retrieve any studies on the effects of carbon dioxide on tea quality, we summarize one recent study outside of the time frame of our review. Li et al. (2017) found that there was a significant increase in concentrations of total catechins and other polyphenols along with theanine and free amino acids with elevated carbon dioxide. Concurrently, levels of caffeine decreased (Li et al., 2017). These chemical findings are in sync with expression levels of biosynthetic genes for catechins and theanine that were up-regulated in tea leaves under elevated carbon dioxide levels while levels of biosynthetic genes for caffeine were down-regulated.

DISCUSSION AND CONCLUSION

While the majority of research examining the effects of climate change on food systems has focused on crop yields (Lobell and

Asner, 2003; Hertel et al., 2010; Lobell et al., 2011), this systematic literature review highlights that multiple environmental variables linked to climate change also significantly impact crop quality as measured by secondary metabolites associated with flavor attributes and health claims for human consumers (Wolfe et al., 2008; Liu, 2013). Specifically, findings provide evidence that shifts in seasonality, water stress, geography, light factors, altitude, herbivory and microbes, temperature, and soil factors that are linked to climate change can result in both increases and decreases up to 50% in secondary metabolites. A gap was found regarding evidence on the direct effects of carbon dioxide on tea quality, highlighting a critical research area for future study. The reviewed studies provides evidence that environmental factors linked to climate change can result in changes in the following biochemical measures of tea quality: phenolic catechins (i.e., epigallocatechin gallate, epicatechin gallate, epigallocatechin, and catechin gallate), total phenolic concentration, antioxidant activity, caffeine, theaflavin, thearubigin, tannins, carotenoids, terpene alcohols (i.e., linalool and geraniol) and other aromatic volatiles, fatty acids, chlorophyll, enzymes, and enzymatic activities. It is important to note that while an increase in many secondary metabolites represents an increase in crop quality, it may represent a decrease in crop quality for other secondary metabolites including those with off flavors or whose increased consumption by human consumers may result in toxicity (Kfoury et al., 2018). While this systematic review provides evidence that multiple environmental parameters are impacting tea quality, the directionality and magnitude of these impacts is not clear with contradictory evidence between studies. Findings build on a recent literature review highlighting the importance of understanding climate change effects on tea production (Marx et al., 2017) by providing evidence on the multi-directionality of changes in crop quality in response to shifts in environmental factors that are shifting with global change. As crop quality is a key determinant of health in the food system, findings have broader implications for other culturally-relevant crops managed and used by people for food, medicinal, and aesthetic purposes including fruits, vegetables, tree nuts, and beverage crops (Ahmed and Stepp, 2016).

The environmental factors with the most consistent evidence in this systematic review were seasonality and water stress. Specifically, a total of 14 out of 18 studies (78%) demonstrated a decrease in concentrations of phenolic compounds or their bioactivity with a seasonal shift from the spring and /or first tea harvest to other seasons. Seven out of ten studies (70%) showed an increase in levels of phenolic compounds or their bioactivity with drought stress. However, for both seasonality and water stress, multiple studies highlighted that while some secondary metabolite compounds increase, other compounds decrease, pointing to the complexity of understanding tea quality. Based on the evidence regarding seasonality and geographic variability highlighted in this study, tea quality is likely to increase during some parts of the year in some geographic areas with climate change (i.e., during periods of drought) while decreasing at other times of the year (i.e., during extended monsoon periods). However, if drought stress exceeds moisture thresholds of tea plants in a warming world, productivity will be jeopardized.

Herbivory and soil fertility were two of the variables that showed the greatest contradictory evidence with mixed impacts on tea quality. A surprising finding was that while many crop species are inhibited by high levels of soluble aluminum (Al) in the soil, we found multiple studies that demonstrated that Al not only stimulated tea growth (Mukhopadyay et al., 2012), but also phytochemical concentration associated with high-quality tea (Chen et al., 2011; Duan et al., 2012; Sae-Lee et al., 2012; Hajiboland et al., 2013). Both herbivory and soil fertility are variables which farmers have the greatest control over, pointing to the importance of agricultural management for climate mitigation and adaptation. Farmers can respond at some capacity to climate-driven shifts on crop quality at the level of individual fields or farms. However, beyond specific thresholds, adaptation would require geographic shifts in production, yet these thresholds are largely unknown for crop quality.

The contradictory evidence found in this systematic review likely results from multiple confounding factors associated with each study including variation in tea variety, cultivar, specific environmental and agricultural management conditions, and differences in methods that make comparisons across studies difficult. For example, previous research indicates that different varieties and cultivars of a specific species such as tea may respond variably to the same environmental factor such as seasonality (Owuor and Chavanji, 1986). Owuor and Chavanji (1986) found that seasonal fluctuations variably impacted the caffeine content of tea plants depending on the type of clone. Controlled experiments are called for that examine the impacts of specific environmental factors on multiple tea varieties and cultivars including climate resilient cultivars. In addition, future research efforts should focus on not just understanding the implications of individual factors on specific attributes of crops, but on modeling the system-wide impacts of simultaneous changes on crop biological systems to better understand the long-term implications of climate change. This includes understanding complex interactions of crops with the environment including dynamic feedback loops in which the presence and concentrations of secondary metabolites are impacted by a cascade of abiotic and biotic conditions such as changes in precipitation, temperature, and pests (Fields and Orians, 2006; Schepp, 2009). Research is further needed to analyze the quality of studies included in this systematic review and similar reviews regarding the effects of climate-related changes on crop quality.

The overall lack of consistency in evidence for climate-driven effects on tea quality, coupled with our inability to quantitatively compare study outcomes because of a lack of comparable measures and information on tea variety, points to the challenge of measuring crop quality and the multidimensional nature and complexity of this parameter. One way to overcome this challenge is to design more widely agreed upon international standards of crop quality for different culturally and economically relevant crops along with implementing global collaborative projects with shared experimental designs to allow for comparison of outcomes across multiple studies on crop quality through space and time.

In addition, the lack of studies examining the interplay of multiple environmental factors on tea quality highlights the need for an ecosystems approach in examining multi-trophic interactions in tea systems as well as implications for consumers and farmer livelihoods. Further research is needed to analyze the quality of studies included in this systematic review. While our synthesis contributes to elucidating the directionality of changes in tea quality in response to environmental change, we were unable to quantitatively compare study outcomes because of a lack of comparable measures. Future work to develop standardized protocols for measuring crop quality to enable monitoring of this parameter and comparisons between studies is also needed.

Multiple agricultural, physiological, and molecular innovations have been identified toward the development of climate resilient tea systems (Ahmed, 2018). At the agricultural level, climate mitigation strategies for tea farms include agricultural diversification, tree planting and maintaining vegetative cover, management of soil organic matter and carbon sequestration, water management, controlling pests and disease, and migration and relocating agroecosystems to more suitable locations (Ahmed and Stepp, 2016; Ahmed, 2018). Physiological and molecular innovations for enhancing climate resilience of tea farms include: (1) cultivation of tea from seed versus from clonal propagules, (2) micropropagation, (3) traditional breeding methods of climate-smart cultivars, (4) genetically modified crops and, (5) sustainable intensification (Ahmed, 2018). Traditional breeding methods of climate-smart tea cultivars including those with enhanced resistance to climate variability such as extreme drought, early- maturing varieties, and biofortified crops that accumulate more minerals and vitamins (Ahmed, 2018). Research is needed to measure the effectiveness of these strategies for enhancing the resilience of shifts in crop quality to climate change. While farmers can respond at some capacity to climate-driven shifts on crop quality at the level of individual fields or farms, changes beyond specific ecological and physiological thresholds would require geographic shifts in production. Future research is called for to elucidate what these thresholds are for crop quality for a range of staple and nutrient-dense crops that support food security and human health.

These findings have notable implications for the sustainability and resilience of agricultural systems in the context of global environmental change. Thus, it is essential to develop and implement evidence-based adaptation strategies for growing crops in ways that are resilient to climate change and support both environmental and human well-being. Based on our reflections on lessons learnt from this systematic review, we recommend the following next steps for advancing the understanding of climate change effects on crop quality as well as for responding to this challenge:

(1) Carry out farm-level research on the effectiveness of various agricultural practices for climate adaptation and mitigation for tea plants and other food and beverage crops including the cost-effectiveness, replicability, and adaptability for different geographic contexts as well as differing scales and models of production. Findings should be applied to design and disseminate climate resilient agricultural guidelines that should be supported by policy including providing economic and social incentives.
(2) Invest in breeding of climate-resilient crop cultivars that includes the evaluation of crop quality to multiple environmental factors and identification of crop quality thresholds.
(3) Design more widely agreed upon international standards of crop quality for multiple culturally and economically relevant crops
(4) Implement long-term global collaborative projects with shared experimental designs and "big data" sharing to allow for comparison of outcomes across multiple studies on crop quality through space and time. These studies should model system-wide impacts of simultaneous environmental changes on agricultural systems and implications for nutrition and human health to better understand the dynamic feedback loops in the food systems and associated social and ecological long-term implications of climate change.
(5) Foster cross-sector collaboration between researchers, practitioners, producers, and policy makers to develop evidence-based adaptation strategies that reduce vulnerability of food systems to shifts in crop quality toward supporting sustainability.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

SA and TG conceived of the study question for the systematic review and guided the systematic review team. SA, TG, and MS designed the systematic review protocol with guidance from RH. All authors contributed to search terms. MS, SA, and MH served on the review panel to identify articles to be included in the study. DK, SA, DS, and AL served on the data extraction panel. SA, CM, and DK created the Figures and Tables. SA and TG wrote the manuscript with contributions from all authors. All authors approved the final manuscript submitted. SA and SC led the submission of the manuscript.

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english/site_1/index.html), (6) Ministry of Education of China Award B08044 (CL and DX; http://old.moe.gov.cn//publicfiles/business/htmlfiles/moe/moe_2792/index.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00939/full#supplementary-material

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Identification of Regulatory Networks of MicroRNAs and Their Targets in Response to *Colletotrichum gloeosporioides* in Tea Plant (*Camellia sinensis* L.)

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Anthracnose disease is caused by *Colletotrichum gloeosporioides*, and is common in leaves of the tea plant (*Camellia sinensis*). MicroRNAs (miRNAs) have been known as key modulators of gene expression in response to environmental stresses, disease resistance, defense responses, and plant immunity. However, the role of miRNAs in responses to *C. gloeosporioides* remains unexplored in tea plant. Therefore, in the present study, six miRNA sequencing data sets and two degradome data sets were generated from *C. gloeosporioides*-inoculated and control tea leaves. A total of 485 conserved and 761 novel miRNAs were identified. Of those, 239 known and 369 novel miRNAs exhibited significantly differential expression under *C. gloeosporioides* stress. One thousand one hundred thirty-four and 596 mRNAs were identified as targets of 389 conserved and 299 novel miRNAs by degradome analysis, respectively. Based on degradome analysis, most of the predicted targets are negatively correlated with their corresponding conserved and novel miRNAs. The expression levels of 12 miRNAs and their targets were validated by quantitative real-time PCR. A negative correlation between expression profiles of five miRNAs (PC-5p-80764_22, csn-miR160c, csn-miR828a, csn-miR164a, and csn-miR169e) and their targets (WRKY, ARF, MYB75, NAC, and NFY transcription factor) was observed. The predicted targets of five interesting miRNAs were further validated through 5'RLM-RACE. Furthermore, Gene Ontology and metabolism pathway analysis revealed that most of the target genes were involved in the regulation of auxin pathway, ROS scavenging pathway, salicylic acid mediated pathway, receptor kinases, and transcription factors for plant growth and development as well as stress responses in tea plant against *C. gloeosporioides* stress. This study enriches the resources of stress-responsive miRNAs and their targets in *C. sinensis* and thus provides novel insights into the miRNA-mediated regulatory mechanisms, which could contribute to the enhanced susceptibility of *C. gloeosporioides* in tea plant.

Keywords: *Colletotrichum gloeosporioides*, *Camellia sinensis*, microRNA, regulatory network, degradome

INTRODUCTION

Plants have evolved to respond to biotic and abiotic stresses through a repertoire of mechanisms, which regulate gene expression to maximize chances of survival in hostile conditions (Dorantes-Acosta et al., 2012). Under biotic stresses, plants trigger two layers of immunity against pathogens, which are pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI) and effector-triggered immunity (ETI). Pathogenic organisms are recognized by conserved PAMPs (elicitors) through pattern-recognition receptors (PRRs) located on the cell surface. Perception of PAMPs triggers basal defense, also known as PTI, which encompasses the immune responses against most pathogens (Jones and Dangl, 2006). Effective pathogens have evolved mechanisms to counteract the basal defense by delivering PTI interfering effector proteins into the plant cells (Chisholm et al., 2006). In turn, many plants have evolved another layer of immunity, ETI. The ETI response is facilitated by proteins encoded by resistance (R) genes, which are typically nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Cui et al., 2015). Most studies of plant immunity have focused on the transcriptional regulation of protein-coding genes. Recently, it has been found that diverse miRNAs are responsive to infection and stress, and function in plant responses to both biotic and abiotic stresses (Xin et al., 2010; Chen et al., 2017).

In recent years, several studies have shown that small non-protein-coding RNAs (sRNAs) are key molecules that regulate diverse eukaryotic biological processes, including transcription, oxidation-reduction, transport, and stress response. Plant microRNAs (miRNAs) and small interfering RNAs (siRNAs) are two major classes of sRNAs and are classified according to their biogenesis (Jones-Rhoades et al., 2006). MiRNAs are derived from single-stranded stem-loop precursor structures, and siRNAs are processed from perfect double-stranded RNA transcripts (Peters and Meister, 2007). Plant miRNAs are an extensive class of newly discovered endogenous small regulatory RNA molecules, which are 20–24 nucleotides in length and negatively regulate gene expression at the post-transcriptional level by guiding target mRNA cleavage or translation inhibition (Jones-Rhoades et al., 2006). MiRNAs play an important role in responses to biotic and abiotic stressors through their interactions with their target mRNAs (Chen et al., 2017). For example, miR393 represses auxin signaling to promote bacterial PAMP-triggered immunity (PTI) (Navarro et al., 2006).

The rapid advances in high-throughput sequencing technologies as well as bioinformatics analyses have provided a highly efficient strategy for discovering conserved and novel miRNAs in several plant species (Xu et al., 2012, Chen et al., 2017), despite the unavailability of plant whole genomic sequences (Xu et al., 2012). To date, 38,589 precursor miRNAs and 48,885 mature miRNAs from 271 species have been deposited in the miRNA database miRBase (Release 22, www.mirbase.org) (Kozomara and Griffiths-Jones, 2014). However, several studies of miRNAs were previously reported (Zhang et al., 2014; Zheng et al., 2015; Liu et al., 2016; Jeyaraj et al., 2017). Tea genome sequence information was recently released by Wei et al. (2018). Identification of miRNA target genes is critical for the elucidation of the pathways they regulate. Degradome sequencing of mRNA combined with gene functional annotation can predict and verify target genes of miRNAs in many plants (Addo-Quaye et al., 2008). This approach for identifying miRNAs and their targets firmly depends on the availability of genomic information and miRNA databases (e.g., miRBase).

The tea plant [*Camellia sinensis* (L.) O. Kuntze] is an economically important and evergreen woody perennial plantation crop that grows mainly in tropical and subtropical climates (Mukhopadhyay et al., 2016). Tea is one of the most popular non-alcoholic beverages in the world and is consumed by over two-thirds of the world population. The tea plant is susceptible to biotic (bacterial, fungal, and viral diseases) and abiotic (cold and drought) stresses (Deng et al., 2013). *Colletotrichum gloeosporioides* is considered to be one of the dominant endophytic taxa of *C. sinensis,* which leads to the fungal disease anthracnose (Fang et al., 2013). As the disease progresses, yellow oval spots will turn to concentric brown rings with scattered dots; it eventually leads to defoliation, affecting both young and old leaves. Unfortunately, many tea plant cultivars are highly susceptible to anthracnose disease, which causes severe damage accompanied by high yield losses (Fang et al., 2013). It was recently reported that symptoms of blight on the leaves of anthracnose infected plants were detected in 30–60% of the *C. sinensis* fields in the Yellow Mountain region in Anhui province of China (Guo et al., 2014).

The infection of hemibiotrophic fungus (*C. gloeosporioides*) displays an initial biotrophic phase, followed by a necrotrophic stage (Münch et al., 2008). The salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signaling pathway is activated against necrotrophic as well as hemibiotrophic pathogens. The SA analog BTH (benzo thiadiazole-7-carbothioic acid S-methyl ester) treatment triggered the accumulation of SA-inducible defense proteins, which in turn activate defense-related genes during anthracnose infection in cowpea seedlings (Latunde-Dada and Lucas, 2001) and cucumber (Deepak et al., 2006). However, the miRNA-mediated gene regulation in response to *C. gloeosporioides* is unknown.

In tea plant, a fewer number of conserved and novel miRNAs were identified relative to other model plants (Zhu and Luo, 2013). Recently, a limited number of tea miRNAs were identified from responses to environmental stresses, such as drought, cold, and insect-induced stress (Zhang et al., 2014; Zheng et al., 2015; Liu et al., 2016; Jeyaraj et al., 2017). However, the miRNA-mediated gene regulatory networks that respond to the biotic *C. gloeosporioides* stress remain unexplored in tea plant. Therefore, in the present study, we employed high-throughput sRNA sequencing technology to identify miRNAs from healthy leaves (the control CK) and leaves treated with *C. gloeosporioides*. Differentially expressed miRNAs between CK and the biotic treatment were identified, and further potential targets of miRNAs were predicted through degradome sequencing. Biological functions of target genes were analyzed along with their interactions with their respective miRNAs, and results were validated by quantitative real-time polymerase chain reaction (qRT-PCR). The predicted cleavage sites in target gene mRNAs of five csn-miRNAs were validated through 5'RLM-RACE. These

results lay the foundation for understanding the regulation of miRNAs and their respective target genes in response to *C. gloeosporioides* stress in tea plant.

MATERIALS AND METHODS

Plant Growth Conditions

Tea plant (*C. sinensis* L. var. Shuchazao) used in the present study were grown in the tea plantation at Anhui Agricultural University, Hefei, China. Three-year-old cuttings were planted in pots (30-cm diameter, 35-cm height) and grown in a green house maintained at 23 ± 3°C with 65 ± 5% room humidity and a 16/8 h (day/night) photoperiod. All experimental plants were irrigated once a day and fertilized once a month. Healthy Shuchazao cuttings with uniform growth (25–30 cm in height) were selected for experiments.

Isolation of Fungal Pathogen

Diseased leaves of Shuchazao were collected from the tea plantation, which is located at Anhui Agricultural University, Hefei City, Anhui Province in China. The pathogenic *C. gloeosporioides* was originally isolated from diseased leaves showing visible anthracnose symptoms. Briefly, the infected leaves with the area brown lesions were cut into small pieces. These small pieces were surface sterilized in 2% NaClO for 3 min, followed by 70% ethanol for 1 min, rinsed twice in sterile water, and then transferred to potato dextrose agar (PDA) containing plate. The culture was incubated at 28°C for 5 days. Single germinating spores were picked up with a sterilized needle and transferred to a new PDA plate, and incubation at 28°C was continued to generate the pure isolates (Cai et al., 2009).

DNA Extraction, PCR Amplification, and Sequencing

The fungal genomic DNA was extracted from fresh mycelia grown in potato dextrose broth (PDB, liquid media) for 5 days, using a modified CTAB protocol as described by Guo et al. (2000). DNA quality and quantity were determined by spectrophotometer and electrophoresis. DNA samples were stored at −80°C for further analysis. In order to identify *C. gloeosporioides*, the ribosomal internal transcribed spacer (ITS) was amplified in a thermocycler (Bio-Rad, Hercules, USA) using ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The 25 µl of reaction mixture consisted of 12.5 µl of Premix Taq™ (Takara, Dalian, China), 1 µl of forward primer, 1 µl of reverse primer, 2 µl of template DNA, and 8.5 µl of double-distilled water. Amplification of ITS regions was performed using the following PCR reaction conditions of 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 45 s, and final extension at 72°C for 5 min. The final PCR products were analyzed on 1% agarose gel and purified using AxyPrep DNA gel extraction kit (AxyPrep, Hangzhou, China) according to the manufacturer's protocol. The purified products were sequenced and analyzed by GenScript (Nanjing, China). To identify the fungus, the resulted sequences were subjected to BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the National Center for Biotechnology Information (NCBI) database. The sequences of ITS region of all isolates had 100% homology with *C. gloeosporioides* (GenBank: JX010223.1) isolates available in the NCBI (Weir et al., 2012).

Pathogen Inoculations and Treatments

For pathogenicity tests, *C. gloeosporioides* was cultured on PDA and incubated at 25°C ± 2°C in darkness for 10 days to promote sporulation. Conidia were harvested and suspended in sterile distilled water. The conidia concentration was determined using a hemocytometer and adjusted to 1×10^6 ml^{-1} for inoculation. The third healthy mature leaves from 3-year-old tea plants were surface-sterilized with 75% ethanol and sterile distilled water. The leaves were wounded on the upper surface with a sterile needle, and 20 µl of conidial suspensions was applied to the wound. An equal volume of sterile water was applied as a mock inoculation. After inoculation, each plant was enclosed in a plastic bag to maintain high relative humidity for conidial germination. The *C. gloeosporioides*–inoculated (CgIL) and mock-inoculated control (CK) leaves were harvested at 1, 4, 7, 10 and 13 days after inoculation. All samples were immediately frozen in liquid nitrogen and then stored at −80°C for further use. Three biological replicates were used for each experiment in this study. The samples were assigned a name with the information of treatment type and treated days, i.e., CgIL4d references the samples collected after 4 days of *C. gloeosporioides* treatment. Three experimental replicates were conducted for the treatment and control.

sRNA Library Construction and High-Throughput Sequencing

Total RNA was isolated from each frozen sample using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The quantity and quality of the total RNA were determined using a Bioanalyzer 2100 (Agilent, CA, USA) and the RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. To identify early response of tea plant to *C. gloeosporioides* infection, equal quantities of total RNA from *C. gloeosporioides*- and mock-inoculated leaves collected at 4 days post infection (4dpi) were used to prepare the sRNA libraries using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). We performed single-end sequencing (36 bp) on an Illumina Hiseq2500 at the LC-BIO (Hangzhou, China) using the vendor's recommended protocol. The sRNA read data from this study have been deposited in the Gene Expression Omnibus (GEO) database; the accession number is GSE119728 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119728).

sRNA Data Analysis and Identification of Conserved and Novel miRNAs

The raw reads generated from the Illumina GAIIX system were processed according to the procedures as described in a previous study (Li et al., 2010) by LC Sciences. In brief, the raw reads were filtered using the Illumina pipeline filter (Solexa 0.3), and then

further processed with the in-house program (ACGT101-v4.2-miR, LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity reads, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences ranging from 18 to 26 nt in length were compared with known plant miRNAs in miRBase (Release 22; http://www.mirbase.org) (Kozomara and Griffiths-Jones, 2014), using BLAST searches to identify known miRNAs with no more than two mismatches. After the analyses, known miRNAs were categorized into four groups (1a, 1b, 2a, and 3a).

The remaining unmatched reads were aligned to a *C. sinensis* genome assembly (Wei et al., 2018) using BLASTn, with no mismatches permitted. The sequences containing hairpin RNA structures were predicated from the flanking 80 nt sequences using RNAfold software (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker, 2003). The following criteria were used for predicting the pre-miRNA secondary structure: i) the number of nucleotides in one bulge in stem was ≤12; ii) the number of base pairs in the stem region of a predicted hairpins was ≥16; iii) the Gibbs free energy (kCal/mol) threshold was ≤−15; iv) the length of the hairpin (up and down stems + terminal loop) was ≥50; v) the length of the hairpin loop was ≤20; vi) the number of nucleotides in one bulge in the mature region was ≤8; vii) the number of biased errors in one bulge in a mature region was ≤4; viii) the number of biased bulges in a mature region was ≤2; ix) the number of errors in a mature region was ≤7; x) the number of base pairs in a mature region of the predicted hairpin was ≥12; and xi) maturity percentage in the stem was ≥80.

The abundances of miRNAs were normalized to reads per million (RPM) for each library. The original counts of miRNA reads were used for differentially expressed miRNAs (DEMs) analysis as reported earlier (Liu et al., 2016). DESequence analysis was conducted to identify the DEMs with changes of at least twofold in abundance ($p \leq 0.05$), relative to the CK control.

Degradome Sequencing and Data Analysis

Total RNA was extracted from the samples using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocols. Total RNA from the three biological replicates of each inoculation time (CK and CgIL) was pooled in equal amounts to generate each degradome library. Two libraries were constructed according to the methods described by Addo-Quaye et al. (2009) and Ma et al. (2010) with minor modifications. Briefly, polyA-enriched RNA was mixed with biotinylated random primers, and then the RNA containing the biotinylated random primers was captured by beads and ligated to 5′ adaptors. The ligated products were used to generate first strand cDNA by reverse transcription. After a short PCR amplification, additional DNA products were produced. Following purification, digestion, ligation, and purification, the cDNA library was sequenced with an Illumina Hiseq2500 (LC-BIO, Hangzhou, China).

Raw sequencing reads were obtained using Illumina's Pipeline v1.5 software and processed to remove adaptor sequences and low-quality sequencing reads. The unique sequencing reads with lengths of 20 and 21 nt were then used to identify potentially cleaved targets with a public software package (CleaveLand3.0 pipeline), as previously described (Addo-Quaye et al., 2008). The degradome reads were mapped to *C. sinensis* reference sequences, including tea genome scaffolds and contigs from whole-genome shotgun sequencing and assembly, tea transcriptome sequences from the NCBI Sequence Read Archive (SRA, GenBank accession no. SRR1979118), EST sequences, genomic survey sequences (GSSs), and nucleotide sequences downloaded from the GenBank nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov). The potential target genes of differentially expressed miRNAs were predicted by Target finder. Alignment score was introduced based on the alignment between each miRNA and its potential target. Mismatched pairs or single nucleotide bulges were each scored as 1 and G:U pairs were scored as 0.5. Mismatched and G:U pair scores were doubled within the core segment (nucleotide pairs at positions 2–13). Furthermore, based on the abundances of the degradome sequences and cleavage sequences, the miRNA targets were classified into five categories (0, 1, 2, 3, and 4) according to the CleaveLand 3.0 pipeline with default parameters (Addo-Quaye et al., 2008).

Functional Analysis of Target Genes and Network Analysis

To better understand the function of the target genes and their corresponding metabolic network during *C. gloeosporioides* stress in tea plant, annotations of target gene functions were performed using the Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) pathway databases. The most abundant DEM targets were annotated based on sequence similarity by performing a $BLAST_X$ search against the GO protein database. Furthermore, target genes were categorized according to their functions under biological processes, molecular functions, and cellular components using GO analysis. The enriched metabolic pathways or signal transduction pathways of potential miRNA target genes were validated using KEGG enrichment analysis to enrich the KEGG terms according to Zhang et al. (2016). Networks between miRNAs and their target genes were subsequently assembled according to the GO analysis results.

Validation of miRNA Expression Profiles and Their Targets

The expression levels of randomly selected miRNAs and their targets were validated by qRT-PCR. Total RNA was isolated from the samples taken at 1dpi, 4dpi, 7dpi, 10dpi, and 13dpi using Trizol reagent (Invitrogen, CA, USA), and the quantity and purity of the total RNA were determined using a Bioanalyzer 2100 (Agilent, CA, USA) and the RNA 6000 Nano LabChip Kit (Agilent, CA, USA). Five hundred nanograms of total RNA from samples was reverse transcribed to cDNA using PrimeScript™ RT Master Mix (Takara, Dalian, China) according to the manufacturer's instructions. The first-strand cDNA was used as a template for qRT-PCR with miRNA and target gene specific primers. For qRT-PCR of miRNAs, the stem-loop RT primers, forward primers, and reverse primers were designed for each individual miRNA according to the criteria described by Varkonyi-Gasic et al. (2007). The primers for mRNA qRT-PCR were designed

using primer premier 5.0 (http://www.premierbiosoft.com/primerdesign/index.html). The amplification was conducted on the CFX96 real-time detection system (Bio-Rad, Hercules, USA) using SYBR Premix Ex Taq™ (Takara, Dalian, China) following the manufacturer's instructions. U6 small nuclear RNA (U6 snRNA) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) genes were used as the references for qRT-PCR of miRNAs and mRNAs, respectively. The expression levels of miRNAs and target genes were determined by calculating fold change using the $2^{-\Delta\Delta Ct}$ method. All qRT-PCR analyses were performed in three biological replicates, each of which consisted of three technical replicates. Detailed information about the primers used in this study is presented in **Supplementary Table 1**.

Verification of miRNA Target Genes by 5'RLM-RACE

The cleavage sites of selected miRNA targets were validated through 5'RLM-RACE using the FirstChoice RLM-RACE Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 10 μg of total RNA was ligated to the 5'RNA adapter using T4 RNA ligase and reverse transcribed to cDNA. Amplification of cleaved miRNA target gene products was performed using target gene specific reverse primers and RNA adapter specific forward primers (**Supplementary Table 1**). The final RLM-RACE products were analyzed on agarose gels and then purified using the DNA gel extraction kit (Corning Life Sciences, Suzhou, China) according to the manufacturer's instruction. Purified products were directly cloned into pEASY-T1 vectors (TransGen Biotech, Beijing, China), transformed into *Escherichia coli* Trans1-T1 competent cells (TransGen Biotech) and sequenced. The sequencing results were analyzed to map the cleavage sites. The primers used to amplify cleavage products of tea miRNA target genes through 5'RLM-RACE are listed in **Supplementary Table 1**.

Statistical Analysis

Fisher's exact test, chi-squared 2×2 test, chi-squared nXn test, Student's t test, or ANOVA was performed based on the experimental designs to compare the expression of miRNAs and degradation of target genes between the CK and CgIL libraries based on normalized deep-sequencing counts. The significance threshold was set to be 0.01 and 0.05 in each test, and the log2 ratio was regarded as a threshold to detect fold changes of miRNA expression and target gene degradation. qRT-PCR data obtained in this study are presented as their means ± standard deviation values. The expression profiles of the miRNAs and target genes identified by qRT-PCR were subjected to Duncan's multiple range tests (DMRT) using DPS software (www.chinadps.net) (Deng et al., 2013).

RESULTS

Pathogenic Fungal Identification and Pathogenicity Tests

For the morphological characterization, diseased tea leaves were sliced into many pieces, surface sterilized, plated on PDA, and incubated at 28°C for 5 days. As a result, a total of seven fungal isolates were recovered from the culture plate. All the cultures on PDA exhibited white and gray color on the front side, while the back side of the colonies was of white and dark gray. For the molecular characterization, genomic DNA of the fungal isolate was amplified using ITS1 and ITS4 primers and sequenced. Based on the ITS rDNA sequence analysis, the species level identity of the fungus was confirmed as *C. gloeosporioides*, which was 100% homology with other *C. gloeosporioides* isolates (GenBank accession number: JX010223.1) (Weir et al., 2012). To identify the disease response of the cultivar of tea plant (Var.Shuchazao) against *C. gloeosporioides* infection, 3-year-old tea plant mature leaves were wounded, inoculated with conidial suspensions of *C. gloeosporioides* (10^6 spores/ml), and then maintained in green house with high relative humidity for conidial germination. In our study, pathogenicity tests showed that the tea plant displayed the typical brown lesions of anthracnose disease around wounded areas at 4, 7, 10, and 13 days after inoculation (**Figure 1**).

Analysis of sRNA Data From Libraries

To identify the miRNAs in the tea plant, we sequenced sRNA in tea leaves isolated from the mock-inoculated control (CK) and the *C. gloeosporioides*-inoculated treatment (CgIL); three independent replicates of each treatment were sequenced using the Illumina GAIIX platform. A total of 66.33 million raw reads were generated, ranging from 10.37 to 12.48 million reads from each library (**Table 1**). After removing 5′ and 3′ adapter sequences, low-quality reads, and RNAs less than 18 nucleotides and longer than 25 nucleotides, the remaining reads were searched against the Rfam database (http://rfam.janelia.org), the Repbase database (http://www.girinst.org/repbase), and tea genome assembly (Wei et al., 2018). Thereafter, sRNAs were classified into different categories according to their annotations as 3′ adapter (ADT) and length filter, junk reads, Rfam, mRNA, repeats, rRNA, tRNA, snRNA, and snoRNA sequences, and other Rfam RNA sequences (**Table 1**). Finally, the total number of clean sequences was reduced to 39.70 million (**Table 1**). The number of unique sRNAs ranged from 1.49 to 4.53 million among the libraries (**Table 1**). The length distribution of sRNAs showed that the top two most abundant sRNAs were 21 and 24 nt (**Table 2** and **Figure 2**). The percentages of unique sRNAs and total sRNAs reads showed drastic differences in their ranges. Comparing reads of small RNAs between CK and CgIL libraries, the abundances of 24 nt total sRNA reads in both libraries were lower than the abundance of unique 24 nt sRNA reads, whereas the percentages of 21 nt total sRNA reads in both libraries were higher than the percentage of unique 21 nt sRNA reads; this indicated that the *C. gloeosporioides* inoculation treatment led to a significant influence on the expression pattern of small RNAs in *C. sinensis*. The abundances of 24 nt unique sRNAs ranged from 33.11% to 68.05%, whereas 21-nt unique sRNA sequences ranged from 8.52% to 22.89% in CK and CgIL libraries (**Table 2** and **Figure 2B**). In summary, 24 nt miRNAs

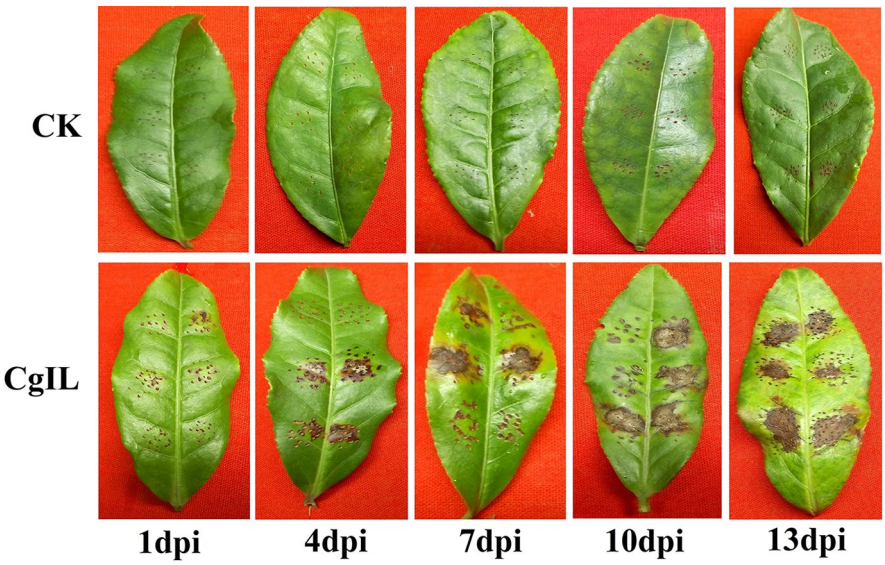

FIGURE 1 | Pathogenicity test of *C. gloeosporioides* on tea plant leaves in CgIL (*C. gloeosporioides*–inoculated leaves) and CK (mock-inoculated control) after 1, 4, 7, 10, and 13 days post inoculations (dpi).

TABLE 1 | Summary of small RNA sequences in the libraries of mock-inoculated control tea leaves at 4dpi (CK1, CK2, CK3) and *C. gloeosporioides*–inoculated infected tea leaves at 4 dpi (CgIL4d1, CgIL4d2, CgIL4d3).

Item	CK1		CK2		CK3	
	Total (%)	Unique (%)	Total (%)	Unique (%)	Total (%)	Unique (%)
Raw reads	10,957,848 (100)	5,095,079 (100)	10,372,329 (100)	4,044,624 (100)	10,469,521 (100)	3,224,037 (100)
3ADT&length filter	941,156 (8.59)	461,151 (9.05)	1,321,950 (12.74)	430,418 (10.64)	3,471,478 (33.16)	961,075 (29.81)
Junk reads	53,707 (0.49)	38,768 (0.76)	42,906 (0.41)	27,760 (0.69)	31,035 (0.3)	17,951 (0.56)
Rfam	163,216 (1.49)	8,793 (0.17)	171,980 (1.66)	8,176 (0.2)	166 201 (1.59)	8,440 (0.26)
mRNA	878,615 (8.02)	60,287 (1.18)	983,984 (9.49)	58,617 (1.45)	694,328 (6.63)	38,605 (1.2)
Repeats	2,757 (0.03)	292 (0.01)	3,879 (0.04)	346 (0.01)	4,056 (0.04)	357 (0.01)
rRNA	136,758 (1.25)	6,706 (0.06)	145,196 (1.4)	6,326 (0.06)	136,326 (1.3)	6,447 (0.06)
tRNA	9,121 (0.08)	636 (0.01)	8,320 (0.08)	514 (0)	10,725 (0.1)	672 (0.01)
snoRNA	4,213 (0.04)	412 (0)	3,707 (0.04)	355 (0)	3,359 (0.03)	322 (0)
snRNA	2,263 (0.02)	293 (0)	1,933 (0.02)	248 (0)	1,422 (0.01)	199 (0)
other Rfam RNA	10,861 (0.1)	746 (0.01)	12,824 (0.12)	733 (0.01)	14,369 (0.14)	800 (0.01)
Clean reads	8,995,659 (82.09)	4,528,076 (88.87)	7,934,269 (76.49)	3,521,692 (87.07)	6,172,590 (58.96)	2,199,975 (68.24)
Item	**CgIL4d1**		**CgIL4d2**		**CgIL4d3**	
	Total (%)	Unique (%)	Total (%)	Unique (%)	Total (%)	Unique (%)
Raw reads	10,734,169 (100)	2,251,034 (100)	12,478,566 (100)	2,039,551 (100)	11,327,463 (100)	2,787,299 (100)
3ADT&length filter	5,451,776 (50.79)	694,095 (30.83)	7,000,101 (56.1)	490,283 (24.04)	2,309,337 (20.39)	457,279 (16.41)
Junk reads	16,324 (0.15)	11,412 (0.51)	17,890 (0.14)	11,765 (0.58)	27,775 (0.25)	18,490 (0.66)
Rfam	172,655 (1.61)	8,306 (0.37)	280,156 (2.25)	10,181 (0.5)	296,893 (2.62)	9,887 (0.35)
mRNA	649,067 (6.05)	32,352 (1.44)	887,460 (7.11)	39,101 (1.92)	1,196,861 (10.57)	51,120 (1.83)
Repeats	2,543 (0.02)	252 (0.01)	3,471 (0.03)	311 (0.02)	3,067 (0.03)	308 (0.01)
rRNA	138,953 (1.29)	5,864 (0.05)	231,694 (1.86)	7,365 (0.06)	253,611 (2.24)	6,859 (0.06)
tRNA	17,039 (0.16)	981 (0.01)	25,444 (0.2)	1,104 (0.01)	20,171 (0.18)	1,076 (0.01)
snoRNA	6,316 (0.06)	518 (0)	7,937 (0.06)	596 (0)	10,082 (0.09)	710 (0.01)
snRNA	1,795 (0.02)	252 (0)	2,336 (0.02)	311 (0)	3,962 (0.03)	464 (0)
other Rfam RNA	8,552 (0.08)	691 (0.01)	12,745 (0.1)	805 (0.01)	9,067 (0.08)	778 (0.01)
Clean reads	4,517,428 (42.08)	1,506,720 (66.93)	4,432,594 (35.52)	1,490,805 (73.09)	7,650,496 (67.54)	2,252,700 (80.82)

were the most abundant, followed by 21 nt miRNA. This finding is highly consistent with those of previous studies on other tea plant cultivars (Zhang et al., 2014; Zheng et al., 2015; Liu et al., 2016) and in other plant species (Liu et al., 2015;Chen et al., 2017). However, some studies have shown 21-nt sRNAs as the most abundant in diverse plant species (Li et al., 2011; Guo et al., 2015). Thus, the 24-nt sRNAs can be important for response to *C. gloeosporioides* infection.

TABLE 2 | The length distribution and abundance of small RNAs in the libraries of mock-inoculated control tea leaves at 4 dpi (CK1, CK2, CK3) and C. *gloeosporioides*–inoculated infected tea leaves at 4 dpi (CgIL4d1, CgIL4d2, CgIL4d3).

Length	CK1		CK2		CK3	
	Total (%)	Unique (%)	Total (%)	Unique (%)	Total (%)	Unique (%)
18	149,518 (1.66)	45,378 (1)	176,549 (2.23)	61,898 (1.76)	424,682 (6.88)	102,351 (4.65)
19	272,043 (3.02)	70,449 (1.56)	294,428 (3.71)	94,231 (2.68)	583,935 (9.46)	125,690 (5.71)
20	344,648 (3.83)	127,282 (2.81)	390,503 (4.92)	159,312 (4.52)	489,998 (7.94)	169,778 (7.72)
21	2,346,628 (26.09)	385,882 (8.52)	2,947,887 (37.15)	448,355 (12.73)	2,527,051 (40.94)	391,997 (17.82)
22	1,102,384 (12.25)	360,653 (7.96)	1,075,665 (13.56)	374,799 (10.64)	727,836 (11.79)	261,189 (11.87)
23	589,500 (6.55)	308,632 (6.82)	410,870 (5.18)	244,774 (6.95)	261,291 (4.23)	140,762 (6.4)
24	3,999,383 (44.46)	3,081,291 (68.05)	2,566,526 (32.35)	2,077,119 (58.98)	1,121,863 (18.17)	977,986 (44.45)
25	191,555 (2.13)	148,509 (3.28)	71,841 (0.91)	61,204 (1.74)	35,934 (0.58)	30,222 (1.37)
Clean reads	8,995,659 (100)	4,528,076 (100)	7,934,269 (100)	3,521,692 (100)	6,172,590 (100)	2,199,975 (100)
Length	**CgIL4d1**		**CgIL4d2**		**CgIL4d3**	
	Total (%)	Unique (%)	Total (%)	Unique (%)	Total (%)	Unique (%)
18	435,568 (9.64)	73,006 (4.85)	434,630 (9.81)	93,833 (6.29)	462,069 (6.04)	74,286 (3.3)
19	643,790 (14.25)	93,701 (6.22)	594,451 (13.41)	117,615 (7.89)	837,351 (10.95)	100,969 (4.48)
20	496,732 (11)	132,355 (8.78)	520,136 (11.73)	157,470 (10.56)	793,105 (10.37)	152,404 (6.77)
21	1,370,503 (30.34)	325,894 (21.63)	1,534,238 (34.61)	341,239 (22.89)	2,358,603 (30.83)	378,465 (16.8)
22	637,184 (14.11)	189,646 (12.59)	583,894 (13.17)	183,113 (12.28)	1,233,251 (16.12)	244,414 (10.85)
23	249,320 (5.52)	95,674 (6.35)	189,275 (4.27)	84,809 (5.69)	543,369 (7.1)	159,761 (7.09)
24	654,980 (14.5)	575,306 (38.18)	551,668 (12.45)	493,639 (33.11)	1,372,726 (17.94)	1,108,633 (49.21)
25	29,351 (0.65)	21,138 (1.4)	24,302 (0.55)	19,087 (1.28)	50,022 (0.65)	33,768 (1.5)
Clean reads	4,517,428 (100)	1,506,720 (100)	4,432,594 (100)	1,490,805 (100)	7,650,496 (100)	2,252,700 (100)

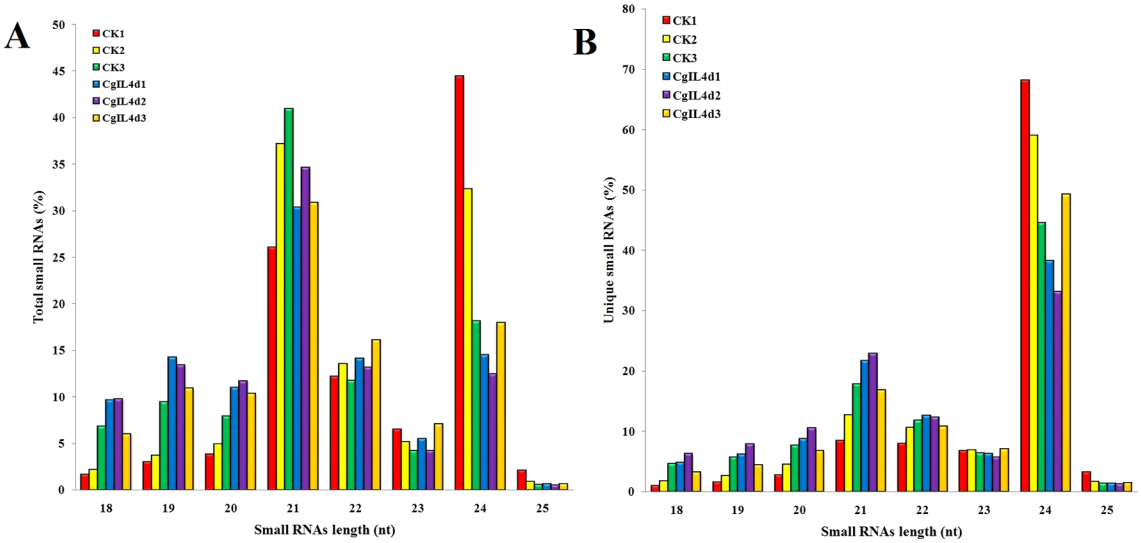

FIGURE 2 | The size distributions and abundances of small RNAs. **(A)** Percentage of total small RNA and **(B)** Percentage of unique small RNA.

Identification of Conserved miRNAs

To identify the conserved miRNAs in tea plant, the sRNA sequences were searched against known miRNAs in miRBase 22.0 by similarity with a maximum of two mismatches and without gaps. A total of 485 conserved miRNAs were found, which corresponded to 488 pre-miRNAs belonging to 89 miRNA families (**Supplementary Table 2** and **Figure 3A**). Among these, three conserved miRNAs, csn-miR166a-3p, csn-miR166a_R+2_2, and csn-miR396b_R+1_1, showed higher abundances of reads in the CK libraries than in the CgIL4d libraries; these may play a specific role during *C. gloeosporioides* infection. Moreover, miRNAs within several conserved miRNA families, such as miR171, miR164, miR394, miR482, and miR535, had abundances ranging from 1,000 to 79,000 in at least one CK or CgIL library. The remaining miRNAs had fewer than 1,000 reads within the six libraries (**Supplementary Table 2**). Differences were found in the number of members within conserved miRNA families. Among the identified families, miR169 and miR171 families contained the most members (29), followed by miR166, miR396, and miR167 families, which had 26, 22, and

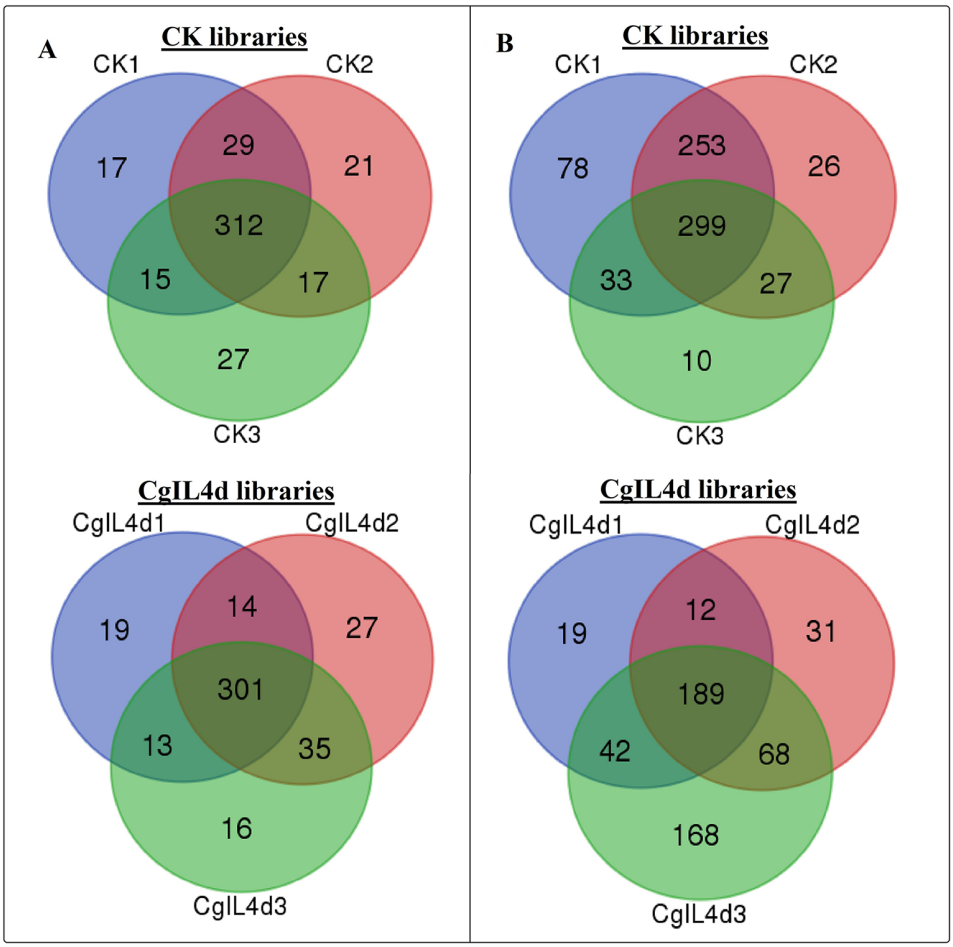

FIGURE 3 | Venn diagrams showing the numbers of conserved **(A)** and novel **(B)** miRNAs identified in six samples. (A) Ser/Thr-Kinase, (B) Auxin response factor 5, (C) NAC domain-containing protein 89, (D) Nuclear transcription factor Y subunit A-3, (E) Transcription factor MYB114, (F) Calcium-dependent protein kinase.

20 members, respectively. In contrast, the majority (38.20%) of miRNA families had only one member (**Figure 4**).

Identification of Novel miRNAs

To identify potentially novel miRNAs, reads that did not match any known miRNAs were further analyzed. All un-annotated unique sRNA sequences were mapped to the scaffold sequences of genome assembly. The stem-loop secondary structure of miRNA precursors was predicted using the mfold software (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (Zuker, 2003). According to aforementioned criteria, we identified 761 novel miRNAs corresponding to 488 pre-miRNAs

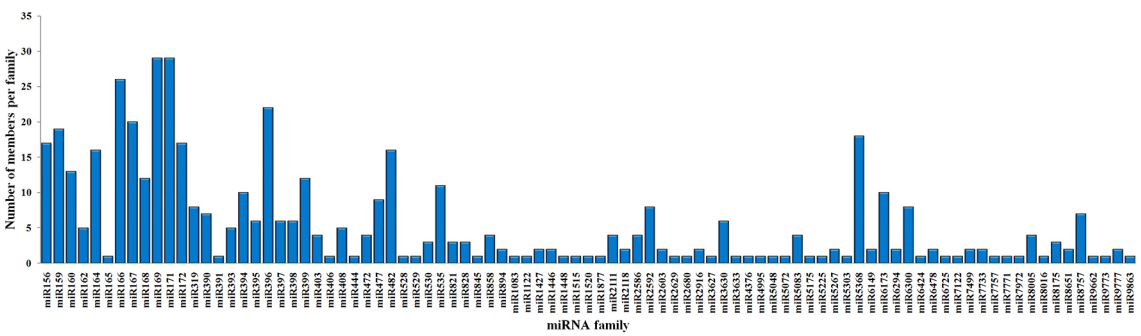

FIGURE 4 | Distribution of members in conserved miRNA families in *C. sinensis*.

in all six libraries (**Supplementary Table 3** and **Figure 3B**). The abundances of four novel miRNAs were significantly different between the CK and CgIL libraries; in addition, among 761 miRNAs, 726 and 529 miRNAs were only detected in the CK and CgIL libraries, respectively (**Supplementary Table 3**). These findings indicate that levels of certain novel miRNAs are specifically repressed by *C. gloeosporioides* stress. The PC-3p-29_74070, PC-3p-23_113175, PC-3p-69_25219, and PC-3p-721_2531 were the most abundant miRNA classes with more than 1,000 reads, and the remaining miRNAs had fewer than 1,000 reads in all six libraries (**Supplementary Table 3**). The sequences of novel miRNAs were 19 to 25 nt in length, and 24 nt reads were the most abundant among the six libraries, followed by 21, 22, 20, 23, 19, and 25 nt. The lengths of novel miRNA precursors ranged from 51 to 220 nt, with an average length of 136 nt. The negative folding free energies of the novel miRNA precursors ranged from −187.6 to −18.1 kcal mol^{-1}, with an average value of −102.85 kcal mol^{-1}. The folding structures of the predicted novel miRNAs are shown in **Supplementary Table 3**.

Differential Expression Analysis of miRNAs

To systematically identify *C. gloeosporioides* stress responsive miRNAs in tea plant, we identified DEMs with at least twofold change in abundance ($p < 0.05$) (**Supplementary Table 4** and **Figure 5**). In total, 88 conserved miRNAs were significantly up-regulated and 151 conserved miRNAs were significantly down-regulated in the CgIL libraries compared with the CK libraries. Of these miRNAs, csn-miR398a was the most significantly up-regulated miRNA (7.65-fold), while csn-miR171j-5p_2ss8CT9TC was the most significantly down-regulated miRNA (−6.28-fold) (**Supplementary Table 4** and **Figure 5**). We also identified 63 up-regulated novel miRNAs and 306 down-regulated novel miRNAs that showed significant differential expression between the CgIL and CK libraries. Of these miRNAs, the most up-regulated miRNA was PC-5p-6208_284 (3.43-fold), while the most down-regulated miRNA was PC-3p-3475_476 (−7.63-fold) (**Supplementary Table 5** and **Figure 5**). Two hundred thirty-six miRNAs (59 conserved and 177 novel miRNAs) were unique to the CK libraries, whereas 115 miRNAs (48 conserved and 67 novel miRNAs) were unique to the CgIL libraries; thus, these differentially expressed miRNAs might play crucial roles in response to *C. gloeosporioides* stress in tea plant.

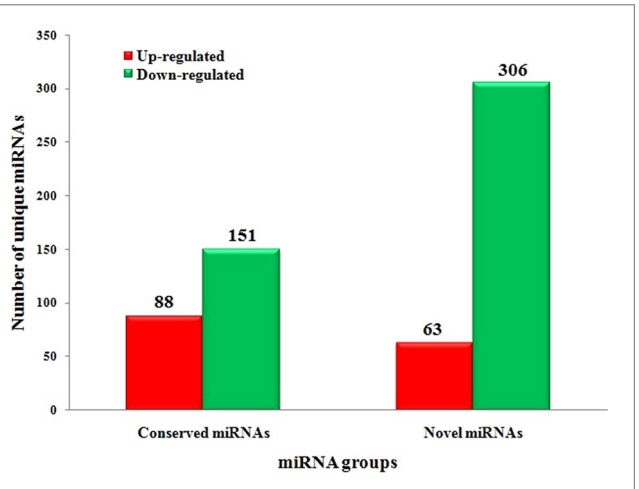

FIGURE 5 | Significantly differentially expressed conserved and novel miRNAs between CgIL4d vs. CK.

Identification of miRNA Targets

To explore the potential miRNA targets and their biological functions during *C. gloeosporioides* stress in tea plant, we performed genome-wide analysis of miRNA-cleaved mRNAs using high-throughput degradome sequencing technology (Addo-Quaye et al., 2008; Gao et al., 2004). A total of 18,056,656 and 14,212,790 reads were identified in the CK and CgIL libraries, respectively (**Table 3**); of these reads, 74.89% and 77.38% could be mapped to the *C. sinensis* genomic assembly, respectively (**Table 3**). Using the CleaveLand3.0 tool, a total of 1,134 conserved and 596 potentially novel miRNA targets were predicted in the degradomes of CK and CgIL, respectively. The abundances of targets were classified in accordance with previous criteria and are shown in **Supplementary Tables 6** and **7** (Li et al., 2010; Addo-Quaye et al., 2008). For CK targets, 205 (13.85%), 19 (1.28%), 551 (37.23%), 71 (4.80%), and 634 (42.84%) were grouped into categories 0, 1, 2, 3, and 4, respectively; meanwhile, for the CgIL targets, 125 (16.13%), 10 (1.29%), 313 (40.39%), 41 (5.29%), and 286 (36.90%) were grouped into categories 0, 1, 2, 3, and 4, respectively (**Supplementary Tables 6** and **7**). We further examined the abundance changes of targets with the treatment, which revealed that 1,487 and 775 targets had significantly changed sites with more than twofold change ($p < 0.05$) in CK and CgIL, respectively (**Supplementary Tables 6** and **7**). These results indicate that most of the predicted targets are efficiently cleaved by their corresponding miRNAs.

In the present study, 1,134 conserved and 596 potentially novel miRNA targets were predicted based on their perfect or near-perfect complementarity to their target gene sequences in the degradomes of CK and CgIL. Of these, 311 and 823 target transcripts were up- and down-regulated by 137 (82 up-regulated and 55 down-regulated miRNAs) and 230 (100 up-regulated and 130 down-regulated miRNAs) differentially expressed conserved miRNAs, respectively, while 131 and 465 targets were potentially up- and down-regulated by 79 (33 up-regulated and 46 down-regulated miRNAs) and 216 (67 up-regulated and 149 down-regulated miRNAs) differentially expressed novel miRNAs, respectively. We defined a predicted miRNA target gene when its expression pattern was in contrast with that of the miRNA, reflecting the fact that mRNA expression was negatively correlated with miRNA expression. Based on these rules, our results indicate that most of the predicted targets of conserved and novel miRNAs are highly negatively correlated with their corresponding miRNAs. These negative correlations positively regulate various metabolic, biological, and cellular processes.

Functional Analysis of miRNA Targets

Gene functional annotation analysis showed that the identified targets include transcription factors, such as auxin response factor (ARF), Myb domain proteins (MYBs), NAC domain transcription factors (NACs), nuclear transcription factor Y (NFYs), and WRKY family transcription factor (WRKYs), which regulate plant growth and development as well as stress responses (**Figure 6**, **Supplementary Tables 6** and **7**). Most of the target genes were protein-coding genes, including serine/threonine-protein kinase (Ser/Thr_kinase), leucine-rich repeat protein kinases (LRR-RLKs), calcium-dependent protein kinase (CDPK), mitogen-activated protein kinase (MAPK), and auxin-responsive protein, which are involved in signal sensing and transduction (**Figure 6**, **Supplementary Tables 6** and **7**). In addition, we identified several target genes related to plant defense, including L-ascorbate oxidase, cytochrome C oxidase, beta-glucosidase, glutathione synthetase, glutathione S-transferase, glutathione reductase, catalase, peroxidase, phenylalanine ammonia-lyase (PAL), and cinnamoyl-CoA reductase (CCR). The other predicted target genes like Calmodulin (CaM) like proteins and ROS appeared to be involved in diverse physiological and metabolic processes, such as plant metabolism, transport, and stress responses (**Supplementary Tables 6** and **7**). In our study, we found that csn-miR394b_L-1_1ss5CA was significantly down-regulated, and its targeted gene encodes CaM like proteins, which may cause influx of calcium after *C. gloeosporioides* infection in tea plant. In addition, csn-miR164a-p3/p5_2ss15CG18CG also targets CaM like proteins (**Supplementary Table 6**). Consistent with our results, Nischal et al. (2012) showed that miR164 inversely regulates the calmodulin-related calcium sensor protein in rice under abiotic stress. Calcium influx is triggered immediately by PAMP perception (Ranf et al., 2011). The extracellular calcium signals generated after pathogen attacks are transmitted to calmodulins (CaM) and calmodulin-binding proteins (CBP), which receive signals from the calmodulins, activate CDPKs, and trigger transcription of specific defense genes (Dodd et al., 2010). Studies have shown that overexpression of CBP in Arabidopsis causes elevated SA accumulation, increased expression of the defense genes, and enhanced resistance to *Pseudomonas syringae* (Wan et al., 2012). Our findings indicate that these miRNAs are likely involved in calcium activated SA biosynthesis in tea plant. We observed that CDPKs were targeted by two up-regulated miRNAs (csn-miR482b-p5_2ss3TA19T and PC-3p-134668_11) and one down-regulated miRNA (csn-miR169e_R-3) after *C. gloeosporioides* infection in *C. sinensis* (**Supplementary Tables 6** and **7**). This result suggested that these miRNAs are involved in protein kinase induced Ca^{2+} signal events, thereby triggering miRNA-mediated regulations.

The GO functional annotation of all predicted miRNA targets were conducted using Blast2GO program (**Supplementary Table 8** and **Figures 7A–C**). In the GO biological process category, the predominant terms were regulation of transcription (16.24%), response to stress (8.80%), biosynthetic processes (5.16%), and signaling pathway (3.66%) (**Figure 7A**). In the GO molecular function category, the most frequent term was enzyme activity (18.79%), followed by DNA binding (13.90%), transcription factor activity (11.97), and kinase activity (6.41) (**Figure 7B**). With regard to the GO cellular component category, nucleus, chloroplast, plasma membrane, and cytoplasm were the most frequent terms (**Figure 7C**). Moreover, a total of 122 KEGG pathways were enriched for the target genes of the 276 conserved and 253 novel differentially expressed miRNAs. Target genes within enriched KEGG pathways were mainly involved in 19 pathways. Among them, translation (14.20%), carbohydrate metabolism (12.95%), signal transduction (11.23%), energy metabolism (9.28%), and amino acid metabolism (6.85%) were the five major pathways. Furthermore, all the KEGG pathways were categorized into five groups including organismal system (5.40%), metabolism (52.80%), genetic information processing (25.99%), environmental information processing (11.70%) and cellular process (4.11%) (**Supplementary Table 9** and **Figure 8**).

Expression Analysis of miRNAs and Their Targets

To understand the physiological importance and the regulatory mechanisms of miRNAs in tea plant, the expression pattern of 12 randomly selected miRNAs and their target genes at different *C. gloeosporioides* inoculation time points (1, 4, 7, 10, and 13 days) was determined through qRT-PCR analysis (**Figure 9**). As shown in **Figure 9**, the qRT-PCR findings showed that the expression pattern of five miRNAs, csn-miR160c, csn-miR164a, csn-miR166g-5p_2ss5GA8GT, csn-miR828a, and csn-miR858b_R-3, were significantly higher at 7 dpi, while other five miRNAs, csn-miR169e, csn-miR399b_1ss21GA, csn-miR408-p3_2ss18GT19GT, csn-miR477g-p5, and PC-5p-80764_22, showed higher expression at 1 dpi. In addition, other two miRNAs, csn-miR156f-p3 and csn-miR398b, exhibited increased levels of expression at 4 and 10 dpi, respectively. On the other hand, the expression levels of eight miRNAs

TABLE 3 | Summary of degradome library data.

Library		Raw reads	Unique raw reads	cDNA mapped reads	Unique cDNA mapped reads	Number of input cDNAs	Number of covered cDNAs
CK (%)	Number	18,056,656	7,027,557	13,522,760	4,862,196	163,291	76,236
	Ratio	–	–	74.89 %	69.19 %	–	46.69 %
CgIL (%)	Number	14,212,790	4,314,069	10,998,394	2,953,113	163,291	61,860
	Ratio	–	–	77.38 %	68.45 %	–	37.88 %

CK, mock-inoculated leaves; CgIL, C.gloeosporioides-inoculated leaves.

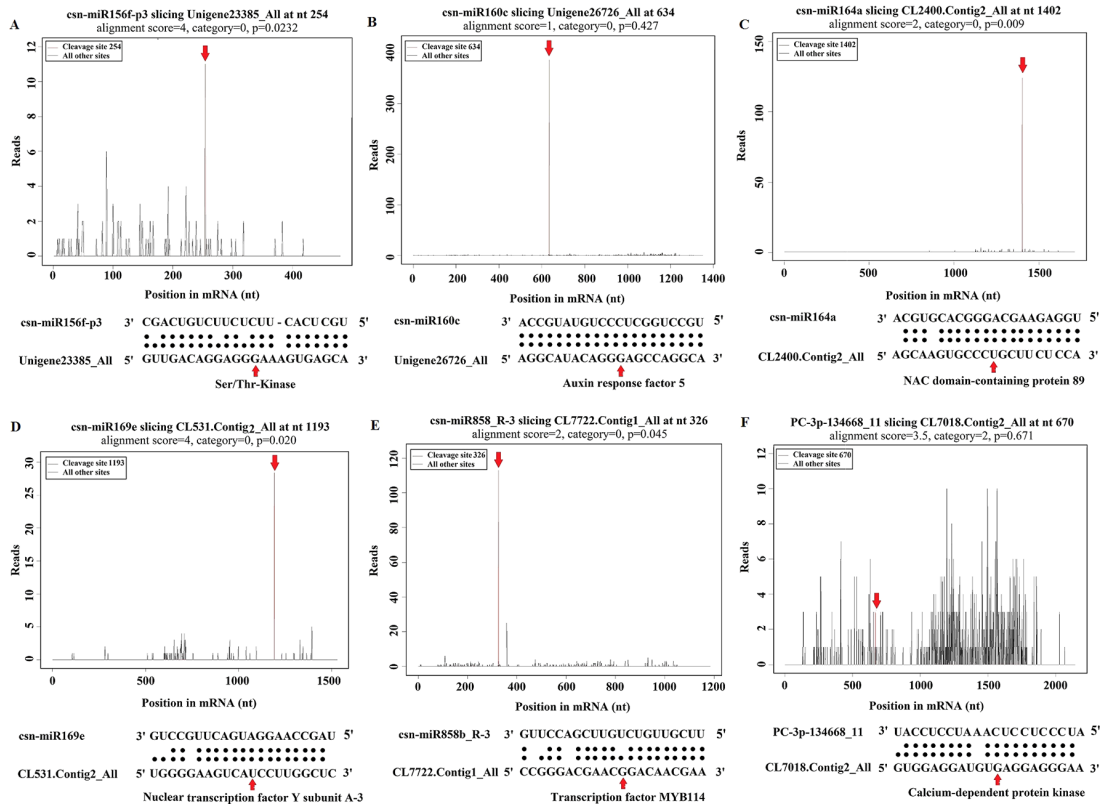

FIGURE 6 | Target plot (t-plot) of representative tea miRNA targets mined from degradome sequencing. **(A)** Ser/Thr-Kinase, **(B)** Auxin response factor 5, **(C)** NAC domain-containing protein 89, **(D)** Nuclear transcription factor Y subunit A-3, **(E)** Transcription factor MYB114, **(F)** Calcium-dependent protein kinase.

(csn-miR156f-p3, csn-miR160c, csn-miR164a, csn-miR166g-5p_2ss5GA8GT, csn-miR169e, csn-miR477g-p5, csn-miR828a, and PC-5p-80764_22) were significantly decreased in CgIL leaves at 13 dpi relative to the mock-inoculated CK control leaves. The opposite expression pattern between miRNAs and corresponding target transcripts were observed in CgIL leaves at different time intervals. For example, the expression levels of csn-miR160c, csn-miR164a, csn-miR477g-p5, csn-miR828a, and csn-miR858b_R-3 in CgIL leaves were decreased, while the expression levels of corresponding target transcript (ARF5, NAC89, PAL, MYB75, and WRKY) were increased at 10 to 13 dpi. In addition, the expression levels of csn-miR169e, csn-miR399b_1ss21GA, and csn-miR408-p3_2ss18GT19GT were significantly decreased in CgIL leaves relative to the control, while the increased expression was observed for their corresponding target transcripts (NFY-A3, LAO and Ser/Thr Kinase) at 1 to 4 dpi.

Correlation Analysis Between miRNA and Target Gene Expression

We have correlated the differential expression of 12 randomly selected miRNAs and their target genes. We found that csn-miR164a, csn-miR169e, csn-miR477g-p5, csn-miR828, csn-miR858b, and PC-5p-80764_22 were negatively correlated with their respective targets. The remaining six miRNAs partially showed positive correlation with their target genes. All the related target genes showing non-additive expression were also significantly enriched in "response to stress" and response to "biotic" in the present study, but negatively correlated gene targets were significantly enriched in the "metabolic process" category. We have also correlated the qRT-PCR expression of these 12 randomly selected miRNA-target genes. Interestingly, the expression of novel miRNA PC-5p-80764_22 exhibited a negative correlation with its target gene WRKY in tea plant samples throughout the entire period of infection (1 to 13 dpi). This shows that this novel miRNA regulates the WRKY gene throughout the infection. At 7 dpi, most of the miRNAs showed negative correlation with their respective targets. However, in general, the correlation between the expression of remaining 11 miRNAs and their respective target genes varied depending on the time points, indicating that additional factors may be involved in regulating these target genes and that miRNA-target gene interactions are extremely complex (**Table 4**).

Experimental Verification of miRNA-Guided Cleavage of Target mRNAs in Tea Plant

To examine the predicted targets of miRNAs, we used 5'RLM-RACE to validate the cleavage sites in the target mRNA. All the

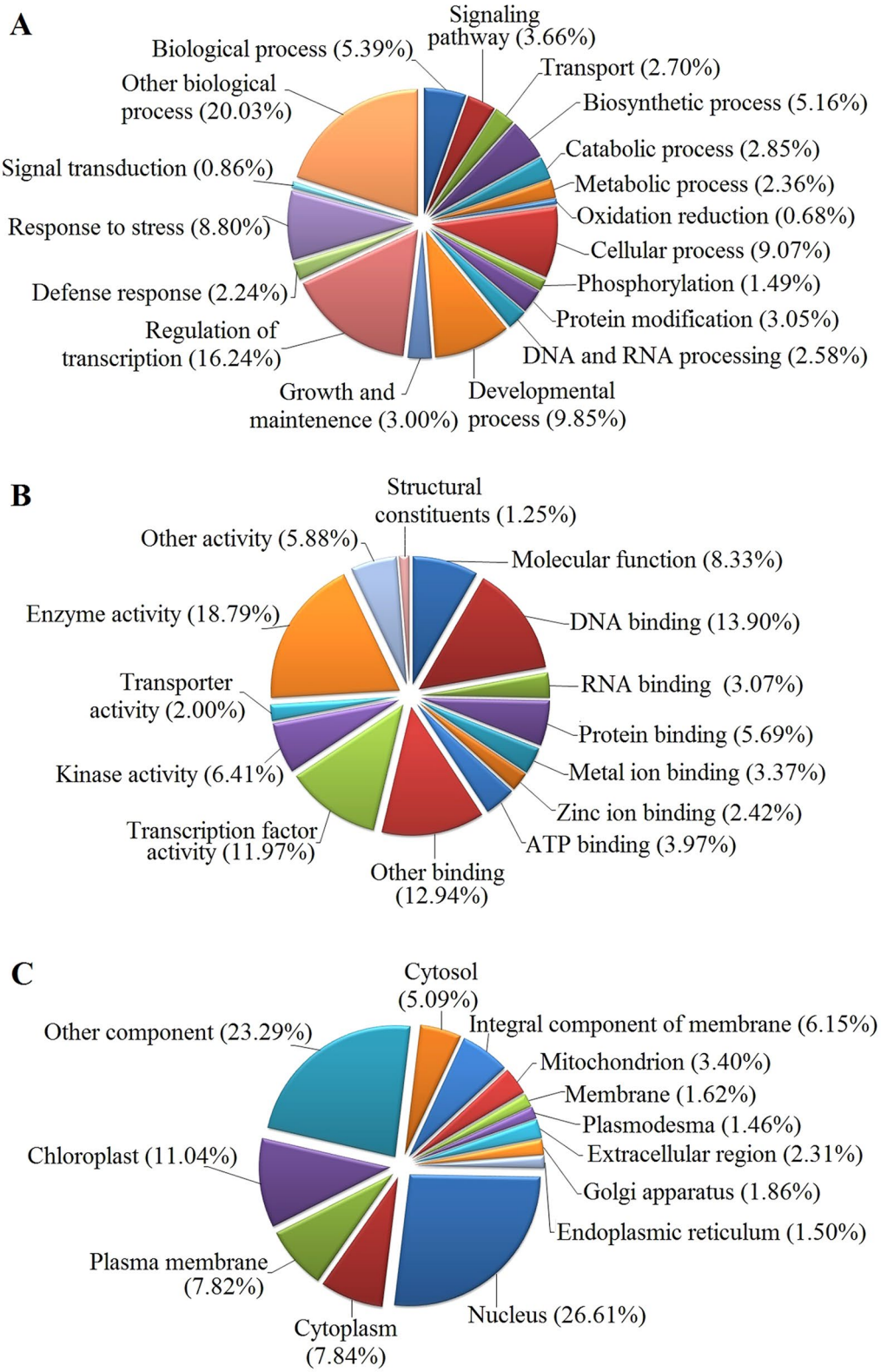

FIGURE 7 | GO analysis of predicted putative target transcripts of all miRNAs. Categorization of miRNA target transcripts was performed according to biological process **(A)**, molecular function **(B)**, and cellular component **(C)**.

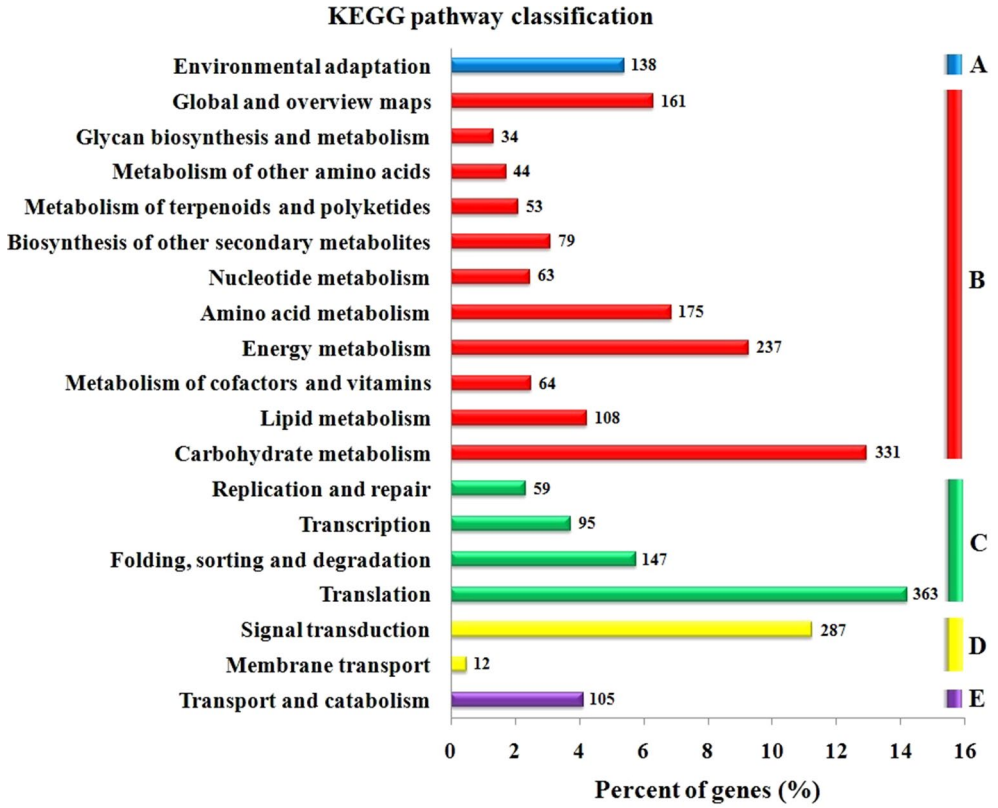

FIGURE 8 | KEGG pathway classification for predicted putative target transcripts of miRNAs between CgIL and CK. The KEGG pathways were categorized into five groups. **(A)** Organismal system, **(B)** metabolism, **(C)** genetic information processing, **(D)** environmental information processing, and **(E)** cellular process.

5'RLM-RACE PCR products amplified from predicted targets of five conserved miRNAs were analyzed on agarose gels, purified, cloned, and sequenced. The sequencing results revealed that the cleavage site of Ser/Thr-protein kinase (Unigene23385_All) and MYB75 (Unigene28964_All) lies between the 12th and 13th bases from the 5' end pairing of csn-miR408-p3_2ss18GT19GT and csn-miR828a, respectively. The cleavage site of ARF (Unigene26726_All) lies between the 11th and 12th bases from the 5' end pairing of csn-miR160c. NFY (CL531.Contig2_All) and MYB114 (CL7722.Contig1_All) were verified as targets of csn-miR169e and csn-miR858b_R-3, respectively. NFY can be regulated by cleavage in the binding region between the 10th and 11th bases from the 5' end pairing of csn-miR169e, and MYB114 can be regulated by cleavage in the binding region between the 8th and 9th bases from the 5' end pairing of csn-miR858b_R-3 (**Figure 10**). These validated sites were consistent with the predicted sites.

Hypothetical Model of miRNA-Mediated Regulation

Briefly, the regulatory network, functional sharing of putative target genes, and expression profile data were used to construct a hypothetical model that illustrates how target genes influence *C. gloeosporioides* pathosystem at the biological level (**Figure 11**). Many conserved and novel miRNAs found in this study were associated with responses to various abiotic and biotic stresses, respectively. In this order, target genes of eight conserved miRNAs (csn-miR8757b, csn-miR396b, csn-miR408, csn-miR398a, csn-miR166e, csn-miR8005a, csn-miR821b, and csn-miR477) and three novel miRNAs (PC-3p-58653_32, PC-3p-180347_8, and PC-3p-184859_7) were confirmed to regulate various antioxidant genes and intensely induced H_2O_2. All these miRNAs may correlate with pathways associated with reactive oxygen species (ROS) and play important roles in stress responses mediated by glycosylating hormones and secondary metabolites. Apart from this, target genes of miR393, miR160, and miR167 are known to regulate downstream gene expression of auxin pathway *via* regulating transport inhibitor response 1 (TIR1) and ARF in response to stress factors and components of stress signal transduction pathways. In contrast, transcription factor genes MYB, NAC, and NF-YA controlled by miRNAs (csn-miR828a, csn-miR858b, csn-miR858a, csn-miR6300, csn-miR164a, and csn-miR169) are identified to be a positive regulator of abiotic and biotic stress response. Most conserved signaling cascade pathway (MAPKs) linked with disease resistance *via* SA signaling pathway was mainly targeted by csn-miR6173 and PC-5p-167506_9. In addition, csn-miR159a

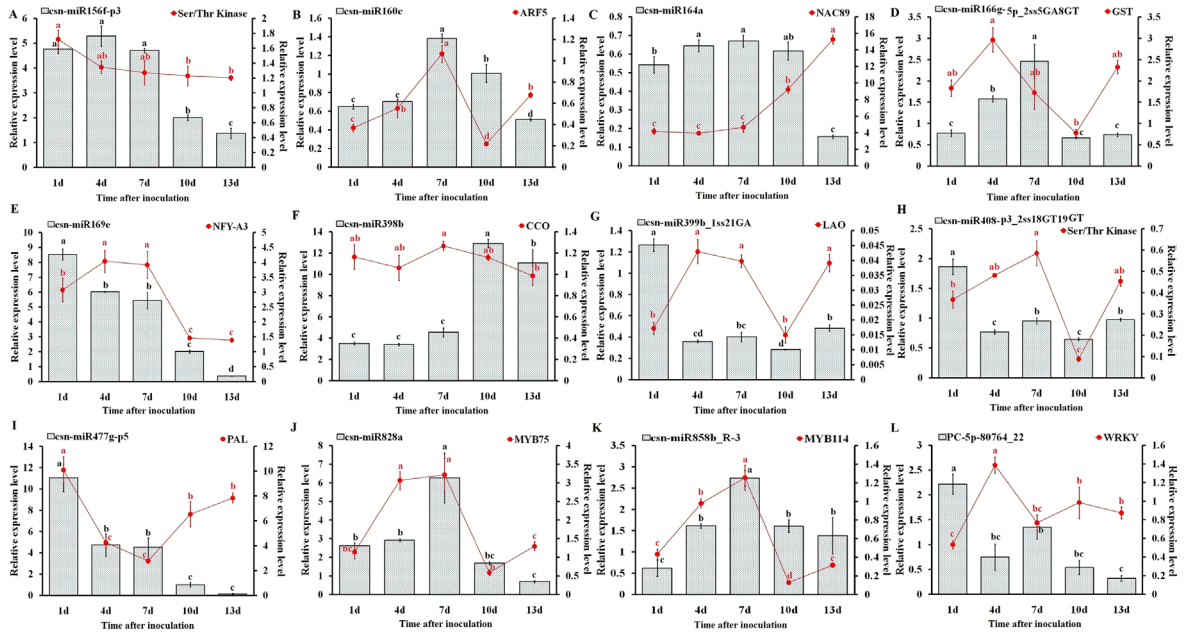

FIGURE 9 | Expression analysis of miRNAs and target genes (A–L). The bars and lines represent the abundances of miRNAs and their corresponding targets in *C. sinensis* leaves exposed to *C. gloeosporioides* at different time intervals by qRT-PCR: csn-miR156f-p3 **(A)**, csn-miR160c **(B)**, csn-miR164a **(C)**, csn-miR166g-5p_2ss5GA8GT **(D)**, csn-miR169e **(E)**, csn-miR398b **(F)**, csn-miR399b_1ss21GA **(G)**, csn-miR408-p3_2ss18GT19GT **(H)**, csn-miR477g-p5 **(I)**, csn-miR828a **(J)**, csn-miR858b_R-3 **(K)**, and PC-5p-80764_22 **(L)**. The Y-axis on the left and right indicate the expression levels of the miRNAs and targets, respectively. U6 SnRNA and GADPH were used as an internal control for miRNAs and targets, respectively. The expression levels of the miRNAs and targets in the control tea plant leaves at different time points (1, 4, 7, 10, and 13 dpi) were set as 1.0. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data represent the mean ± SD values of three biological replicates. Different letters above the bars represent significant differences at $p < 0.05$. Means followed by the same letter over the bars are not significantly different at the 0.5% level according to DMRT analysis.

TABLE 4 | Correlations between the expression of 12 tea miRNAs and their target genes during *C. gloeosporioides* infection.

miRNA/Target	Leaf (r values)				
	1 dpi	4 dpi	7 dpi	10 dpi	13 dpi
csn-miR156f-p3/ STK1	**0.94**	**0.88**	-0.63	**0.99**	**0.46**
csn-miR160c/ARF	**0.50**	-0.97	-0.68	-0.58	-0.31
csn-miR164a/NAC	-0.41	-0.21	**0.92**	-0.27	-0.29
csn-miR166g-5p/GST	**0.95**	**0.90**	-0.04	**0.85**	**0.24**
csn-miR169e/NFY	-0.89	**0.49**	**0.92**	-0.24	-0.89
csn-miR398b/Cox	**0.61**	-0.81	-0.58	**0.62**	-0.25
csn-miR399b/LAO	**0.50**	**0.82**	-0.39	**0.47**	-0.53
csn-miR408-p3/STK2	-0.97	**0.70**	-0.36	-0.22	**0.99**
csn-miR477g-p5/PAL	-0.99	**0.44**	**1.00**	-0.92	**0.84**
csn-miR828a/MYB75	**0.95**	**0.71**	-0.49	-0.99	-0.87
csn-miR858b_R-3/MYB114	**0.57**	**0.61**	-0.33	**0.94**	**0.91**
PC-5p-80764_22/WRKY	-0.95	-0.94	-0.96	-0.68	-0.92

"1 dpi, 4 dpi, 7 dpi, 10 dpi, 13 dpi" indicate the number of days post inoculation; "values bolded with +" indicate positive correlation, "values with -" indicate negative correlation; "r" indicates correlation coefficient values. Results of expression correlation between miRNA and its target genes during CgIL infection were analyzed by Pearson's correlation analysis.

targeted a transcriptional activator of pathogenesis-related (PR) genes (PTI6), which controls the SA-mediated pathway associated with programmed cell death during the hypersensitivity response. The target genes of three conserved miRNAs such as miR396, miR5368, and miR156a and four novel miRNA, namely PC-5p-80764_22, PC-3p-1581_1049, PC-3p-106557_16, and PC-3p-70583_26, may belong to WRKY transcription factor, LRR protein kinase, Ser/Thr-kinase, and LRR receptor, which are well-known to play important roles in defense responses and disease resistance in plant–pathogen interactions. The target genes of csn-miR164a and csn-miR168 are a beta-glucosidase that triggers a plant-defense-related product in response to fungal pathogens. csn-miR394 and csn-miR164a target genes encode CaM like proteins, which receive signals from the calmodulins and lead to calcium activated SA biosynthesis through CDPKs and trigger transcription of

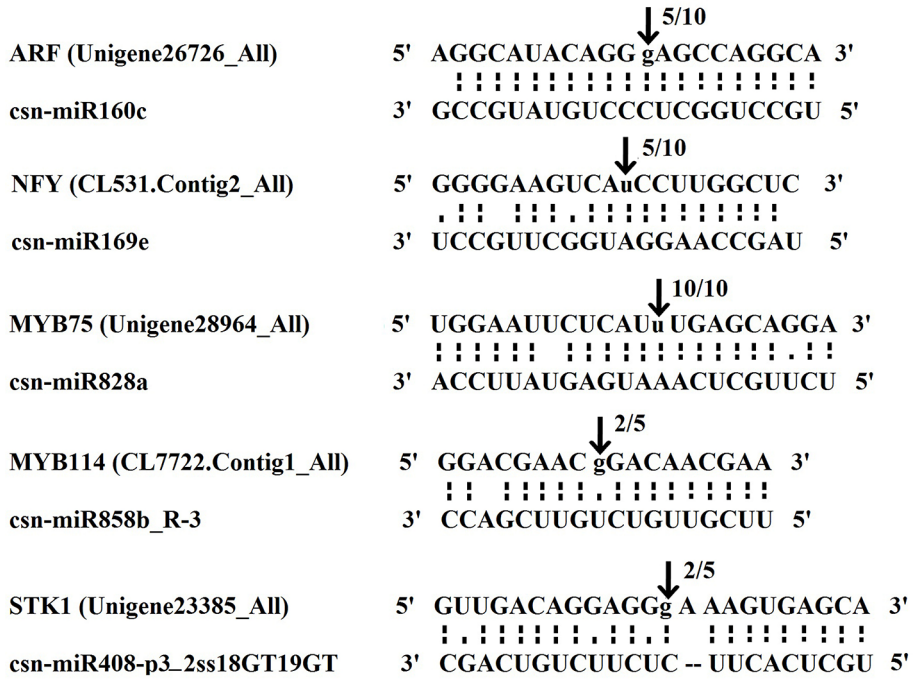

FIGURE 10 | The mRNA cleavage sites of miRNAs identified by 5' RLM-RACE. Each top strand depicts a miRNA complementary site, and each bottom strand depicts the anti-parallel miRNA. The arrows indicate the 5' termini of mRNA fragments isolated from tea plant, as identified by the RLM-RACE product, with the frequency of clones shown above. The numbers indicate the fraction of cloned PCR products terminating at different positions.

specific defense genes (Dodd et al., 2010). The hypothetical model reveals that association of miRNAs and target genes can influence different metabolic pathways and cellular processes, which leads to various biological responses. All these mutual effects allow tea plant to be susceptible with *C. gloeosporioides* infection-related stress at the biological level (**Figure 11**).

DISCUSSION

During plant fungal infection, miRNAs are thought to be involved in both PTI and ETI mechanisms to regulate various gene expressions (Weiberg et al., 2014). Anthracnose disease caused by *C. gloeosporioides* is one of the most destructive fungal diseases in tea plant (Fang et al., 2013). Little is known about the roles of miRNAs in tea plant under biotic stress of *C. gloeosporioides* infection, although miRNAs and their target genes have been identified in tea plant during abiotic stresses such as cold and drought (Zhang et al., 2014; Liu et al., 2016). Here, we identified *C. gloeosporioides*-responsive miRNAs and investigated their target mRNAs using high throughput sequencing in tea plant.

We identified miRNAs consisting of 485 conserved and 761 novel miRNAs in this study. Among these, some of the conserved miRNA families are of interest and have been reported to be involved in biotic stress responses. For example, miR159, miR164, miR166, miR168, miR171, miR394, miR396, miR482, and miR535 showed higher abundances, while other miRNA families (miR156, miR160, miR169, miR319, miR393, miR395, miR396, and miR408) showed lower abundances after CgIL treatment. Previous reports revealed that miR156, miR159, miR160, miR164, and miR171 families are involved in response to *Pseudoperonospora cubensis* infection in cucumber (Jin and Wu, 2015), while miR156 and miR408 are involved in the response to viral infection in tabacum (Chen et al., 2017). In addition, some miRNA families such as miR159, miR164, miR169, and miR171 play important roles in regulation of the basal defense response during leaf rust and powdery mildew infection in wheat (Kumar et al., 2017). Thus, we proposed that these miRNA families might play a role in the response to *C. gloeosporioides* infection in tea plant.

Among the identified 239 known and 369 novel DEMs under *C. gloeosporioides* stress in this study, the majority were down-regulated, while others were induced under *C. gloeosporioides* stress. Many conserved miRNAs in our study showed similar expression patterns in response to disease infection across different plant species, suggesting functional conservation in this particular biological process. For instance, the expression levels of miR156, miR159, miR164, miR168, miR172, miR393, miR396, miR398, miR403, and miR472 were significantly altered in tea plant after infection; expression levels of these miRNAs also changed in response to fungal infection in cotton, rice, wheat, and pine (Xin et al., 2010; Yin et al., 2012; Li et al., 2014; Lu et al., 2007). Notably, our study initially identified some conserved miRNAs, which were responsive to *C. gloeosporioides* in tea plant, such as csn-miR3630-3p, csn-miR5368-p5, csn-miR7733, csn-miR7771-p5, and csn-miR9777. These miRNAs

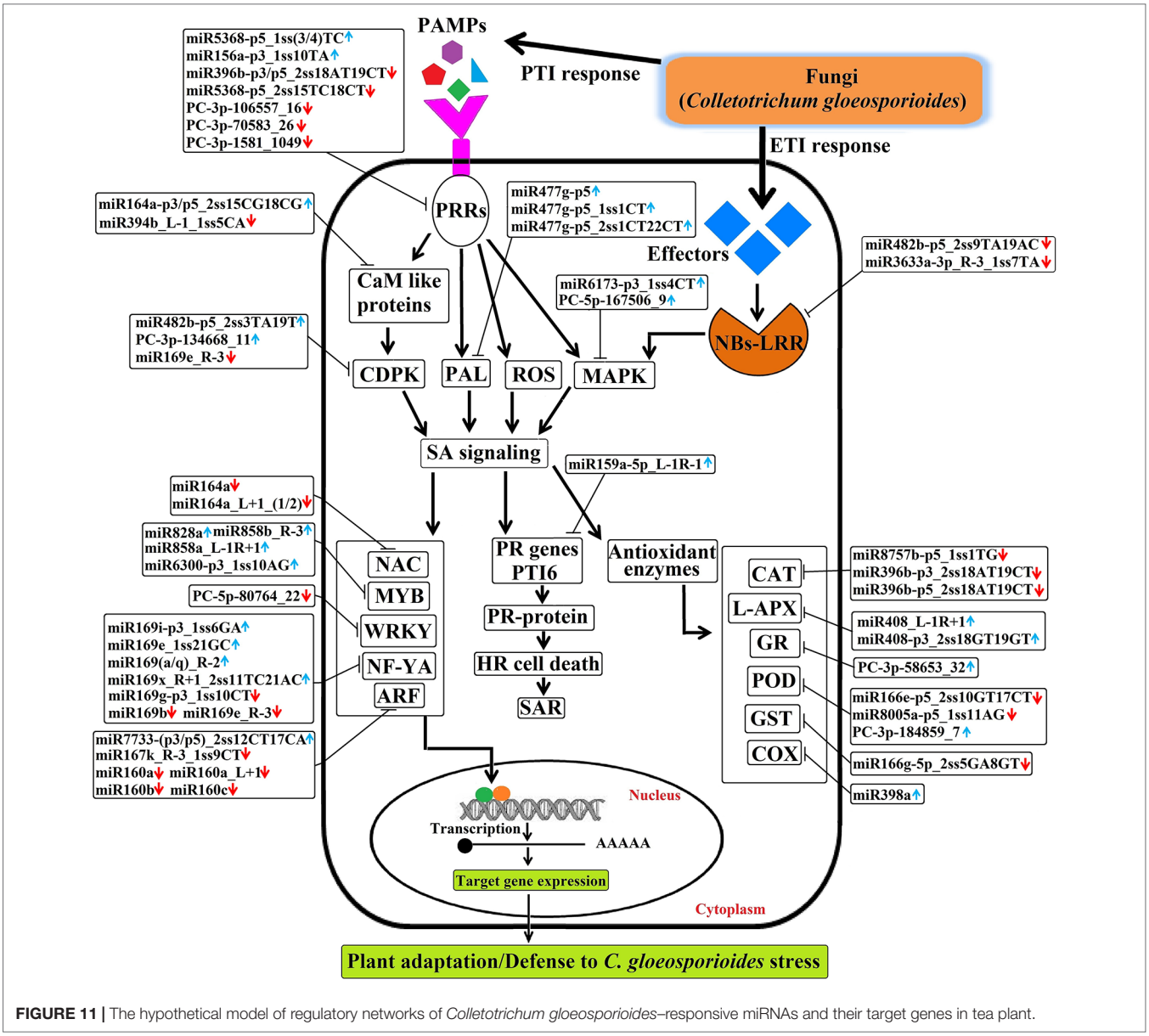

FIGURE 11 | The hypothetical model of regulatory networks of *Colletotrichum gloeosporioides*–responsive miRNAs and their target genes in tea plant.

may contribute to biotic stress response in tea plant and are of interest for further investigation.

C. gloeosporioides–responsive miRNAs Targeting Defense Responsive Genes

Plants conflict with the majority of the invaders like fungi and virus by activating intricate immune responses, which usually result in a disease resistance response. On the other hand, pathogens have normally developed habits to bypass plant defenses, and susceptibility to pathogens reappears. In addition to this random immune failure of the host, other immune-response independent processes allow further entry of the invading pathogen and contribute to plant pathogen susceptibility (Dobon et al., 2015). In this study, we found over 1,500 miRNA targets with diverse functions in response to *C. gloeosporioides*. For a better understanding, we propose a regulatory network in tea plant, derived from integrating our results with previous findings (**Figure 11**). Being a hemibiotrophic fungi, *C. gloeosporioides* initially act as biotrophic phase during which the host's immune system and cell death are actively suppressed and allow invasive hyphae to spread throughout the infected tea plant tissues. This is followed by a necrotrophic phase during which toxins are secreted by the pathogen to induce host cell death and create tea plant susceptibility to this fungi. Time-course experiments carried out by Vargas et al. (2012) also revealed the biotrophic growth in susceptible maize leaves; the hemibiotrophic fungus *Colletotrichum graminicola* induced classical plant defense responses, such as the accumulation of ROS and phenolic compounds. They hypothesized that it is the switch to necrotrophy that enables the fungus to evade the plant immune system and allows pathogenicity.

Characteristic fungus–plant interfaces that accumulate effectors in biotrophy were previously reported in hemibiotrophs, the rice blast fungus *Magnaporthe oryzae* (Mosquera et al., 2009; Khang et al., 2010), and the crucifer anthracnose fungus *Colletotrichum higginsianum* (Kleemann et al., 2012). During the biotrophic phase, fungus must obtain nutrients from living host cells and tissues and secrete limited amounts of effectors to suppress the host immune system. PTI is the first layer of innate immunity and is initiated by plants in response to pathogen infection (Stergiopoulos and de Wit, 2009). C. *gloeosporioides* species exhibits a broad geographic range and has the capacity to cause severe damage on plants, resulting in large economic losses in agriculture (Rabha et al., 2016). Generally, *Colletotrichum* sp. delivered effector to the host was tightly coupled to early biotrophic expression of effector genes, which implies biotrophy-associated functions, such as the maintenance of biotrophy and/or suppression of postinvasion defenses until the switch to necrotrophy (Irieda et al., 2016). Likewise, during initial infection, *C. gloeosporioides* synthesize and secrete effector proteins, which actively pass signals to host. Activation of PRRs, such as leucine rich repeat (LRR) protein kinase, serine/threonine (Ser/Thr) protein kinase, and LRR receptor like Ser/Thr protein kinase by PAMPs, leads to phosphorylation and activation of LRR; this in turn leads to activation of a number of downstream signaling components for first line of PTI defense signaling (Jones and Dangl, 2006). Interestingly, our results showed that the LRR protein kinase can be targeted by csn-miR396b-p3/p5_2ss18AT19CT (down-regulated) and csn-miR5368-p5_1ss(3/4)TC (up-regulated). The Ser/Thr protein kinase can be targeted by three down-regulated miRNAs (csn-miR5368-p5_2ss15TC18CT, PC-3p-106557_16, and PC-3p-70583_26), and LRR receptor like Ser/Thr protein kinase can be targeted by csn-miR156a-p3_1ss10TA (up-regulated) and PC-3p-1581_1049 (down-regulated) after *C. gloeosporioides* infection in tea plant (**Supplementary Tables S5** and **S6**). Therefore, we provide evidence that these miRNAs function to target LRR protein kinase, Ser/Thr-kinase, and LRR receptor like Ser/Thr protein kinase receptors, thereby triggering a first line of PTI defense. The immune system enables the perception of the pathogen attack as the infection process starts, being the expression of susceptibility conditioned by the magnitude and/or timing of defense responses.

Plant susceptibility to hemibiotrophs is a genetically complex process and comprises the coordinated action of a wide range of hormones, including ET, JA, SA, and abcisic acid (ABA) (Thomma et al., 1998; Berrocal-Lobo et al., 2002; Adie et al., 2007; García-Andrade et al., 2011). Study by Diniz et al. (2017) has shown that there is a stronger activation of ERF1 gene at the beginning of the necrotrophic phase of hemibiotrophic fungi *Colletotrichum kahawae* in the susceptible variety of *Coffea arabica*, which suggests the involvement of ET in tissue senescence. ET pathway activation in the susceptible variety may be related with tissue damage promoted by the fungal necrotrophic phase. In this study, we found that a novel miRNA (PC-5p-66414_28) significantly targets ET insensitive 3, which activates the ET pathway; thereby we suggest that in later infection stages, *C. gloeosporioides* might deliver effectors or phytotoxins that manipulate the tea plant to produce ET for entering the necrotrophic stage of infection and therefore overcome plant defenses.

As a consequence of the biotrophic phase, fungi that require living host tissues fail to survive and infect plant. So, later, these fungi enter the necrotrophic phase, which mainly depends on ETI. ETI is triggered by the recognition of pathogen effectors by plant R proteins that leads to a hypersensitive response (HR) and localized host cell death (Jones et al., 2006). The largest class of R proteins belongs to the conserved family of nucleotide binding site LRR (NBS-LRR) proteins (Tameling and Takken, 2007). Rice blast fungus (*M. oryzae*) effectors are recognized by two divergently transcribed NBS-LRR genes, RGA4 and RGA5, which impart resistance against *M. oryzae* infection in rice (Cesari et al., 2013). The expression of tomato miR482, which targets some NBS-LRR protein genes, suppresses bacterial and viral pathogens (Shivaprasad et al., 2012). Notably, we identified NBS-LRR in tea plant as a potential target of csn-miR482b-p5_2ss9TA19AC and csn-miR3633a-3p_R-3_1ss7TA (**Supplementary Table 6**). We also found that more miRNAs are targeting the HR related PTI6 protein. HR at the site of infection also activates systemic acquired resistance (SAR), which provides protection against pathogens throughout the plant (Durrant and Dong, 2004; Chen et al., 2017). SAR requires the signal molecule SA, which induces the accumulation of PR proteins (Van Loon and Van Strien, 1999). Nonexpresser of PR gene1 (NPR1) is the central regulator of the SA signaling pathway and functions as a co-activator for SA-responsive genes. NPR1 monomers are then translocated into the nucleus where they interact with TGA-bZIP transcription factors (Zhou et al., 2000), leading to an activation of SA-dependent gene expression (Fan and Dong, 2002). In our study, we found that the transcriptional activator of PR genes, PTI6, was targeted by an up-regulated conserved miRNA (csn-miR159a-5p_L-1R-1) (**Supplementary Table 6**). Based on this result, we suspect that the PR gene transcription activator targeted by this miRNA should regulate PR genes, which encode antimicrobial proteins; thereby it is suggested that *C. gloeosporioides* enter the necrotrophic phase and activate R protein-mediated ETI to cause HR cell death, which leads to effector-triggered susceptibility (ETS). Our results emphasize the hypothesis that a cell wall integrity surveillance system evolved to sense the presence of a pathogen and to transduce signals into a rapid transcriptional reprogramming of the affected cell. This transcriptional reprogramming might serve to promote fungal growth to facilitate susceptibility of tea plant.

C. gloeosporioides–Responsive miRNAs Targeting Transcription Factors

MiRNAs have important functions in plant stress responses by targeting multiple transcription factors, which, in turn, regulate the expression of various downstream genes involved in the stress response (Shukla et al., 2008). In our study, we found that some miRNAs and their corresponding target genes are involved in the regulation of transcription factors, hormone signaling, and crosstalk between defense-related signaling hormones (ABA, SA, JA, ET, and auxin) as well as phenylpropanoid biosynthesis; this is consistent with previous studies, which showed that SA signaling is an integral part of both the PTI and ETI defense responses (Yi et al., 2014, Chen et al., 2017). We also found that miRNAs target multiple

transcription factors such as MYB, WRKY, NAC, and NF-YA. MYB is a transcription factor family in plants, which is involved in the regulation of a wide range of molecular events (Ambawat et al., 2013). Many studies suggest that PAMP triggers MYB induction, which would result in SA accumulation (Lee et al., 1995; Seo and Park, 2010). Our results showed that csn-miR828a, csn-miR858b_R-3, csn-miR858a_L-1R+1, and csn-miR6300-p3_1ss10AG target the MYB transcription factor, which is consistent with previous findings for heat response in cotton (Guan et al., 2014; Wang et al., 2016). WRKY is another well-known transcription factor family in plants, which is involved in various stress response networks (Banerjee and Roychoudhury, 2015). We found that WRKYs were targeted by down-regulation of the novel miRNA PC-5p-80764_22. NAC and its family members act as major transcriptional regulators of plant development and plant responses to biotic and abiotic stresses (Feng et al., 2014). Recently, the miR164 family was shown to target six NAC family members under various conditions of stress in several plant species (Bhardwaj et al., 2014; Feng et al., 2014). In addition, Jeyaraj et al. (2017) found that miR164 mainly targets NAC genes in response to biotic stress (geometrid attack) in tea plant; in accordance, we found that down-regulation of csn-miR164a and csn-miR164a_L+1_(1/2), which target NAC genes, was involved in the regulation of NACs. Members of the nuclear factor Y (NF-YA) transcription factor family have been reported to be key regulators of plant development, phytohormone signaling, and drought tolerance (Ren et al., 2016). Recently, Luan et al. (2015) found that the miR169 family members regulate the expression of NF-YA genes *via* transcript cleavage in response to abiotic stress in maize leaves. In addition, Ni et al. (2013) reported that NF-YA, a target gene of miR169, is a positive regulator of plant tolerance to drought stress. Interestingly, we observed significant up-regulation of csn-miR169i-p3_1ss6GA, csn-miR169e_1ss21GC, csn-miR169(a/q)_R-2, and csn-miR169x_R+1_2ss11TC21AC, as well as significant down-regulation of csn-miR169g-p3_1ss10CT, csn-miR169b, and csn-miR169e_R-3 under *C. gloeosporioides* stress in tea plant.

Some highly conserved pathogen-responsive miRNAs, including miR393, miR160, and miR167, play important roles in regulating perception and signaling of auxin (Yang et al., 2013). miR393 down-regulates transport inhibitor response 1 (TIR1) and represses transcription of auxin binding F-box proteins (Navarro et al., 2006), while miR160 and miR167 down-regulate five different ARF transcripts by guiding cleavage of their cognate mRNAs (Yang et al., 2013). It was previously demonstrated that many types of stresses, including bacterial and fungal infection, could up-regulate miR393 and repress auxin signaling by keeping TIR1 at a low level, thereby increasing AUX/IAA-ARF heterodimerization (Sunkar et al., 2012). However, we observed that csn-miR393a targeting TIR1 was down-regulated while ARF was targeted by an up-regulated miRNA [csn-miR7733-(p3/p5)_2ss12CT17CA] and five down-regulated miRNAs (csn-miR167k_R-3_1ss9CT, csn-miR160a, csn-miR160a_L+1, csn-miR160b, and csn-miR160c) after *C. gloeosporioides* infection. Thus, we speculated that miR393, miR160, and miR167 may work together, but may also involve other regulators to regulate the auxin pathway in tea plant during *C. gloeosporioides* infection.

C. gloeosporioides–Responsive miRNAs Involved in MAPK Activation

The MAPK pathway is one of the most conserved signaling cascade pathways in plants and regulates a plethora of cellular processes including normal growth and development and plant defense responses against abiotic and biotic stresses (Nakagami et al., 2005). The PAMP receptor LRR protein kinase and ROS production cause downstream activation by phosphorylating a complete MAPK cascade (Suarez-Rodriguez et al., 2007). Thereafter, the activated MAPK regulates stress responses by phosphorylation of specific MYB and WRKY transcription factors (Andreasson et al., 2005). SA and various pathogen-derived elicitors were shown to induce the MAPKs and SA-induced protein kinase (SIPK) (Zhang and Klessig, 1997). Expression of constitutively active forms of MAPK and SIPK leads to multiple gene expression and HR-like cell death (Yang et al., 2001). Studies have reported that the silencing of MAPK attenuates plant resistance to bacteria and virus pathogen (Jin et al., 2002; Liu et al., 2004). Interestingly, it was observed that miR531 targets most members of the MAPK cascade gene family in *Oryza sativa* (Raghuram et al., 2014). We revealed in our study that csn-miR6173-p3_1ss4CT and PC-5p-167506_9 were up-regulated and targeted the MAPKs (**Supplementary Table S6 and S7**). This finding suggests that these miRNAs are involved in the MAPK induced signaling cascade, thereby triggering the miRNA-mediated transcriptional regulation of WRKYs and MYB against *C. gloeosporioides* stress in tea plant.

C. gloeosporioides–Responsive miRNAs Regulates Other Targets

Apart from various transcription factor genes, signal transducing genes, and kinases, *C. gloeosporioides* also target the ROS scavenging system in tea plant. Rapid production of ROS is the early change associated with pathogen infection in plants, which causes the development of the antioxidant system through a group of enzymes as well as non-enzymatic components, such as ascorbate and glutathione (Silveira et al., 2015). Increased activities of antioxidant enzymes under pathogen attacks are observed in several crops, such as sorghum (*Sorghum bicolor*), soybean (*Glycine max*), cucumber (*Cucumis sativus*), and common bean (*Phaseolus vulgaris*) (Datnoff et al., 2007; Resende et al., 2012; Polanco et al., 2014). We observed that among the reported genes encoding antioxidant enzymes, catalases isozyme1 (*CAT*) was targeted by three down-regulated miRNAs (csn-miR8757b-p5_1ss1TG, csn-miR396b-p3_2ss18AT19CT, and csn-miR396b-p5_2ss18AT19CT). *L-APX*, which encodes L-ascorbate peroxidase, was targeted by two up-regulated miRNAs (csn-miR408_L-1R+1 and csn-miR408-p3_2ss18GT19GT). *GR*, encoding glutathione reductase, was targeted by an up-regulated miRNA (PC-3p-58653_32) (**Supplementary Tables 6** and **7**). *GST*, encoding glutathione S-transferase, is known to play a key role in ROS detoxification and reduction in response to oxidative burst after pathogen infection (Bhattacharjee, 2012). Consistent with earlier results, the present study observed that csn-miR166g-5p_2ss5GA8GT, the miRNA that targets *GST*, was down-regulated. *COX*, which encodes cytochrome c oxidase, is a crucial component of the mitochondrial

respiratory chain and is of utmost importance for providing cellular energy (Radin et al., 2015). A previous study reported that miR398 targets *COX* subunit V and helps plants to cope with various stresses (Sunkar and Zhu, 2004). Here, we found that *COX* subunit V was targeted by csn-miR398a (up-regulated); peroxidase was targeted by three conserved miRNAs (csn-miR166e-p5_2ss10GT17CT, csn-miR8005a-p5_1ss11AG, and csn-miR821b-p5_2ss14TG20AT) and two novel miRNAs (PC-3p-180347_8 and PC-3p-184859_7) (**Supplementary Tables 6** and **7**). Therefore, we inferred that miRNAs regulate antioxidant genes that are involved in the ROS responses in tea plant against *C. gloeosporioides* infection.

Apart from these, nitric oxide activates the SA biosynthesis pathway by inducing phenylalanine ammonia lyase (PAL), which is a key enzyme in biosynthesis of SA. PAL is one of the most extensively studied enzymes in the response pathways of plant biotic and abiotic stress (MacDonald and D'Cunha, 2007). Saravanakumar et al. (2007) have shown that the accumulation of peroxidase and PAL increases in response to pathogen infection in the tea plant. Studies have shown that *C. gloeosporioides* and *Colletotrichum camelliae* infection in various plants up-regulated the expression of PAL (Wang et al., 2016). In this study, we found that the expression of PAL was highly regulated by the miR477 family (csn-miR477g-p5, csn-miR477g-p5_1ss1CT, and csn-miR477g-p5_2ss1CT22CT) after *C. gloeosporioides* infection in tea plant (**Supplementary Table 6**). Thus, ROS scavenging system also plays an important role in *C. gloeosporioides* stress responses mediated by regulating the glycosylating hormones and secondary metabolites in tea plant. We speculate that during plant infection, the production and recognition of specific ligand peptides might act as pathogen effectors to facilitate the suppression of the plant's immune system and orchestrate the reprogramming of the infected tissue so that it becomes a source of nutrients that are required by the pathogen to support its growth and development, which could contribute to the enhanced susceptibility. Thus, fungi might have adapted to these plant processes to improve the invasion of the host.

CONCLUSIONS

In the present study, we used high-throughput sequencing to identify 485 conserved miRNAs and 761 novel miRNAs from mock- and *C. gloeosporioides*–inoculated tea plant leaves. Our results show that several important miRNAs are differentially expressed in *C. gloeosporioides*–infected leaves. Through degradome sequencing analysis, a total of 1,134 targets for conserved and 596 targets for novel miRNAs were identified. GO annotation revealed that the top ranked miRNA targeting genes were involved in diverse biological processes, including regulation of transcription, response to stress, biosynthetic processes, and signaling pathway. KEGG pathway analysis showed that the potential target genes of the miRNAs were mainly involved in translation, carbohydrate metabolism, signal transduction, energy metabolism, and amino acid metabolism. Moreover, negative correlations between expression levels of identified miRNAs and their corresponding targets were validated through qRT-PCR, and we verified the potential target genes (ARF, NFY, MYB75, MYB114, and STK1) for selected miRNAs by 5'RLM-RACE. These target genes were actively involved in the regulation of auxin pathway, ROS scavenging pathway, SA mediated pathway, receptor kinases, and transcription factors, which, in turn, actively intricate to various biological responses against *C. gloeosporioides* stress. A hypothetical model of the *C. gloeosporioides*–responsive miRNA regulatory network and their target genes in tea plant was derived from the data and illustrated schematically. These results will facilitate a comprehensive understanding of *C. gloeosporioides* stress in tea plant, and help to elucidate miRNA-mediated molecular mechanisms underlying tea plant responses to *C. gloeosporioides* and provide insights into the functional role of miRNAs and their targets in tea plant susceptible to this fungus.

DATA AVAILABILITY

The sRNA read data sets supporting the results in this study have been deposited in the Gene Expression Omnibus (GEO) database; the accession number is GSE119728 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE119728). Tea transcriptome sequence data are deposited in NCBI Sequence Read Archive (SRA) under GenBank accession no. SRR1979118.

AUTHOR CONTRIBUTIONS

AJ participated in sample collection, performed the experiments and data analysis, interpreted the results, and drafted the manuscript. XW and SL participated in manuscript revision critically. SW, RZ, and AW participated in sample collection and qRT-PCR experiments. CW provided guidance on the experimental design and received funding. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01096/full#supplementary-material

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Functional Analysis of 3-Dehydroquinate Dehydratase/ Shikimate Dehydrogenases Involved in Shikimate Pathway in *Camellia sinensis*

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Polyphenols play an important role in the astringent taste of tea [*Camellia sinensis* (L.)] infusions; catechins in phenolic compounds are beneficial to health. The biosynthesis of gallic acid (GA), a precursor for polyphenol synthesis, in tea plants remains unknown. It is well known that 3-dehydroquinate dehydratase/shikimate dehydrogenase (DQD/SDH) is a key enzyme for catalyzing the conversion of 3-dehydroshikimate (3-DHS) to shikimate (SA); it also potentially participates in GA synthesis in a branch of the SA pathway. In this study, four *Cs*DQD/SDH proteins were produced in *Escherichia coli*. Three *Cs*DQD/SDHs had 3-DHS reduction and SA oxidation functions. Notably, three *Cs*DQD/SDHs showed individual differences between the catalytic efficiency of 3-DHS reduction and SA oxidation; *Cs*DQD/SDHa had higher catalytic efficiency for 3-DHS reduction than for SA oxidation, *Cs*DQD/SDHd showed the opposite tendency, and *Cs*DQD/SDHc had almost equal catalytic efficiency for 3-DHS reduction and SA oxidation. *In vitro*, GA was mainly generated from 3-DHS through nonenzymatic conversion. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis showed that *CsDQD/SDHc* and *CsDQD/SDHd* expression was correlated with GA and 1-*O*-galloyl-β-D-glucose accumulation in *C. sinensis*. These results revealed the *Cs*DQD/SDHc and *Cs*DQD/SDHd genes are involved in GA synthesis. Finally, site-directed mutagenesis exhibited the mutation of residues Ser-338 and NRT to Gly and DI/LD in the SDH unit is the reason for the low activity of *Cs*DQD/SDHb for 3-DHS reduction and SA oxidation.

Keywords: *Camellia sinensis*, 3-dehydroquinate dehydratase/shikimate dehydrogenase, gallic acid, shikimate pathway, site-directed mutagenesis

INTRODUCTION

The shikimate (SA) pathway contributes to the production of a wide range of intermediates and aromatic amino acids vital for secondary metabolites and protein biosynthesis of microorganisms and plants. The SA pathway in plants consists of seven enzymatic reaction steps, beginning with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate

to form 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) and ending with the synthesis of chorismate from 5-enolpyruvylshikimate 3-phosphate (EPSP) (**Figure 1**) (Sprenger, 2006; Bentley and Haslam, 2008). Chorismic acid, the end-product of SA pathway, is essential for the formation of aromatic amino acids (L-tyrosine, L-tryptophan, and L-phenylalanine), which can be further catalyzed into several secondary metabolites, such as phenylalanine, lignin, flavonoids, chlorogenic acid, indole acetic acid, and alkaloids (Sprenger, 2006; Maeda and Dudareva, 2012). Tyrosine and phenylalanine are two crucial precursors that can be catalyzed by phenylalanine ammonia-lyase (PAL) to activate the phenylalanine pathway and accumulate flavonoids, polyphenols, and lignin. Furthermore, increasing *FaSKDH* expression can activate the phenylpropanoid pathway to promote flavonoid and polyphenol accumulation in strawberry fruit (Xu et al., 2014; Nagpala et al., 2016).

The SA pathway is important to plant growth, development, and defense; and silencing the DAHP synthase gene (*DAHPS*) or 3-dehydroquinate dehydratase/shikimate dehydrogenase (*DQD/SDH*) in potato, tomato, and tobacco plants results in slow growth and reduces the production of secondary metabolites, such as lignin and chlorogenic acid (Dyer et al., 1989; Kasama et al., 2007). In plants, DQD/SDH, a bifunctional enzyme, is crucial in the third and fourth reversible reactions in the SA pathway; SA can be formed from 3-dehydroshikimate (3-DHS) and NADPH under catalysis by DQD/SDH, and 3-DHS can also be generated from SA and $NADP^+$ under catalysis by DQD/SDH (Kasama et al., 2007; Maeda and Dudareva, 2012). Analysis of the crystal structure of *Arabidopsis* DQD/SDH showed the functional difference between DQD and SDH sites, which bind with the substrates SA and tartrate to form complexes (Singh, 2006), respectively. Metabolic flux through the SA pathway can be controlled by increasing the effective concentration of the intermediate 3-DHS at each of the two sites. The ratio of SDH to DQD catalytic efficiency is 9:1; therefore, 3-DHS can be quickly converted to SA without accumulation in the stroma of spinach chloroplasts (Fiedler and Schultz, 1985).

Polyphenols in tea plants, including phenolic acids, catechins, and flavonol derivatives, not only determine the mouthfeel of tea infusions but also provide health benefits. Tea plants have abundant ester catechin and epigallocatechin gallate (EGCG) content, which range up to 12%. Gallic acid (GA) is an essential precursor for galloylated catechin biosynthesis (**Figure 1**), and the amount of GA is a key limiting factor for the formation of EGCG and epicatechin gallate (ECG) (Liu et al., 2012). In addition, GA derivatives, including hydrolysable tannins (HTs) and mainly galloylquinic acid (GQA), highly accumulate in vegetables, *Rhus typhina*, and *Camellia sinensis* (Haslam and Cai, 1994; Niemetz and Gross, 2005; Jiang et al., 2013). They are responsible for the unique flavor of plant-derived foods and show a wide range of biological activities, including antioxidant, antiviral, anti-inflammatory, antibacterial, anticancer, and immune-regulation activities (Vit et al., 2008; Steinmann et al., 2013).

GA biosynthesis has been studied for more than 50 years since the 1960s (Dewick and Haslam, 1969). By using the isotope-labeling method, studies have provided an indication regarding GA biosynthesis: GA is mainly derived from the dehydrogenation of 3-DHS by the action of SDH to produce 3,5-didehydroshikimate. This compound tautomerizes to form the redox equivalent GA. Studies have also verified this speculation in plants: GA could be synthesized from 3-DHS with $NADP^+$ as the cofactor when the crude enzyme of birch leaf was used (Ossipov et al., 2003). Transgenic *Nicotiana tabacum* lines overexpressing *Juglans regia SDH* exhibited a 500% increase in

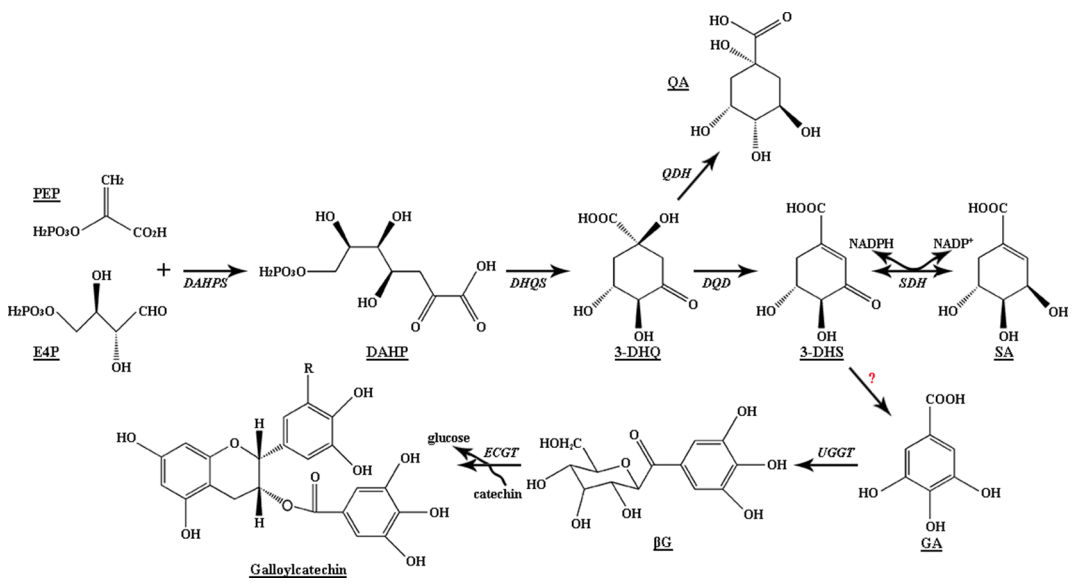


FIGURE 1 | Biosynthetic pathway of gallic acid and shikimic acid in plants. DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQS, 3-dehydroquinate synthase; DQD, 3-dehydroquinate dehydratase; SDH, SA dehydrogenase; QDH, quinate dehydrogenase; UGGT, UDP-glucose: gallate 1-*O*-galloyltransferase; ECGT, epicatechin: 1-*O*-galloyl-β-D-glucose *O*-galloyltransferase.

GA accumulation (Muir et al., 2011). In the persimmon fruits of pollination-constant and nonastringent (PCNA)-type mutants, downregulated *SDH* expression was correlated with the reduction in epigallocatechin content, which confirmed the correlation of SDH with proanthocyanidin (PA) content (Akagi et al., 2009). Recently, two of the four *Vv*SDH genes, namely, *Vv*SDH3 and *Vv*SDH4, were validated to be involved in GA biosynthesis in Vitis vinifera (Bontpart et al., 2016).

Recently, an early intermediate of the SA pathway, 3-DHS, was identified to be a crucial precursor for GA synthesis in plants. Alternatively, a study found that GA could be produced through the SA pathway and phenylpropanoid pathway simultaneously. Ishikura and coworkers revealed that GA was derived from not only labeled SA in young leaves but also labeled L-phenylalanine in mature and autumn leaves of *Acer buergerianum* and *Rhus succedanea* (Ishikura et al., 1984); this finding indicated that the biosynthetic pathway of GA is diverse and changes according to the developmental stage of the plant.

Studies related to the key gene involved in GA biosynthesis in tea plants are scant. In this study, we screened out four *CsDQD/SDH* genes from the tea genome (designated as *CsDQD/SDHa*, *CsDQD/SDHb*, *CsDQD/SDHc*, and *CsDQD/SDHd*). The functions of *CsDQD/SDHs* expressed in *Escherichia coli* were surveyed, and after purification, the capacity of recombinant proteins *in vitro* to produce GA was determined using enzymatic analysis. We also evaluated the relationship between the expression patterns of these four *CsDQD/SDH* genes and the contents of GA and its derivatives in various organs of tea plants. The results may provide a basis for understanding the biosynthesis of GA and its derivatives.

METHODS AND MATERIALS

Plant Material

Camellia sinensis cv. Shuchazao (variety approval number: CHN20022008) samples were obtained from the experimental tea garden of Anhui Agricultural University, Hefei, China. Leaves (bud, first leaf, and second leaf), young stems, and tender roots were immediately frozen in liquid nitrogen and stored at −80°C until use.

Chemicals

DL-Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), $NADP^+$, NADPH, GA, SA, 3-DHS, NaCl, NaOH, Tris, maltose, HCl, isopropyl β-D-thiogalactoside (IPTG), Bis-Tris propane HCl (BTP-HCl), tryphone, and yeast extract were obtained from Sigma Aldrich (St. Louis, MO, USA). Ultra-performance liquid chromatography (UPLC)-grade methanol, acetic acid, acetonitrile, and phosphoric acid were purchased from Tedia Co., Ltd. (Fairfield, OH, USA).

CsDQD/SDH Cloning and Sequence Analysis

Total RNA was isolated from 50 mg of various tea tissues using the RNAiso Plus kit and RNAiso-mate (Takara, Dalian, China), and cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. The sequences of *CsDQD/SDHa* (National Center for Biotechnology Information (NCBI) accession number: MH000201, 1,605 bp), *CsDQD/SDHb* (NCBI accession number: MH000202, 1,560 bp), and *CsDQD/SDHc* (NCBI accession number: MH000203, 1,599 bp) were obtained from the NCBI database, and the sequence of *CsDQD/SDHd* (NCBI accession number: MH000204, 1,578 bp) was obtained from the Sequence Read Archive database at NCBI under Bioproject ID PRJNA283013 for Huangjinya. The open reading frames (ORFs) of *CsDQD/SDHs* were cloned with high-fidelity DNA polymerase using *C. sinensis* cv. Shuchazao cDNA as the template and were constructed into a pEASY-Blunt Simple Cloning Kit vector (New England Biolabs, MA, USA) to sequence the full-length gene. The forward and reverse primers for cloning into the pMAL-c2x expression vector (New England Biolabs) were inserted to incorporate the restriction site for *Bam*HI and *Pst*I before the start codon and after the stop codon, respectively (primers are listed in **Supplementary Table S1**).

Multiple DQD/SDH protein sequences (**Supplementary Table S2**) were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) 12.0 and NCBI (https://www.ncbi.nlm.nih.gov/). Amino acid sequences were aligned, and phylogenetic analysis was performed using DNAMAN 6.0 (Lynnon Corporation, San Ramon, CA, USA), Primer 5.0 (Primer, Canada), and MEGA 5.0 (Mega, Raynham, MA, USA) (Tamura et al., 2011). The tree nodes were statistically evaluated using the bootstrap method, with 1,000 bootstrap replicates conducted. A neighbor-joining tree with the evolutionary distance was computed using the ρ-distance model and protein sequences alignment with a gap open penalty of 10 and a gap extension penalty of 0.2.

CsDQD/SDH Expression in *Escherichia coli* and Purification of Recombinant Proteins

CsDQD/SDH ORFs were cloned into the pMAL-c2x (New England Biolabs, MA, USA) expression vector and then expressed in *E. coli* NovaBlue (DE3) competent cells (Novagen, Schwalbach, Germany) to express recombinant proteins, and the proteins purified according to the manufacturer's protocol (New England Biolabs). The recombinant *E. coli* strains expressing *Cs*DQD/SDHs were shaken in Luria–Bertani (LB) medium (1 L of LB culture medium containing 5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl, adjusted to pH 7.0 before sterilization) containing 1 $\mu L \cdot ml^{-1}$ of ampicillin to an OD_{600} of 0.6, and the cells were shaken at 37°C at 220 rpm. Thereafter, 1 $mmol \cdot L^{-1}$ of IPTG was added to the culture medium, and protein expression in strains was induced at 24°C for 24 h. After centrifugation at 7,200 rpm for 15 min, supernatants were discarded, and the precipitates were resuspended in column buffer (200 mM of NaCl, 20 mM of Tris, 1 mM of EDTA, and 1 mM of DTT, pH 7.5). The cells were ultrasonically disrupted for approximately 20 min and then centrifuged at 6,500 rpm for approximately 15 min at 4°C (SCIENTZ-llD, NingBo, China). Supernatants were purified using a maltose column; finally, the target proteins were eluted

using maltose column buffer (20 mM of maltose in column buffer, pH 7.5). The target proteins were concentrated using 50-kD ultrafiltration concentration pipes. Recombinant protein concentrations were determined using Coomassie Brilliant Blue G-250, and their concentrations were confirmed by running the proteins on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis gel; they were then stored at −80°C in 50% glycerol.

Enzyme Activity and Product Analysis

To analyze the *in vitro* activity of the candidate *Cs*DQD/SDHs for 3-DHS reduction and SA oxidation, reactions were conducted in a 100-μL reaction solution consisting of 100 mM of BTP-HCl buffer (pH 7.5), 1 mM of NADPH or $NADP^+$ as the cofactor donor, 1 mM of 3-DHS or 1 mM of SA as the substrate, and 10 μg of purified recombinant *Cs*DQD/SDHs protein at 30°C for 30 min. Reactions were stopped by mixing the reaction solutions with 2.3 M of HCl. Reaction samples lacking recombinant proteins were used as blank controls.

The high-performance liquid chromatography (HPLC) system from Agilent Technologies (RHMo Alto, CA, USA) was used in this study to detect 3-DHS and SA (UV maximum absorption wavelengths were 234 and 211 nm, respectively). The HPLC system comprised a Venusil XBP C18 reverse phase column (4.6 × 251 mm^2, Agela Technologies), quaternary pump with a vacuum degasser, thermostated column compartment, and autosampler. The elution profile was as follows: 100% eluent A (1% phosphoric acid in water) for 0–24 min and termination at 25 min at 0.2 $mL{\cdot}min^{-1}$ flow rate (eluent B: 100% acetonitrile).

To analyze the *in vitro* activity of *Cs*DQD/SDHs to produce GA, assays were performed in a buffer containing 100 mM of BTP-HCl buffer (pH 9.0), 1 mM of 3-DHS, 1 mM of $NADP^+$, and 10 μg of purified recombinant *Cs*DQD/SDHs protein at 30°C for 30 min. Reactions were stopped by mixing the reaction solutions with an equal volume of 100% methanol. GA (UV maximum absorption wavelengths were 280 nm) was analyzed using a reverse-phase HPLC LC10Avp system (Shimadzu, Kyoto, Japan). The column was eluted using a mobile phase consisting of eluent A (1% acetic acid) and eluent B (100% acetonitrile) at room temperature. The elution profile was as follows: starting with 100% A (1% acetic acid), a linear gradient from 1% to 5% B (100% acetonitrile) for 0–8 min, 5–10% B for 8–13 min, 10–1% B for 13–14 min, and termination at 15 min at a flow rate of 0.2 $ml{\cdot}min^{-1}$ (eluent B: 100% acetonitrile).

All products were analyzed using UPLC–triple quadrupole mass spectrometry (QQQ)–tandem mass spectrometry (MS/MS) with an Agilent 20RBAX RRHD Eclipse Plus C18 column (particle size, 1.8 mm; length, 100 mm; and internal diameter, 2.1 mm) at a flow rate of 0.4 $ml{\cdot}min^{-1}$ following previously published protocols (Jiang et al., 2013; Zhao et al., 2017). All reactions in each experiment were three bio-replications.

Enzymatic Kinetic Analysis of Recombinant *CsDQD*/SDH Proteins

Optimal pH analysis was performed using 3-DHS and SA as substrates at varying pH. The buffers for the pH test were 100 mM of citric acid with pH ranging from 4 to 7, 100 mM of BTP-HCl buffer with pH ranging from 6 to 9, and 100 mM of sodium carbonate with pH ranging from 8 to 11. The reaction was maintained at 30°C for 30 min, and the reaction was stopped by adding 40 μL of 2.3 M of HCl.

In vitro, the kinetic parameters of the recombinant enzymes were obtained from hyperbolic Michaelis–Menten saturation curves for substrates. For the measurement of the K_M and V_{max} of *Cs*DQD/SDHs, 3-DHS, SA, $NADP^+$, and NADPH were used as acceptor substrates. The linear phase of the reaction was conducted in BTP-HCl buffer (pH 8.5) with 1.5 mM of 3-DHS and $NADP^+$ (0–500 μM), 1.5 mM of $NADP^+$ and 3-DHS (0–500 μM), 1.5 mM of SA and NADPH (0–500 μM), and 1.5 mM of NADPH and SA (0–500 μM) at 30°C for 3 min. Three biological replicates were used per reaction.

Functional Unit Reorganization and Site-Directed Mutagenesis

To identify the key unit and amino acid residues responsible for 3-DHS reduction and SA oxidation, *Cs*DQD/SDHa was categorized as two mutant proteins named *Cs*DQDa (from Lys-91 to Phe-316 in the *At*SDH protein sequence) and *Cs*SDHa (from IIe-328 to Gly-588 in the *At*SDH protein sequence). Referring to the model of *Arabidopsis thaliana* protein crystal structure, the amino acid residue sites of Gly-338, Gly-381, Asp-483, Leu-484, and Asp-485 in *Cs*DQD/SDHb were mutated to Ser-338, Thr-381, Asn-483, Arg-484, and Thr-485, respectively; the mutant protein was named MT*Cs*DQD/SDHb.

Site-directed mutagenesis was performed using a gene site-directed mutagenesis kit. The plasmid pMAL-c2X harboring *Cs*DQD/SDHs was used as templates to obtain the site-directed mutants of *Cs*DQDa, *Cs*SDHa, and MT*Cs*DQD/SDHb. Oligonucleotide sequences specifically designed for mutagenesis are listed in **Supplementary Table S1**.

Purification and analysis of the mutant recombinant proteins were performed using the same protocols as those used for native proteins. The quantitative measurement of the recombinant enzyme products was performed using the aforementioned method, and three biological replicates were used for each experiment.

Expression of GA and GA-Related Compounds and Their Accumulation in Tea Plants

GA, βG, ECG, and EGCG were extracted from various organs of tea plants as follows: 1 g of the dry weight sample (bud, first leaf, second leaf, stem, and root) was ground in liquid nitrogen; it was then extracted with 2 ml of the extraction solution (0.2% HCl added to 80% methanol and 20% water) at room temperature through ultrasonic extraction for 10 min and centrifugation for 15 min at 4,000 rpm (Wang et al., 2018). The precipitate was resuspended in the extraction solution and re-extracted twice as previously described; supernatants were filtered through a 0.22-μm filter membrane. Three biological replicates were analyzed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

GA, βG, ECG, and EGCG contents were detected using an UPLC–MS/MS system equipped with a quaternary pump with a vacuum degasser, thermostated column compartment, autosampler, diode array detector (DAD), and QQQ purchased from Agilent Technologies (Palo Alto, CA, USA). Samples were analyzed using UPLC–QQQ–MS/MS with an Agilent 20RBAX RRHD Eclipse Plus C18 column (particle size, 1.8 mm; length, 100 mm; and internal diameter, 2.1 mm) at a flow rate of 0.4 $mL \cdot min^{-1}$ following previously published protocols (Jiang et al., 2013; Zhao et al., 2017).

Expression Pattern of *CsDQD/SDHs* in Tea Plants

The expression levels of glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), as the reference gene, were standardized against *CsDQD/SDHs* expression levels. Total RNA was extracted using TRIzol and then reverse transcribed into cDNA. All primers were designed using Primer 5.0 and were detected using PCR. The qRT-PCR system contained 200 ng of cDNA template, 10 μL of IQ SYBR Green Supermix (Takara), and 0.8 μL of each gene-specific primer, and the reaction volume of 20 μL was attained through the addition of RNase-free H_2O. The expression levels are represented as a mean value of three replicates. Relative *CsDQD/SDH* expression was deduced from the cycle threshold (CT) based on the $2^{-\Delta\Delta Ct}$ method. $\Delta CT = CT_{target} - CT_{internal\ standard}$ and $-\Delta\Delta CT = -(\Delta CT_{target} - \Delta CT_{control})$, where CT_{target} and $CT_{internal\ standard}$ are the cycle threshold (CT) values for the target and housekeeping genes, respectively. The Pearson correlation coefficient of the *CsDQD/SDH* expression profiles was also calculated.

RESULTS

Cloning and Protein Sequence Analysis of Four *CsDQD/SDH* Genes

The four *CsDQD/SDH* genes were screened from the NCBI and tea genome database and successfully cloned from the tea plant cDNA library. Their ORF lengths are 1,605, 1,560, 1,599, and 1,578 nucleotides, and their encoded proteins are 534, 519, 532, and 525 amino acid residues in length, respectively, with predicted molecular weights of 57.568, 56.411, 58.241, and 56.776 kDa, respectively, and calculated isoelectric points (pIs) of 6.26, 6.1, 6.73, and 6.8, respectively.

Analysis using DNAMAN 6.0 showed that the protein sequence of DQD/SDHs in plants shared 54.17% consistency (**Supplementary Figure 1**). The amino acid sequence of *Cs*DQD/SDHa shared 64.11% and 66.79% identity with *Cs*DQD/SDHc and *Cs*DQD/SDHd. However, *Cs*DQD/SDHb showed only 49.44%, 48.41%, and 48.67% identity with *Cs*DQD/SDHa, *Cs*DQD/SDHc, and *Cs*DQD/SDHd, respectively.

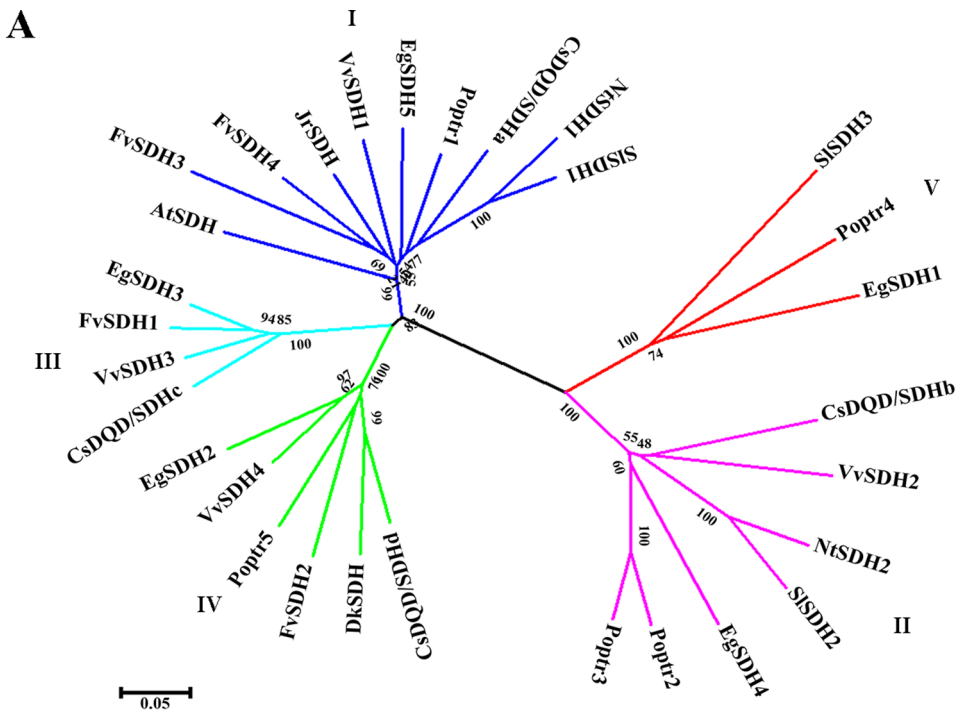
Some characterized DQD/SDH protein sequences were extracted from the NCBI database and Phytozome 12.0, and a phylogenetic tree was constructed using MEGA 5.0 (**Figure 2A**). The result showed that the plant DQD/SDH proteins can be divided into five groups. Bontpart et al. (2016) divided *Vv*SDH proteins into four groups. The *Cs*DQD/SDH proteins in this study were divided into four groups, similar to *Vv*SDH proteins. **Figure 2B** presents the enzyme characteristics of each DQD/SDH protein identified from *Arabidopsis thaliana* (Singh, 2006), *Nicotiana tabacum* (Kasama et al., 2007), *Vitis vinifera* (Bontpart et al., 2016), and *Populus trichocarpa* (Guo et al., 2014). Relevant studies have indicated that the DQD/SDH proteins in the first, third, and fourth groups derived from showed both 3-DHS reduction and SA oxidation activities. However, the enzymatic activities of several DQD/SDH proteins in the second group could not been detected. *Vv*SDH2 only showed a very low SA oxidation activity (Bontpart et al., 2016). Whether DQD/SDH proteins participate in GA production needs further investigation and supporting evidence, although research has proved that *Vv*SDH3 and *Vv*SDH4 are involved in GA production *in vitro* (Bontpart et al., 2016).

The DQD/SDH protein sequence alignments in **Figure 2** show that the plant DQD/SDH proteins consist of two functional units: the DQD unit (**Supplementary Figure 2A**) and the SDH unit. The DQD unit can catalyze the conversion of 3-dehydroquinate to 3-DHS (Michel et al., 2003; Vogan, 2003), whereas the SDH unit can catalyze the NADPH-dependent reduction of 3-DHS to SA (Padyana and Burley, 2003; Ye et al., 2003). According to the structure of *At*DQD/SDH (Singh, 2006), residues Lys-241 and His-214 as well as Arg-279 in the DQD unit function as key catalytic groups and a binding group, respectively. Residues Lys-385 and Asp-423 have been proposed to be a catalytic dyad in the SDH unit, and Ser-336 has been proposed to be its key binding group. Moreover, Asn-483, Arg-484, and Thr-485 formed the NRT motif responsible for binding the cofactor NADP(H). The Ser-338 Ala mutant of *At*SDH affected substrate binding and its catalysis. These amino acid residues were conserved in plant DQD/SDHs of the first, third, and fourth groups, except for *Cs*DQD/SDHc (where His-214 was replaced by Gln).

Notably, the corresponding catalytic residues His-214 and Arg-279 in the DQD unit and Ser-338 in the SDH unit of *Cs*DQD/SDHb and *Vv*SDH2 belonging to the second group were replaced by Tyr, Gln, and Gly, respectively. The position of NRT was replaced by DI/LD in *Cs*DQD/SDHb and *Vv*SDH2. These results may contribute to the difficulty in detecting the enzymatic activities of DQD/SDH proteins in the second group.

Enzyme Assays and Product Identification

To detect a recombinant protein activity, the four *Cs*DQD/SDHs were fused to maltose-binding protein and expressed in NovaBlue (DE3) strains. The NADPH-dependent reduction of 3-DHS (**Supplementary Figure 3A**) and the $NADP^+$-dependent oxidation of SA (**Supplementary Figure 3B**) by the recombinant proteins of *Cs*DQD/SDHa were measured, and the corresponding products in these reactions were detected quantitatively through UPLC–QQQ–MS/MS (**Supplementary Figure 3C**). Quantitative results indicated that among these four recombinant proteins, *Cs*DQD/SDHa exhibited the highest 3-DHS reduction and SA oxidation activities, and *Cs*DQD/SDHb exhibited the lowest SA oxidation activity but had no 3-DHS reduction activity (**Figure 3**).



B

	3-DHS reduction	SA oxidation	GA production	
●CsDQD/SDHa				
NtSDH1	+	+	ns	I
Poptr1	+	+	ns	
VvSDH1	+	+	+	
AtSDH	+	+	ns	
VvSDH3	+	+	+	III
●CsDQD/SDHc				
Poptr5	+	+	ns	IV
VvSDH4	+	+	+	
●CsDQD/SDHd				
NtSDH2	−	−	ns	II
●CsDQD/SDHb				
VvSDH2	−	+	−	
Poptr3	−	−	ns	
Poptr2	−	−	ns	
Poptr4	−	−	ns	V

+: there is a corresponding activity; -: there is no corresponding activity; ns: not studied.

FIGURE 2 | Neighbor-joining tree analysis. **(A)** A neighbor-joining tree was constructed from the four *Cs*DQD/SDHs sequenced in this study and the 26 sequences available in public databases (NCBI and Phytozome 12) using MEGA 5.0 software. **(B)** A simplified neighbor-joining tree was constructed to display the enzyme characteristics of each DQD/SDH protein from *Arabidopsis thaliana*, *Nicotiana tabacum*, *Vitis vinifera*, and *Populus trichocarpa*. NCBI, National Center for Biotechnology Information; DQD/SDH, 3-dehydroquinate dehydratase/shikimate dehydrogenase.

GA production was found in *Cs*DQD/SDHs assays. SA generation occurred immediately in the 3-DHS reduction reaction catalyzed by *Cs*DQD/SDHa and reached its maximum value after 15 min, whereas GA was detected after 20 min in time course of the reaction (**Figure 4A**). A similar pattern was observed for SA oxidation by *Cs*DQD/SDHa; the increase in GA content was consistent with the decrease in the product 3-DHS (**Figure 4B**). On the basis of these results, we speculate that GA may be directly generated from 3-DHS with $NADP^+$ as the coenzyme. However, a significant increase in GA production was not detected in the time course of the *Cs*DQD/SDHa assay when using 3-DHS and the coenzyme $NADP^+$ as substrates (**Figure 4C**). To further verify that GA was directly generated from 3-DHS, enzymatic or nonenzymatic *Cs*DQD/SDH assay was conducted using 3-DHS and the coenzyme $NADP^+$ as substrates, and the assay revealed GA was directly generated from 3-DHS in the enzymatic SA oxidation reaction (**Figure 5**).

Determination of Kinetic Parameters

To determine the optimum pH, assays were performed at 30°C for 30 min with citric acid buffer (100 mM, pH 4–7), BTP-HCl buffer (100 mM, pH 6–9), and sodium carbonate buffer (100 mM, pH 8–11), individually. The results showed that the 3-DHS reduction and SA oxidation activities of *Cs*DQD/SDHa, *Cs*DQD/

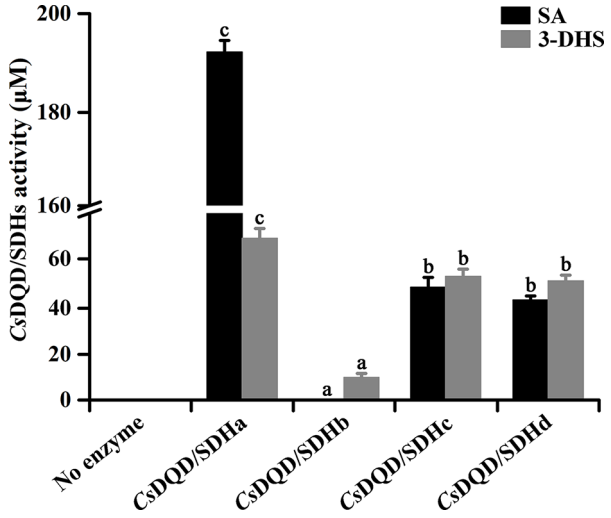


FIGURE 3 | Quantification of 3-DHS and SA production catalyzed by recombinant *Cs*DQD/SDHs *in vitro*. The reaction products in enzymatic assays were quantified using HPLC ($\lambda_{3\text{-DHS}}$ = 234 nm, λ_{SA} = 211 nm). Data are shown as the mean of three replicates ± SD. Different letters represent statistically differ at $P < 0.05$ according to one-way ANOVA of Duncan's test. 3-DHS, 3-dehydroshikimate; SA, shikimate; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.

SDHc, and *Cs*DQD/SDHd were higher in alkaline buffer than in acidic buffer (**Supplementary Figure 4**).

In vitro, the kinetic parameters of both substrates (3-DHS and SA) and cofactors (NADP$^+$ and NADPH) were measured at 30°C for 3 min in a buffer with pH 8.5 (**Table 1**). For 3-DHS reduction, compared with *Cs*DQD/SDHc ($K_{M(3\text{-DHS})}$ = 330.933 μM) and *Cs*DQD/SDHd ($K_{M(3\text{-DHS}}$ = 465.971 μM), *Cs*DQD/SDHa ($K_{M(3\text{-DHS})}$ = 286.576 μM) had the highest affinity for 3-DHS. The catalytic efficiency of *Cs*DQD/SDHa ($k_{cat}/K_{M(3\text{-DHS})}$ = 1,412.281 $S^{-1}{\cdot}M^{-1}$) was almost three times more than that of *Cs*DQD/SDHc ($k_{cat}/K_{M(3\text{-DHS})}$ = 506.596 $S^{-1}{\cdot}M^{-1}$) and 14 times more than that of *Cs*DQD/SDHd ($k_{cat}/K_{M(3\text{-DHS})}$ = 99.546 $S^{-1}{\cdot}M^{-1}$). For SA oxidation, *Cs*DQD/SDHc ($K_{M(SA)}$ = 199.653 μM) showed the highest affinity for the substrate SA in comparison with *Cs*DQD/SDHa ($K_{M(SA)}$ = 272.782 μM) and *Cs*DQD/SDHd ($K_{M(SA)}$ = 610.643 μM). However, the catalytic efficiency of *Cs*DQD/SDHa ($k_{cat}/K_{M(SA)}$ = 562.168 $S^{-1}{\cdot}M^{-1}$) and *Cs*DQD/SDHc ($k_{cat}/K_{M(SA)}$ = 535.744 $S^{-1}{\cdot}M^{-1}$) were comparable and approximately four times more than that of *Cs*DQD/SDHd ($k_{cat}/K_{M(SA)}$ = 138.482 $S^{-1}{\cdot}M^{-1}$). In summary, *Cs*DQD/SDHa compared with *Cs*DQD/SDHc and *Cs*DQD/SDHd had the highest catalytic efficiency for 3-DHS reduction and SA oxidation.

Notably, *Cs*DQD/SDHs showed considerable individual differences between the catalytic efficiency of 3-DHS reduction and SA oxidation; *Cs*DQD/SDHa had higher catalytic efficiency for 3-DHS reduction than for SA oxidation, *Cs*DQD/SDHd showed the opposite tendency, and *Cs*DQD/SDHc had almost equal catalytic efficiency for 3-DHS reduction and SA oxidation. In addition, *Cs*DQD/SDHd had higher affinity for NADP$^+$. These results suggest that *Cs*DQD/SDHc and *Cs*DQD/SDHd function differently in tea plants, such as efficient GA generation.

Truncation and Site-Directed Mutagenesis

To determine the function of the SDH unit and the DQD unit, two units were expressed in *Escherichia coli*, respectively, and the recombinant proteins were named *Cs*DQDa and *Cs*SDHa. The results of the enzyme assay indicated that compared with the *Cs*DQD/SDHa recombinant protein, the *Cs*SDHa recombinant protein had an almost identical enzymatic activity for NADPH-dependent reduction of 3-DHS and a lower enzymatic activity for NADP$^+$-dependent oxidation of SA, whereas the *Cs*DQDa recombinant protein was completely inactive (**Figure 6A**).

The sequence alignments in **Supplementary Figure 2** showed that the key residues Ser-338 and NRT in the SDH unit of *Cs*DQD/SDHb were replaced by Gly and DI/LD, respectively; this may be a reason that the enzymatic activities of DQD/SDH proteins in the second group were difficult to detect. To verify this postulation, the substrate and cofactor binding sites of *Cs*DQD/SDHb were analyzed using site-directed mutagenesis. Referring to the model of *A. thaliana* protein crystal structure, the residue sites of Gly-338, Gly-381, Asp-483, Leu-484, and Asp-485 in *Cs*DQD/SDHb were mutated to Ser-338, Thr-381, Asn-483, Arg-484, and Thr-485, respectively; the mutant protein was named MT*Cs*DQD/SDHb. The results showed that MT*Cs*DQD/SDHb had a similar reduction activity of 3-DHS and had six times higher oxidation activity of SA than *Cs*DQD/SDHb (**Figure 6B**), suggesting that the mutation of residues Ser-338 and NRT to Gly and DI/LD in the SDH unit is the reason for the low activity of *Cs*DQD/SDHb, respectively.

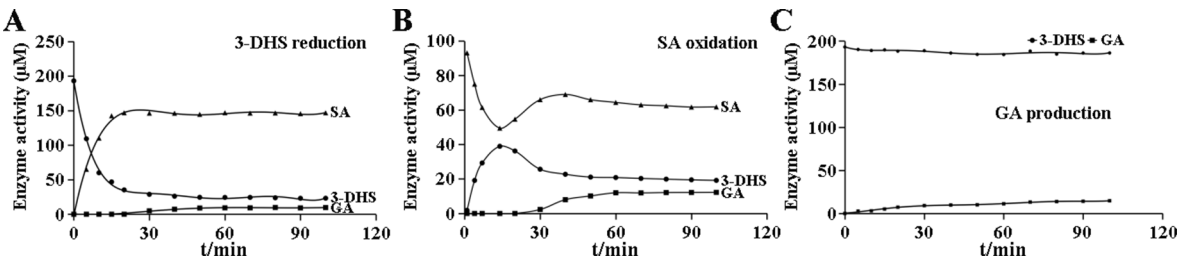


FIGURE 4 | Enzymatic reaction time of the recombinant protein *Cs*DQD/SDHa *in vitro*. **(A)** The time course of the 3-DHS reduction reaction catalyzed by the *Cs*DQD/SDHa enzyme. **(B)** The time course of the SA oxidation catalyzed by the *Cs*DQD/SDHa enzyme. **(C)** GA production in a reaction catalyzed by the *Cs*DQD/SDHa enzyme. 3-DHS, 3-dehydroshikimate; SA, shikimate; GA, gallic acid.

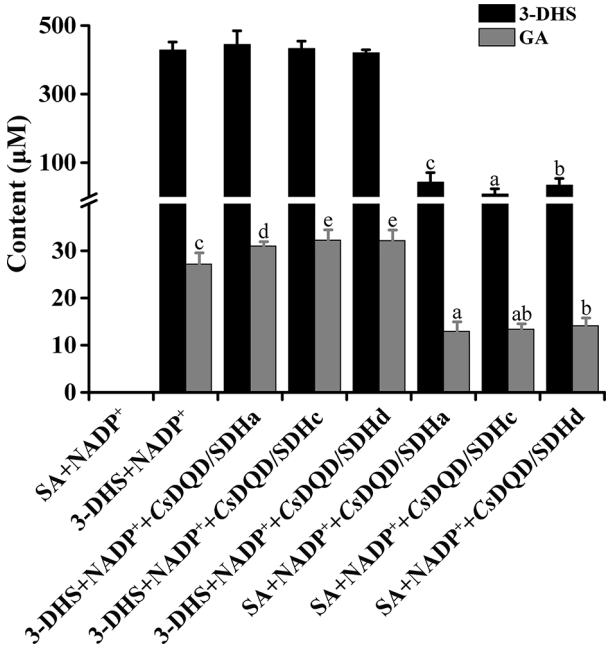


FIGURE 5 | GA generation capacity from *Cs*DQD/SDHa, *Cs*DQD/SDHc, and *Cs*DQD/SDHd and without enzyme as the control. The reaction products in the enzymatic assays were quantified using HPLC ($\lambda_{3\text{-DHS}}$ = 234 nm, λ_{GA} = 280 nm). Data are shown as the mean of three replicates ± SD. Different letters represent statistically differ at $P < 0.05$ according to one-way ANOVA of Duncan's test. GA, gallic acid; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.

Expression Pattern of CsDQD/SDHs and Accumulation of GA, βG, ECG, and EGCG in Tea Plants

The relative expression pattern of the four *CsDQD/SDHs* in diverse plant parts (bud, first leaf, second leaf, stem, and root) was determined using qRT-PCR (**Figure 7A**). The results revealed that *CsDQD/SDHa* and *CsDQD/SDHb* showed similar expression patterns in buds, young leaves, stems, and roots and showed the highest expression in first leaves. *CsDQD/SDHc* and *CsDQD/SDHd* were highly expressed in the bud and tender leaves, and their expression decreased from the bud to the root, with the least expression in roots.

The contents of GA and its glucose ester, βG, were higher in buds and young leaves than in other tissues. A positive correlation was observed between the expression pattern of *CsDQD/SDHc* and *CsDQD/SDHd* and the accumulation of GA, βG, ECG, and EGCG (**Figure 7B**). The Pearson correlation coefficient (**Figure 7C**) indicating that the expression pattern of *CsDQD/SDHc* was more positively related to the synthesis of GA, βG, and galloylated catechins.

DISCUSSION

Early studies have also uncovered the importance of the SA pathway in plant secondary compound synthesis, including lignin and pigments such as anthocyanins. Research related to the key gene involved in GA biosynthesis in tea plants is scant but indicates that *DQD/SDHs* are the candidate target genes. GA serves as a precursor for the biosynthesis of galloyl-type HTs and galloylated PAs (Haslam and Cai, 1994). HTs play crucial roles in tea plant herbivore deterrence and influence tea's bitterness and astringency. A critical step in HT production is βG generation. For βG formation, *Cs*UGT84A22 requires GA as a substrate (Cui et al., 2016), which is produced from 3-DHS of the SA pathway. In addition, UGT84A23, UGT84A24, and DQD/SDH are localized in the cytoplasm in *Punica granatum* (Ono, 2016); therefore, it is conceivable that GA production in cytosolic from *Cs*DQD/SDHs may be supplied to *Cs*UGT84A22-catalyzed reactions. PAs, also known as condensed tannins, are polymers of flavan-3-ol units such as catechins and galloylated catechins (Winkel-Shirley, 2001). The galloylated catechins EGCG and PA polymers are the dominant flavonoids in tea leaves and roots, respectively

TABLE 1 | Michaelis–Menten kinetic parameters of recombinant *Cs*DQD/SDHs for each substrate.

Enzyme	Substrate	Product	K_M (μM)	V_{max} (nKat·μg^{-1})	k_{cat} (S^{-1})	k_{cat}/K_M ($S^{-1}\cdot M^{-1}$)
***Cs*DQD/SDHa**	NADPH	SA	270.306	31.545	0.202	746.282
	3-DHS		286.576	63.291	0.405	1412.281
	$NADP^+$	3-DHS	138.031	18.248	0.117	845.397
	SA		272.782	23.981	0.153	562.168
***Cs*DQD/SDHb**	NADPH	SA	nd*			
	3-DHS		nd			
	$NADP^+$	3-DHS	nd			
	SA		nd			
***Cs*DQD/SDHc**	NADPH	SA	267.244	7.987	0.052	193.409
	3-DHS		330.933	25.907	0.168	506.596
	$NADP^+$	3-DHS	114.553	6.579	0.043	371.655
	SA		199.653	16.529	0.107	535.744
***Cs*DQD/SDHd**	NADPH	SA	248.798	7.758	0.049	196.708
	3-DHS		465.971	7.353	0.046	99.546
	$NADP^+$	3-DHS	46.342	0.937	0.006	127.616
	SA		610.643	13.405	0.085	138.482

nd, no activity detectable or too low to determine kinetic properties.

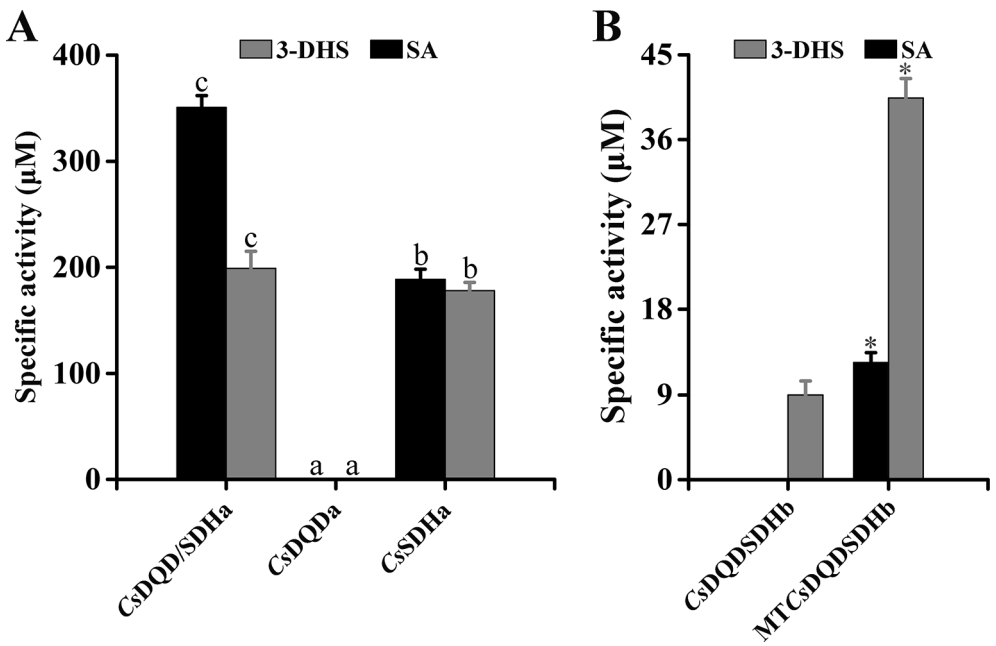


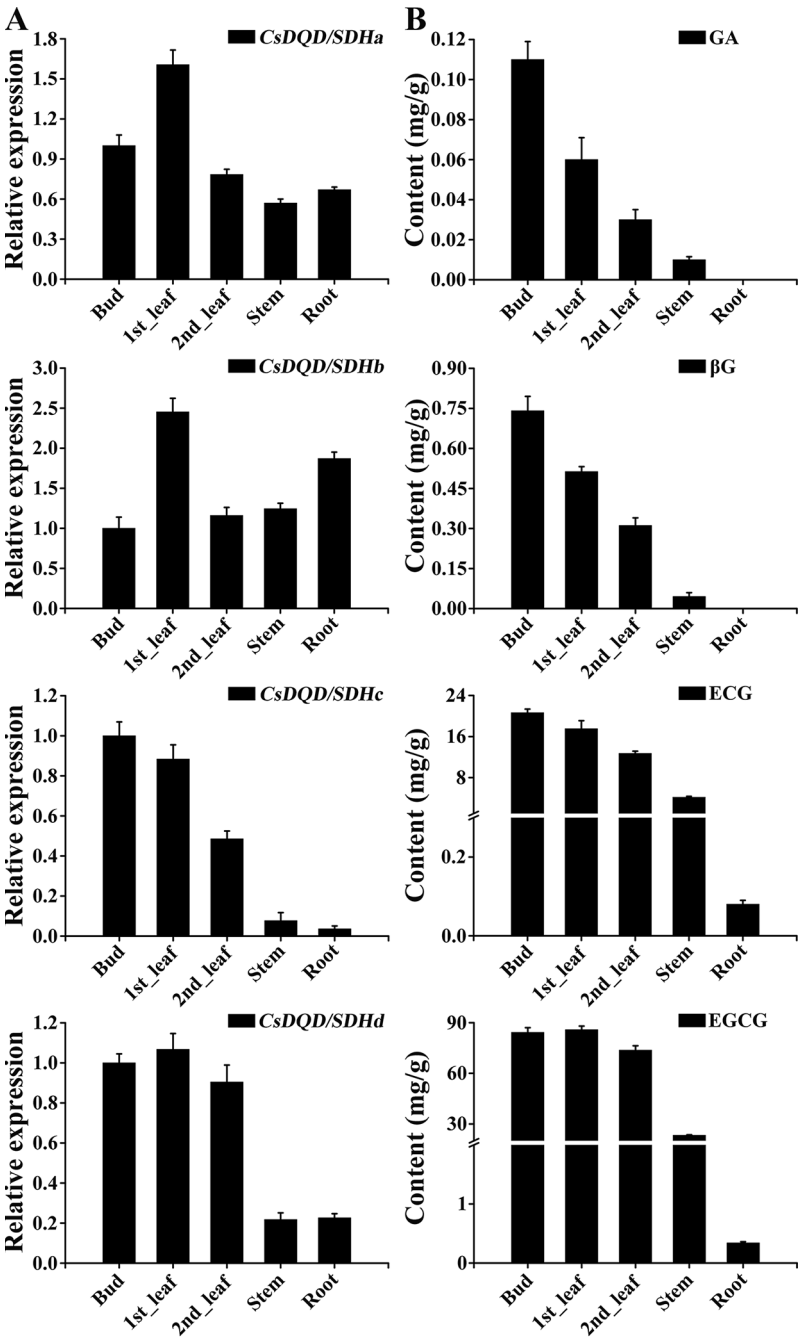
FIGURE 6 | Effect of key unit and site-directed mutagenesis on the activity of *Cs*DQD/SDHa and *Cs*DQD/SDHb, respectively. **(A)** Specific activity analysis of the *Cs*DQDa and *Cs*SDHa truncated mutant proteins. **(B)** Specific activity analysis of the purified MT*Cs*DQD/SDHb mutant protein. Data are presented as the means of three independent assays. Data are shown as the mean of three replicates ± SD. Different letters in **(A)** are statistically different at $P < 0.05$ according to one-way ANOVA of Duncan's test. Asterisks in **(B)** indicate significant difference based on Tukey's test ($P < 0.05$). ANOVA, analysis of variance.

(Jiang et al., 2013; Jiang et al., 2015). Previous studies have shown that a UDP-glycosyltransferases and a serine carboxypeptidase-like (SCPL) acyltransferase participate in the biosynthesis of galloylated flavanol-3-ol and galloyl-type HTs (Gross, 2008).

In plants, DQD/SDH is an essential bifunctional enzyme involved in the SA pathway producing chorismic acid, which is converted into several secondary metabolites (Herrmann, 1995; Maeda and Dudareva, 2012). As a bi-functional or tri-functional enzyme, the DQD/SDH protein plays vital roles in controlling 3-DHS reduction, SA oxidation, and GA synthesis. Plant *DQD/SDH* belongs to the multigene family and may play different roles in 3-DHS reduction, SA oxidation, and GA synthesis (Kasama et al., 2007). Enzyme–substrate kinetic analysis indicated that *Cs*DQD/SDHa had higher catalytic efficiency for 3-DHS reduction than for SA oxidation; therefore, we can assume that *Cs*DQD/SDHa directs carbon flux towards aromatic acids and biosynthesis in the main trunk of the SA pathway, effectively converting 3-DHS into SA. Ding et al. revealed that RNAi-mediated reduction of NtDHD/SHD-1 (in the first group with *Cs*DQD/SDHa) expression resulted in severe metabolic and phenotypic alterations in tobacco plants (Kasama et al., 2007), highlighting the essentiality of DQD/SDHs (from the first group) for plant growth and development. *Cs*DQD/SDHd showed the opposite tendency compared with *Cs*DQD/SDHa, whereas *Cs*DQD/SDHc had almost equal catalytic efficiency for 3-DHS reduction and SA oxidation. All these findings imply that *Cs*DQD/SDHc and *Cs*DQD/SDHd have different functions compared with *Cs*DQD/SDHa *in vivo*; for instance, they might be more suitable for SA oxidation and result in continuous GA generation. In grape berry, the highest expression of *Vv*SDH3 and *Vv*SDH4 occurred in parallel with galloylated PA accumulation (Bogs et al., 2005; Kennedy et al., 2010). *Cs*DQD/SDHc and *Cs*DQD/SDHd generate high amounts of galloylated flavan-3-ols in tea plants (Jiang et al., 2013). Moreover, the phenomenon of the consistency of the expression pattern of *CsDQD/SDHc* and *CsDQD/SDHd* with the accumulation pattern of GA, *βG*, gallocatechin gallate (GCG), and EGCG in tea plants showed that the *CsDQD/SDHc* and *CsDQD/SDHd* genes are involved in GA synthesis. In addition, *Dk*SDH, which clustered with *Cs*DQD/SDHd into the fourth group, was downregulated in nonastringent persimmon fruits (with low galloylated PA levels) compared with astringent persimmon fruits (Ikegami et al., 2005).

The function of *Cs*DQD/SDH in GA synthesis remains unclear. GA could be spontaneously generated from 3-DHS in the enzymatic or nonenzymatic *Cs*DQD/SDHs assay when using 3-DHS and the coenzyme $NADP^+$ as substrates.

Plant DQD/SDH belongs to the second group, which is unique. In tea plants, among the four proteins, the *Cs*DQD/SDHa, *Cs*DQD/SDHc, and *Cs*DQD/SDHd proteins exhibited a mainstream activity for the substrates 3-DHS and SA with the cofactors of NADPH and $NADP^+$, respectively, whereas *Cs*DQD/SDHb exhibited a very low mainstream activity. Site-directed mutagenesis suggested that mutation of residues Ser-338 and NRT to Gly and DI/LD in the SDH unit, respectively, is the reason for the low activity of *Cs*DQD/SDHb for 3-DHS reduction and SA oxidation. Notably, the Poptr2 and Poptr3 proteins from *P. trichocarpa* belonging to the second group quinate dehydrogenase (QDH) activity; these proteins are



C

Genes	GA	βG	ECG	EGCG
CsDQD/SDHa	0.533	0.643	0.679	0.676
CsDQD/SDHb	-0.232	-0.136	-0.096	-0.051
CsDQD/SDHc	0.936*	0.986**	0.983**	0.920*
CsDQD/SDHd	0.724	0.858	0.913*	0.943*

*: There was a significant correlation at 0.05 level (bilateral);
**: There was a significant correlation at 0.01 level (bilateral).

FIGURE 7 | Expression patterns of four *CsDQD/SDH* genes and the accumulation profiles of GA and its derivatives in different tissues of tea plants. **(A)** Expression profiles of the *CsDQD/SDHa*, *CsDQD/SDHb*, *CsDQD/SDHc*, and *CsDQD/SDHd* genes in different organs. **(B)** Quantitative analysis of GA, βG, ECG, and EGCG contents in different tea organs. Data are presented as the means of three independent assays. **(C)** Analysis of the Pearson correlation coefficient of the expression pattern of *CsDQD/SDHs* and the content of GA, βG, ECG, and EGCG. GA, gallic acid; ECG, epicatechin gallate; EGCG, epigallocatechin gallate.

involved in the synthesis of quinate and its derivatives (Guo et al., 2014). GQA, a conjugate of quinic acid (QA) and GA, highly accumulates in tea plants (Jiang et al., 2013). Chlorogenic acid, a QA derivative, is involved in antimicrobial and antiherbivore activities, and it mainly accumulates in the roots of carrot (Cole, 2010b), sweet potato (Mcclure, 1960), and lettuce plants (Cole, 2010a); it is induced by microbe and herbivore infestation. Because of its high sequence identity with Poptr2 and Poptr3, *Cs*DQD/SDHb must be a favorable candidate enzyme for the biosynthesis of QA and its derivatives, and a study examining this postulation is ongoing.

In conclusion, experimental evidence from enzyme assays and kinetic analysis indicates that *CsDQD/SDHc* and *CsDQD/SDHd* are favorable candidate genes for GA biosynthesis in tea plants. This study advances our understanding of GA, and its metabolism is tightly connected to the SA pathway.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the manuscript/ **Supplementary Files**.

AUTHOR CONTRIBUTIONS

KH, ML, LG, and TX conceived and designed research. KH, ML, YL, and YZ performed the real-time PCR experiments. KH, MZ and GZ analyzed the data. YW, LZ, and XD revised the English writing of the manuscript. YW, LZ, and LG revised the English writing of the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01268/full#supplementary-material

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Seasonal Theanine Accumulation and Related Gene Expression in the Roots and Leaf Buds of Tea Plants (*Camellia Sinensis* L.)

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Theanine, a unique and abundant non-proteinogenic amino acid in tea, confers to the tea infusion its umami taste and multiple health benefits. Its content in new tea shoots is dynamic in winter and spring. However, its seasonal accumulation pattern and the underlying regulation mechanism of tea plants remain largely unknown. In this study, we measured the theanine contents in the roots and leaf buds of 13 tea cultivars at four time points from winter to spring (Dec. 12, Mar. 1, Mar. 23, and Apr. 13). We found theanine accumulated significantly in the roots to as high as ~6% dry weight. We found theanine content in the roots was constant or slightly decreased on Mar. 1 compared with Dec.12 but increased consistently on Mar. 23 and then decreased on Apr. 13 in all 13 cultivars. In the leaf buds, theanine content kept increasing from Mar. 1 to Mar. 23 and decreasing from Apr. 13 in most of the 13 cultivars, meaning it was probably both season- and developmental stage-dependent. The expression of theanine biosynthesis and amino acid transporter genes in the roots and buds at the four time points was then examined. The correlation analyses between the gene expression and theanine content suggested the expression of theanine-biosynthesis genes was generally and negatively correlated with theanine content; however, the expression of amino acid transporter genes including *CsLHT* was generally and positively correlated with theanine contents. Finally, we showed that *CsLHT* has theanine transport activity. Taken together, this study provided insight into the seasonal regulation of theanine biosynthesis and transport in tea plants during winter and spring.

Keywords: *Camellia sinensis*, theanine, biosynthesis and transport, seasonal regulation, theanine transporter, CsLHT

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INTRODUCTION

Tea plants are unique; an abundance of health-promoting and pleasant flavor-conferring components, including catechins, caffeine, aroma, and theanine, accumulate in a single tea leaf. This makes tea one of the most popular beverages in the world. Theanine, a non-proteinogenic amino acid, is the most abundant free amino acid in tea, and it confers the tea infusion its umami taste (Ashihara, 2015). Recently, it was shown that theanine derivatives are also critical components of the aroma of oolong tea and large-leaf yellow tea (Guo et al., 2018; Guo et al., 2019). Impressively, many health-promoting effects of theanine, such as its anti-anxiety, anti-tumor, neuron-protecting, and

memory improving properties alongside its antagonistic effect on the negative action of caffeine among other things, have been studied intensively (Sharma et al., 2018). Therefore, theanine content in tea leaves is highly related to the quality and price of teas, especially green teas (Yamaguchi and Ninomiya, 2000).

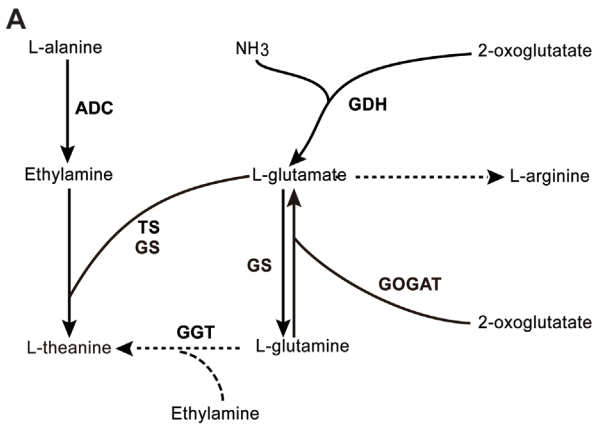
The substrates for theanine biosynthesis are glutamate (Glu) and ethylamine (EA). The enzyme catalyzing theanine synthesis is theanine synthetase (TS) (Sasaoka et al., 1965). Recently Wei and his colleagues identified the *TS* gene (*CsTSI*) in the tea plant (*Camellia sinensis* L.) (Wei et al., 2018). However, Cheng et al. showed that the glutamine synthetases (GS) of tea plants and *Arabidopsis* can also catalyze theanine biosynthesis from Glu and EA (Cheng et al., 2017). In bacteria, theanine can be synthesized from glutamine (Gln) and EA by gamma-glutamyl*transpeptidase* (GGT) (Suzuki and Kumagai, 2002; Suzuki et al., 2002; Yao et al., 2006; Shuai et al., 2010).

EA is derived from the decarboxylation of alanine catalyzed by alanine decarboxylase (AIDA) (Takeo, 1978). Arginine decarboxylase (ADC) was suggested to act as AIDA in tea plants (Shi et al., 2011). Glu is synthesized from Gln and α-ketoglutarate under the catalysis of glutamate synthase (GOGAT). Glu can also be synthesized from ammonium and α-ketoglutarate by Glu dehydrogenase (GDH) in tea plants (Takeo, 1979). The biosynthesis pathway and the enzymes involved in the process are illustrated in **Figure 1A**. Genes coding for enzymes in theanine biosynthesis have been widely identified in tea plants along with the completion of the genome sequencing (Xia et al., 2017; Wei et al., 2018).

Theanine catabolism is catalyzed by theanine hydrolase in tea plants (Tsushida and Takeo, 1985). This process occurs mainly in leaves and is regulated by light (Kito et al., 1968). However, the gene encoding theanine hydrolase has not been identified yet.

In general, plants assimilate nitrogen (N) into Glu, Gln, Asp, and Asn as the main transport forms of N (Fisher et al., 1998). Tea plants are unique for the assimilation and storing of most N in the form of theanine in their roots (Oh et al., 2008). Theanine can account for 60–70% of free amino acid content in the roots and new shoots of tea plants (Takeo, 1981). Tea plants mainly transport Gln, Theanine, and Glu from root to shoot (Oh et al., 2008). Thus, theanine is also a transport in the form of N in tea plants. However, transporters that mediate theanine transport have not been identified in tea plants. As theanine has a similar structure to Gln, amino acid transporters transporting Gln likely have theanine transport activity.

In plants, there are two superfamilies of amino acid transporters: the amino acid/auxin permease (AAAP) and the Amino acid-Polyamine-Choline transporter (APC) family (Tegeder et al., 2007). The AAAP superfamily consists of amino acid permeases (AAPs), lysine, and histidine transporters (LHTs), proline transporters (ProTs), ɣ-aminobutyric acid transporters (GATs), auxin transporters (AUXs), and aromatic and neutral amino acid transporters (ANTs). Vesicular aminergic-associated transporters (VAATs) were recently identified and were shown to modulate amine neurotransmission in animals (Larhammar et al., 2014). The homologs of animal VAATs were identified in the potato genome (Ma et al., 2016). Amino acid transporter-like (ATLs) were also identified in potato and soybean (Cheng et al., 2016; Ma et al., 2016). Both VAATAs and ATLs were classified into the AAAP superfamily. The APC superfamily includes cationic amino acid transporters (CATs), amino acid/choline transporters (ACTs), and polyamine H^+-symporters (PHSs) (Tegeder et al., 2007). Recently, it is reported that members of the Usually Multiple Acids Move In and out Transporters family (UMAMIT) can also transport amino acids (Muller et al., 2015; Besnard et al., 2016, 2018). Members of AAPs, LHTs, ANTs, CATs, and UMAMITs are reported to have varied affinities for Gln. The conserved homologs of these amino acid transporters are the candidate transporters mediating theanine transport from root to new shoot as theanine has a similar structure to Gln.



B

Abbreviation	Full name
ZC102	Zhongcha 102
ZC302	Zhongcha 302
AH3	Anhui 3
FZ2	Fuzao 2
ZN113	Zhenong 113
ZN117	Zhenong 117
MSBH131	Mingshan baihao 131
LJ43	Longjing 43
YS	Yingshuang
DG	Dangui
SDC	Shidacha
WC91	Wancha 91
TGY	Tieguanyin

FIGURE 1 | Putative theanine biosynthesis pathway in tea plant and cultivars used in this study. **(A)** Putative theanine biosynthesis pathway and the enzymes in tea plant. GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; TS, theanine synthetase; GS, glutamine synthetase; GGT, glutamyltranspeptidase; *ADC*, alanine decarboxylase. **(B)** The 13 tea cultivars used in this study. All cultivars were 8-year-olds and were grown in the same tea plantation under the same management.

Previous studies have suggested that theanine is primarily synthesized in the root in the winter (Konishi and Kasai, 1968; Konishi and Takahashi, 1969). Ruan et al. (2012) also observed that theanine concentration in xylem sap increased in the spring. However, how the biosynthesis and transport are regulated during winter and spring is largely unknown. In this study, we investigated the dynamic changes of theanine content and expression of related genes were in the roots and buds of 13 cultivars at four time points of tea leaf bud development. The results demonstrated theanine accumulation patterns in the roots and buds during winter and spring and also provided insight into the regulation of theanine biosynthesis and transport.

MATERIALS AND METHODS

Plant Samples

Leaf buds and unlignified lateral roots were collected from 13 tea plant (*Camellia sinensis* L.) cultivars grown in the Guohe Tea Plantation (Lujiang, Anhui, China) on December 12, 2017 (Dec. 12), March 1, 2018 (Mar. 1), March 23, 2018 (Mar. 23), and April 13, 2018 (Apr. 13). Leaf buds of the representative shoots within the top crown area were sampled. These representative shoots were at a similar developmental stage as the same cultivar. These cultivars included Zhongcha 102 (ZC102), Zhongcha 302 (ZC302), Anhui 3 (AH3), Fuzao 2 (FZ2), Zhenong 113 (ZN113), Zhenong 117 (ZN117), Mingshanbaihao 131 (MSBH131), Longjing 43 (LJ43), Yingshuang (YS), Dangui (DG), Shidacha (SDC), Wancha 91 (WC91), and Tieguanyin (TGY). These tea plant cultivars were all 8-year-old stem cutting clones and the growth status was similar. These tea plants were routinely managed: they were pruned for ~50 cm and given 450 kg of inorganic compound fertilizer (N: P_2O_5: K_2O, 15:15:15) and 150 kg urea per hectare in May, 2017; given 2,250 kg soybean cake fertilizer and 450 kg urea per hectare in October, 2017. The collected samples were immediately frozen in liquid nitrogen and stored at -80°C.

Determination of L-Theanine Content

Theanine was exacted and detected as previously described (Tai et al., 2015) with slight modification. The samples were grounded into a fine powder in liquid nitrogen before being freeze-dried. Then, 50 mg root or 200 mg bud was used to extract theanine, using 5 ml double distilled water, by boiling at 100°C for 20 min and cooling to room temperature. After centrifuging at 6,000 rpm for 10 min, the supernatant was filtered through a 0.22-μm membrane and analyzed by a Waters e2695 HPLC system equipped with 2489 UV/Vis detector. A reverse-phase C18 column (5 μm, 250 mm × 4.6 mm) was used and the column temperature was set at 28°C. The wavelength was set at 210 nm. The mobile phase A was water and phase B was 100% acetonitrile. The gradient elution was as follows: 0 min, 100% A, 0% B; 7 min, 100% A, 0% B; 9 min, 40% A, 60% B; 15 min, 100% A, 0% B; 20 min, 100% A, 0% B. The HPLC system was injected with 10 μl of the extraction for measurement. The amount of theanine was determined according to a calibration curve of authentic theanine standard, purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Total RNA Extraction and Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted using a RNAprep Pure Plant Kit (Polysaccharides&Polyphenolics-rich) (TIANGEN, China) according to manufacturer instructions. First-strand cDNA was synthesized using the HiScript® II One Step RT-PCR Kit with gDNA Eraser (Vazyme, China). The 20-μl quantitative real time polymerase chain reaction (qRT-PCR) analysis contained 0.4 μl of forward and reverse primers (10 μM), 1 μl of eight-fold diluted first strand cDNA as template, and 10 μl of SYBR Green Supermix (Vazyme, China). Reactions were performed in a 96-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s on a Bio-Rad CFX96. A melting curve was generated for each sample after each run to ensure the purity of the amplified products. No-template controls were included for each primer pair in each run. Relative transcript levels were calculated based on the $2^{-\Delta Ct}$ method (Schmittgen et al., 2000), using *CsGAPDH* as an internal control. All primers used for qRT-PCR are listed in **Table S4**.

Identification and Selection of Theanine Biosynthesis and Transport-Related Genes for qRT-PCR Analysis

Theanine biosynthesis pathway genes, including *CsGDHs*, *CsGOGAOTs*, *CsADCs*, *CsTSI*, *CsGGT*, and *CsGSs* were identified and named by Wei et al. (2018). Theanine transport-related genes were identified and named by sequence homology with genes encoding amino acid transporters with glutamine transport activity in *Arabidopsis*. The nucleotide sequences of all these genes were downloaded from a tea plant genome database (Xia et al., 2019, http://teaplant.org/).

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD) of three independent biological replicates. The correlation coefficient between theanine content and gene expression was analyzed *via* Pearson correlation.

Yeast Expression, Transformation, and Theanine Transport Measurement

The ORF of *CsLHT* was amplified by PCR from first-strand cDNA of RNA extracted from roots of tea plant (*Camellia sinenesis*, *cv*, Shuchazao) and cloned with HindIII/EcoRI in the yeast expression vector pYES2. The yeast strain 22Δ10α mutant (23344c background) was transformed with pYES2-*CsLHT6* or the empty vector pYES2 using the yeast transformation kit (ZYMO RESEARCH, USA). Transformants were selected on yeast base media (DifcoTM Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate, BD, USA) lacking uracil and supplemented with 2 mM ammonia sulfate.

For theanine transport activity determination, yeast cells were grown on a YNB medium to OD_{600} = 0.8. Yeast cells were collected and washed three times. Before theanine feeding, cells were cultured in YNB liquid medium with no nitrogen source for 1 h, at 30°C. This yeast solution was aliquoted into 4 ml tubes and

added to 2 mM or 5 mM theanine for 5 min. The collected yeast cells were washed four times with deionized water. Then, 1 ml deionized water was used to resuspend cells and heated at 98°C for 1 h to extract theanine. After centrifugation at 13,000 rpm for 5 min, the supernatant was collected and filtered through a 0.22 μm membrane for a HPLC-based measurement of theanine.

RESULTS

Theanine Accumulation in the Roots of 13 Tea Cultivars During Winter and Spring

To investigate the dynamic theanine accumulation in roots during winter and spring, we measured theanine contents in the roots of 13 tea cultivars on Dec. 12, Mar. 1, Mar. 23, and Apr. 13. The cultivars and the abbreviations of their names are listed in **Figure 1B**. These four time points are representative dates of winter time (Dec.12), leaf bud sprout (Mar. 1), leaf bud harvested for high-quality green tea production (Mar. 23), and leaf quality decreasing for green tea production (Apr. 13), respectively, in the major zone of green tea production in China.

Interestingly, we observed a consistently changing pattern of theanine content in the roots of these 13 cultivars (**Figures 2A**, **B**): slightly decreased on Mar. 1 compared with Dec. 12; obviously increased and reached the highest on Mar. 23 in all these cultivars; deceased on Apr. 13. These results suggested theanine accumulation in the roots of the 13 cultivars was dynamically and consistently regulated.

Expression of Theanine Biosynthesis Pathway Genes in Roots During Winter and Spring

To explore the regulation of the dynamic changes of theanine accumulation in roots, we examined the expression of theanine biosynthesis pathway genes in five cultivars with high (AH3), medium (DG, TGY and Shuchazao [SCZ]), and low (ZN113) levels of theanine in the roots. The contents of theanine in the roots of SCZ at the four time points were reported by Dong et al. (2019). These theanine-biosynthesis genes included three *CsGDHs*, three *CsGOGAOTs*, three *CsADCs*, *CsTSI*, *CsGGT*, and four *CsGSs*, which were named by Wei et al. (2018).

The expression of genes encoding CsGDHs and CsGOGATs, which catalyze Glu synthesis, was examined first. We found *CsGDHs* were generally increased in spring (Mar. 1, Mar. 23, and Apr. 13) compared with Dec. 12, especially that of *CsGDH2* and *CsGDH3* in these cultivars (**Figure 3A**). Interestingly, *CsGDH4* expression increased on Mar. 1 or Mar. 23 and then reduced on Apr. 13 in most of these cultivars. This changing pattern of *CsGDH4* expression was similar to that of theanine content (**Figure 2B**). By contrast, *CsGOGAT1* expression was highest on Mar. 1 and then reduced on Mar. 23 in four out of five cultivars; next, it increased on Apr. 13 in three out of five cultivars. Thus, the changing pattern of *CsGOGAT1* expression was opposite to that of theanine content during these four time points. In addition, *CsGOGAT2* and *CsGOGAT3* expression was relatively stable during winter and spring. These results indicated that different homologs of *CsGDHs* and *CsGOGATs* respond differently to seasonal factors, suggested that there exist varied roles in glutamate and theanine synthesis during winter and spring in tea plants.

CsADCs were proposed to catalyze EA synthesis (Shi et al., 2011). *CsADC1* and *CsADC2* expression was then examined. Interestingly, *CsADC1* expression was generally induced on Mar. 1 and was then gradually repressed on Mar. 23 and Apr. 13, whereas *CsADC2* expression was continuously induced on Mar. 1 and Mar. 23 and then repressed on Apr. 13 in most of these cultivars (**Figure 3B**). Thus, the expression of *CsADC2* generally followed the pattern of theanine content in the roots.

CsTSI and CsGSs are the characterized enzymes catalyzing theanine biosynthesis from Glu and EA (Cheng et al., 2017; Wei et al., 2018). CsGGT is a putative enzyme catalyzing Gln and EA into theanine. Compared with other theanine biosynthesis pathway genes, the expression of *CsGSs*, *CsTS1*, and *CsGGT* was relatively stable in the roots of these cultivars at these four time points (**Figure 3C**), suggesting these genes do not obviously respond to seasonal factors during winter and spring.

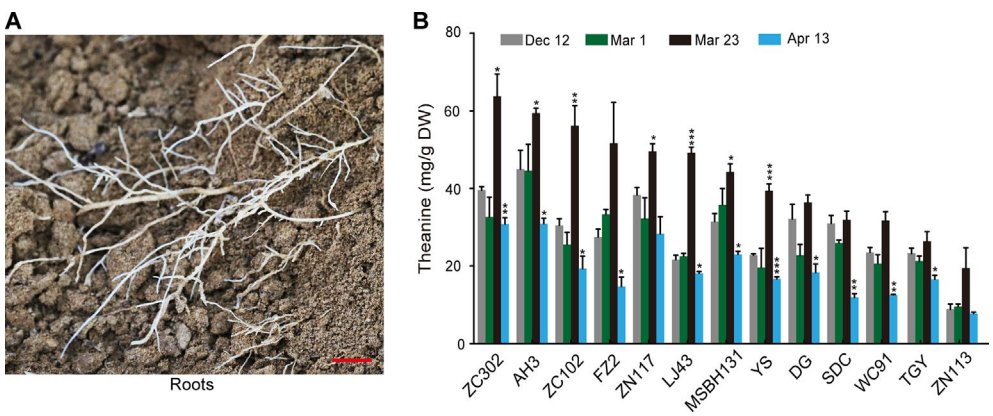


FIGURE 2 | Theanine content in the roots of 13 cultivars at four time points. **(A)** Representative root sample collected. Scale bar: 1 cm **(B)** Theanine content in the roots. Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01, ***p 0.01).

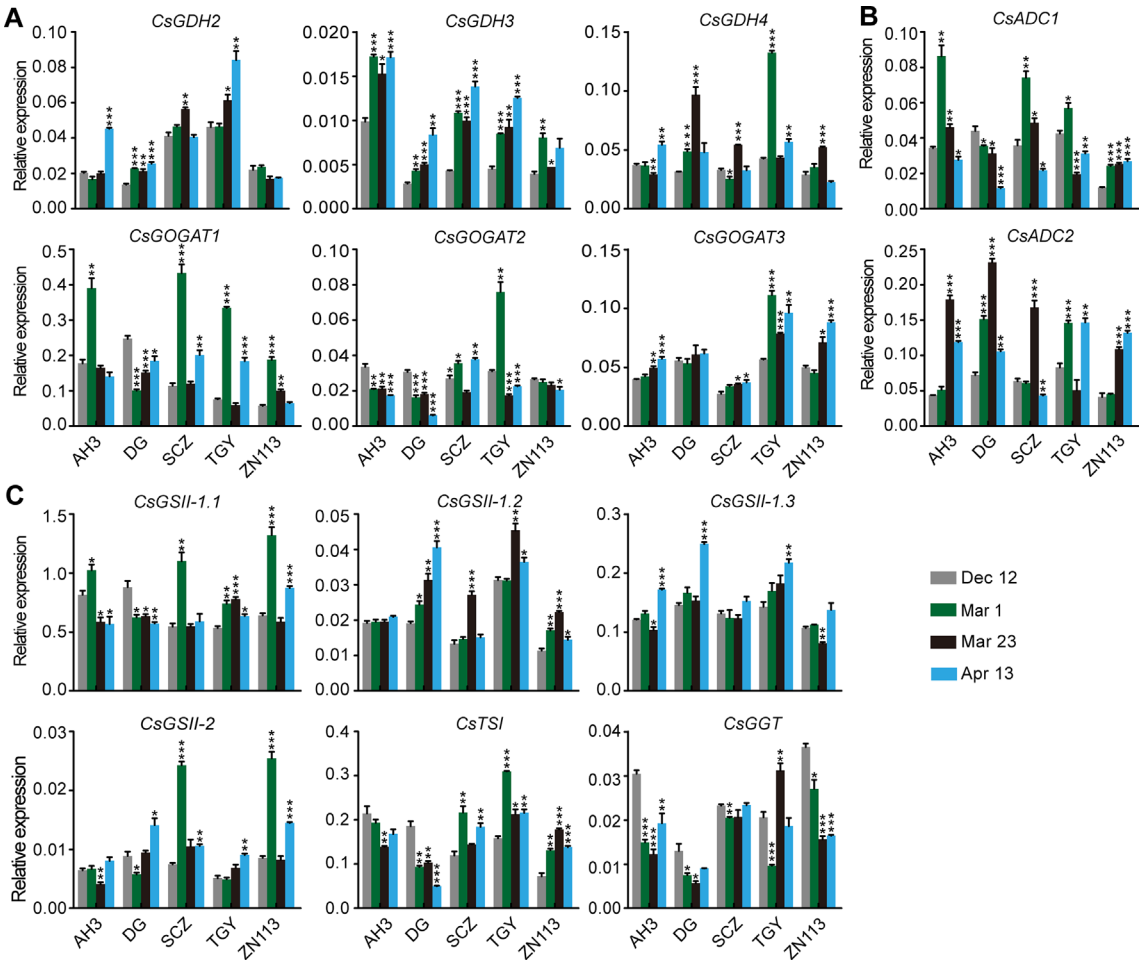


FIGURE 3 | Relative expression of genes in theanine biosynthesis pathway in the roots of five representative cultivars at the four time points. **(A)** Relative expression of *CsGHDs* and *CsGOGATs*. **(B)** Relative expression of genes *CsADCs*. **(C)** Relative expression of *CsGSs*, *CsTSI*, and *CsGGT*. *CsGAPDH* was used as an internal control.Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01, ***p 0.01).

Correlation Between the Expression of Theanine Biosynthesis Pathway Genes and the Theanine Contents in Roots at Each Time Point

To get further insight into the regulation of theanine biosynthesis in the roots, we next analyzed the correlation between the gene expression and the theanine contents in the roots of AH3, DG, SCZ, TGY, and ZN113 at each time point. The correlation coefficient and *p* values are listed in **Table S1**. Here, we found the expression of these theanine biosynthesis pathway genes was generally and positively correlated with the theanine contents, with 10 out of 14 genes showing positive correlation on Dec. 12 (**Figure 4A**). In great contrast, the expression of most of these genes showed negative correlation with theanine contents on Mar. 1 and Mar. 23. Interestingly, on Apr. 13, most of these genes returned to a positive correlation with theanine contents. Given that the theanine level was relatively lower on Dec. 12, increased in Mar., and decreased on Apr. 13, the pattern of correlation between gene expression and theanine contents suggested theanine biosynthesis is generally feedback regulated in the roots.

Although the expression of the genes examined was generally feedback related with theanine contents in the roots, we found *CsGDH3* and *CsGOGAT1* always positively, and *CsGSII-2* and *CsGOGAT3* always negatively, correlated with theanine content in the roots at all four time points (**Figure 4B**). These results suggested the positive role of *CsGDH3* and *CsGOGAT1* and negative role of *CsGSII-2* and *CsGOGAT3* in theanine synthesis in the roots.

Further, we observed that the expression of *CsGOGAT2* and *CsTSI* showed positive, reduced positive or negative, and higher negative correlation with the theanine contents in the roots on Dec. 12, Mar. 1, and Mar. 23, respectively. Subsequently, *CsTSI* showed a positive correlation with theanine contents on Apr. 13 (**Figure 4B**) when theanine contents decreased in the roots (**Figure 2B**). These results suggested *CsGOGAT2* and *CsTSI* was involved in the feedback regulation of theanine biosynthesis.

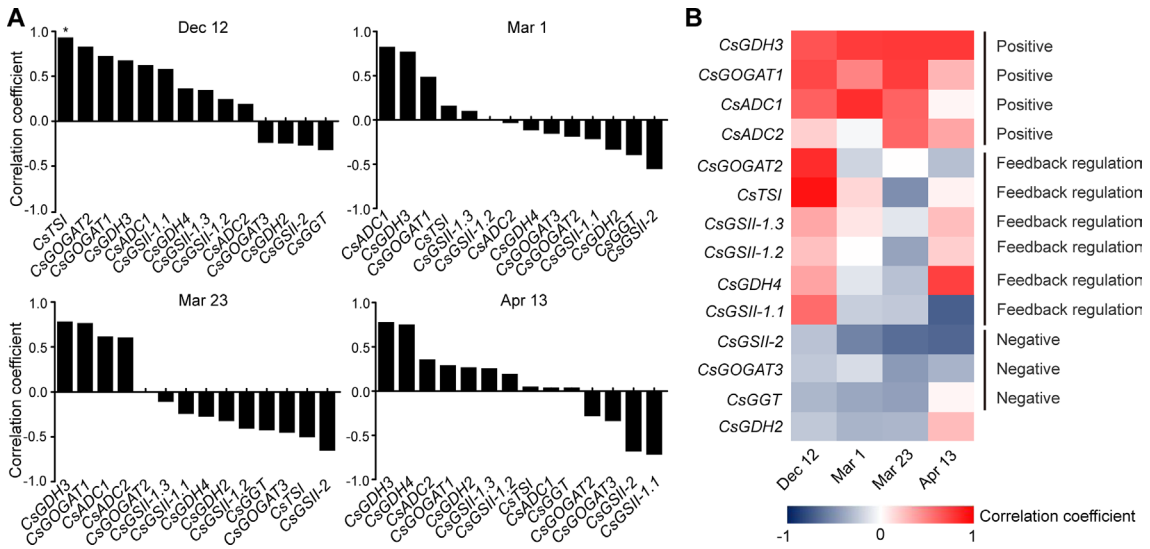


FIGURE 4 | Correlation coefficient between the expression of theanine-biosynthesis pathway genes and the theanine content in the roots of five representative cultivars at each time point. **(A)** Correlation coefficients at each time point. Asterisks represent statistical significance determined by Student's t-test (*p 0.05). **(B)** Heat map of the Correlation coefficients.

Dynamic Accumulation Pattern of Theanine in the Buds of the 13 Cultivars During Winter and Spring

Leaf buds contain the highest concentration of theanine within the tissues in shoots (Feldheim et al., 1986) and are the main tissue harvested for high-quality green tea production. To explore how theanine accumulation in leaf buds changes during winter and spring, we next measured the theanine content in the buds of the 13 cultivars at the four time points (Dec. 12, Mar. 1, Mar. 23 and Apr. 13) (**Figure 5A**). We observed that theanine content in the buds was low (~5 mg/g dry weight) on Dec. 12 in all these cultivars (**Figure 5B**). The content increased on Mar. 1, reached the highest levels on Mar. 23, and decreased on Apr. 13 in most of these cultivars. These results indicated theanine content in the buds generally started to increase in early Mar. and increase to the highest levels in late Mar. or early Apr. before decreasing. This is probably why the quality of green tea produced in Mar. and early Apr. is the highest, only for it to decrease from the middle of Apr.

However, we found that, in ZN117, MSBH131, DG, and TGY, theanine contents in the buds continued to increase on Apr. 13 (**Figure 5B**). In addition, in ZC302, ZN113, and FZ2, theanine content in buds just slightly decreased on Apr. 13 compared with that of Mar. 23. In contrast, in YS, ZN102, LJ43, WC91, AH3, and SDC, theanine content greatly decreased on Apr. 13. We hypothesized that this inconsistency was caused by the different developmental stages of the buds of these cultivars. To test this hypothesis, we evaluated the development stages of buds on Mar. 23 in these cultivars. We defined bud developmental stages I, II, III, and IV, as illustrated in **Figure 5C**. Interestingly, we found most of the buds of YS, ZN102, LJ43, and WC91 were in Stage III and IV, suggesting an early sprout time for the buds of these cultivars. However, most of the buds of ZN117, MSBH131, DG, TGY, ZC302, ZN113, and FZ2 were in Stage I and II (**Figure 5D**), suggesting a late sprout time for the buds of these cultivars. Therefore, the dynamic change of theanine content in these buds is highly associated with both seasons and bud developmental stages.

Expression of Theanine Biosynthesis Pathway Genes in Buds During Winter and Spring

Although theanine is mainly synthesized in the root, Deng and colleagues showed that tea plant shoots also have a weak theanine synthesis capacity (Deng et al., 2009). To explore the molecular mechanism of the dynamic changes of theanine in the buds, we next investigated the expression of theanine biosynthesis pathway genes in the buds of AH3, ZN113, TGY, DG, and SCZ. Theanine content in the buds of SCZ at the four time points was reported by Dong et al. (2019).

We observed that the expression of *CsGDH4*, *CsGOGAT1*, *CsGOGAT3*, *CsTSI*, and *CsGGT* was suppressed on Mar. 1 and Mar. 23 and was increased on Apr. 13 (**Figures 6A–C**). These results—indicated in the changed pattern of the expression of these genes—were generally opposite to those of the theanine content in the buds (**Figure 5B**) and implied that the expression of these genes was probably feedback regulated by theanine content in the buds.

In contrast to the feedback-regulated genes, *CsGSII-1.2* was gradually repressed in the buds from Dec. 12 to Apr. 13 (**Figure 6C**). *CsGSII-2* expression, however, was stable on Dec. 12, Mar. 1, and Mar. 23 and was induced on Apr. 13 in all five cultivars. Therefore, *CsGSII-1.2* and *CsGSII-2* expression in the buds was likely regulated by seasonal factors, including temperature and light, but was not regulated by the theanine

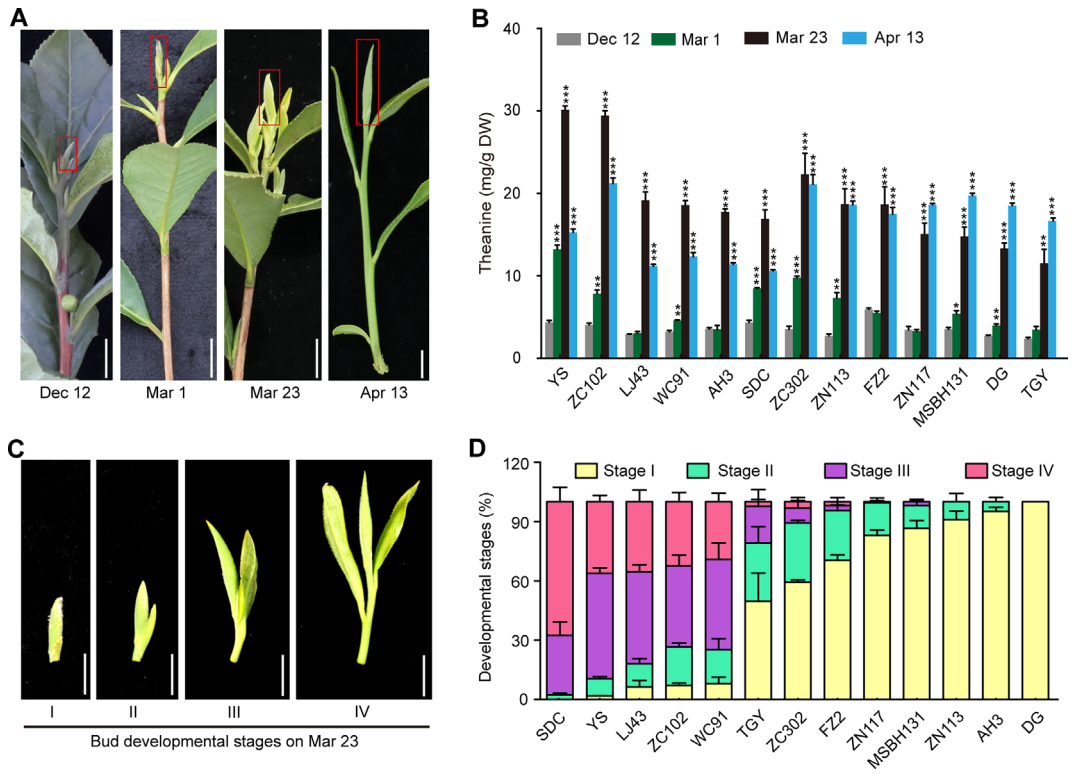


FIGURE 5 | Theanine contents in the buds of 13 cultivars at four time points and the developmental stages of the buds on Mar. 23. **(A)** Representative bud samples collected at four time points. Red boxes highlighted the buds collected. **(B)** Theanine content in the buds at the four time points. Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01, ***p 0.01). **(C)** Buds of 13 cultivars on Mar. 23 were classified into four developmental stages, as shown. **(D)** Statistic results of the buds of the 13 cultivars at four developmental stages on Mar. 23. Data represent means ± SD of three biological replicates. Scale bars in **(A)** and **(C)**, 1 cm.

content in buds. In addition, *CsGOGAT2* and *CsGSII-1.1* expression in the buds was generally stable at all these four time points (**Figures 6A**, **C**). This result suggested *CsGOGAT2* and *CsGSII-1.1* was likely not regulated by theanine content in the bud.

Correlation Between the Expression of Theanine Biosynthesis Pathway Genes and Theanine Content in the Buds at Each Time Point

To investigate the regulation of theanine-biosynthesis gene expression in the buds, we also analyzed the correlation between gene expression and theanine content at each time point. The correlation coefficient and *p* values are listed in **Table S2**. Similarly to the situation in the roots, most of the genes had a positive correlation with theanine content in the buds (**Figure 7A**), when theanine content in the buds was low; next, 10 and 12 out of 14 genes had a negative correlation on Mar. 1 and Mar. 23, when theanine content in the buds increased, respectively. Furthermore, the number of genes that had a negative correlation reduced to six on Apr. 13 when theanine content decreased. These results suggested the expression of theanine biosynthesis pathway genes was also generally feedback regulated by the theanine content in buds.

Next, we analyzed the correlation of individual genes. We observed that *CsGDH2/3/4*, *CsGOGAT1/2*, *CsADC1/2*, and *CsGGT* showed positive correlation when theanine content was low, negative correlation when theanine content increased, and positive correlation when theanine content decreased in the buds (**Figure 7B**). These results suggested that the expression of these eight genes was typically feedback regulated by theanine levels in the buds.

Interestingly, *CsGSII-1.1/2/3* was almost always negatively correlated with theanine contents in the buds (**Figure 7B**), suggesting a negative role of these three genes in theanine biosynthesis in the buds. Finally, the fact that no gene showed continuously positive correlation with theanine contents in the buds supported the notion that buds have a minor role in theanine biosynthesis in tea plants.

Expression of Amino Acid Transporter Genes in the Roots During Winter and Spring

Amino acid long-distance transport is mediated by amino acid transporters (Tegeder, 2014). However, the molecular mechanism of theanine root-to-shoot transport is still largely unknown. To explore this mechanism, we next examined the expression of amino acid transporter genes in the roots of the five

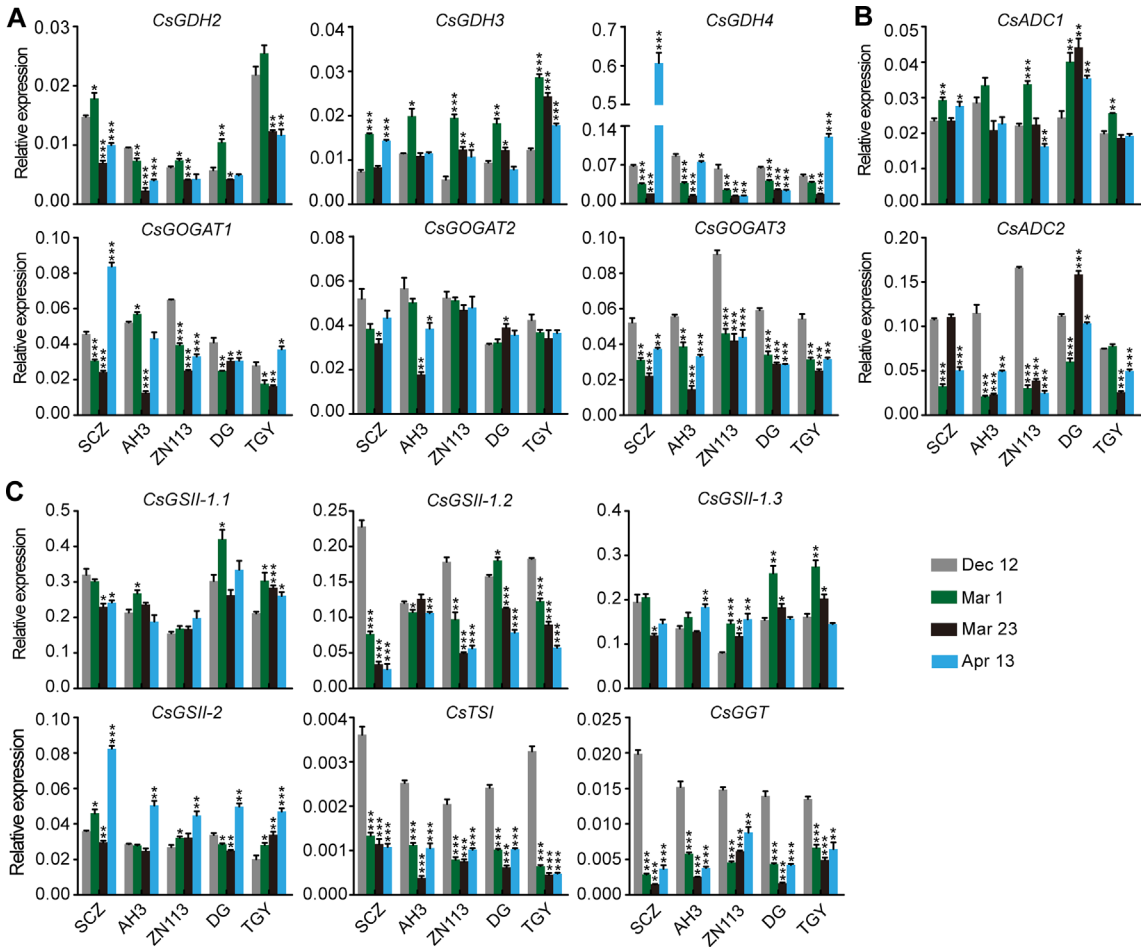


FIGURE 6 | Relative expression of genes in theanine-biosynthesis pathway in the buds of five representative cultivars, at the fourtime points. **(A)** Relative expression of *CsGHDs* and *CsGOGATs*. **(B)** Relative expression of genes *CsADCs*. **(C)** Relative expression of *CsGSs*, *CsTSI*, and *CsGGT*. *CsGAPDH* was used as an internal control. Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01, ***p 0.01).

cultivars. These examined amino acid transporter genes included members of AAP, LHT, ProT, PUT, AUX, UMAMIT, VAAT, and ATL families.

We found that the expression of *CsAAP9*, *CsVAAT*, *CsAUX*, and *CsATL* was gradually induced in the roots of most of these cultivars through the four time points (**Figure 8**). By contrast, the expression of *CsProT* and *CsPUT2* was gradually repressed. Although *CsLHT* was expressed at different levels in these cultivars, its expression levels were relatively stable through the four time points. Similarly, *CsAAP2* expressed differentially but stably in these cultivars on Dec. 12, Mar. 1, and Mar. 23, but was great repressed on Apr. 13.

It is worth noting that theanine content in roots as slightly decreased on Mar. 1, increased on Mar. 23, and then decreased on Apr. 13 (**Figure 2B**). Thus, the change in the expression of these genes was generally different from that of theanine content in the roots. One exception was *CsUMAMIT*; this was generally induced when theanine content decreased and was repressed when theanine content increased in the roots (**Figure 8**), suggesting feedback regulation of its expression by theanine content in the root.

Correlation of Amino Acid Transporter Gene Expression With Theanine Content in the Roots During Winter and Spring

To further investigate the regulation of theanine transport, we next analyzed the correlation of *CsAATs* expression with theanine content at each time point in the roots of the five representative cultivars. The correlation coefficient and *p* values are listed in **Table S3**. Different from theanine biosynthesis pathway genes, most of the *CsAATs* showed positive correlation with theanine content in the roots at all these four time points (**Figures 9A**, **B**).

When analyzed individually, we found *CsPUT2* was always highly and positively correlated with theanine content in the roots at these four time points (**Figures 9A**, **B**), suggesting a continuous positive role of CsPUT2 in theanine transport. Although *CsLHT*

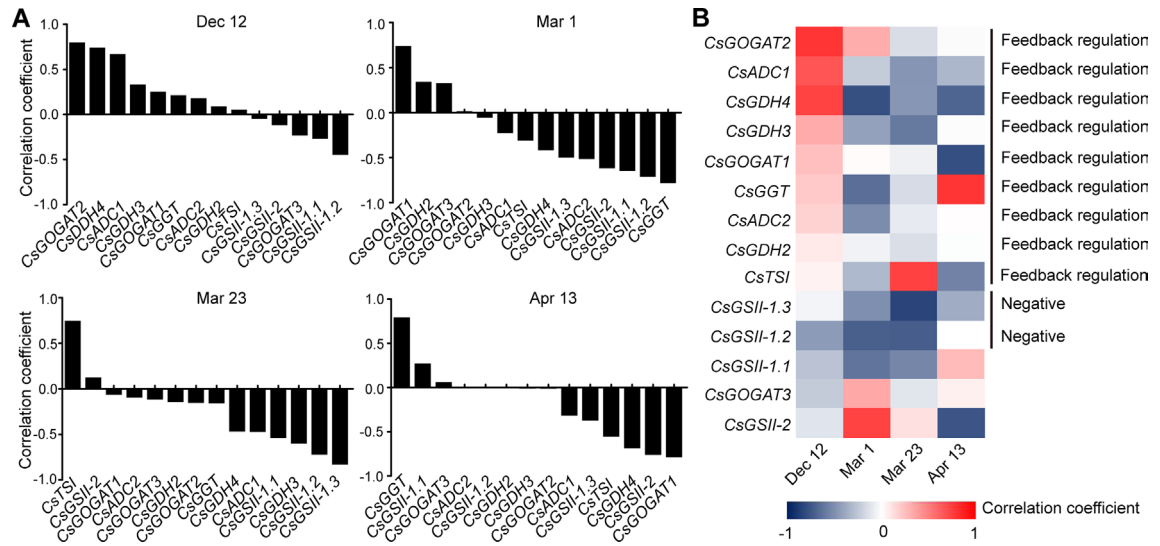


FIGURE 7 | Correlation coefficient between the expression of theanine-biosynthesis pathway genes and the theanine content in the buds of five representative cultivars at each time point. **(A)** Correlation coefficients at each time point. Statistical analysis was performed and there was no statistical significance, but the correlation pattern is consistent and valuable. **(B)** Heat map of the correlation coefficients.

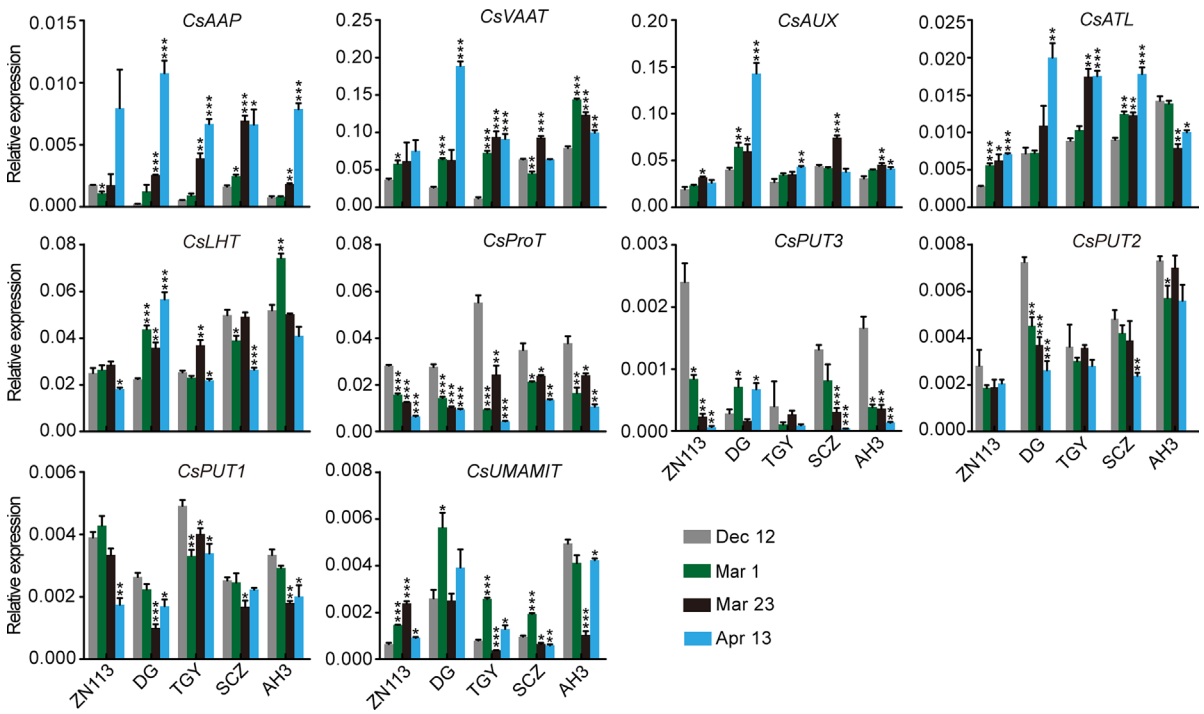


FIGURE 8 | Relative expression of amino acid transporter genes in the roots of five representative cultivars, at the four time points. *CsGAPDH* was used as an internal control. Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01, ***p 0.01).

and *CsVAAT* had positive correlation at all the four time points, they had higher positive correlation in Mar. and lower positive correlation on Apr. 13. This result suggested the positive role of CsLHT and CsVAAT in theanine transport was feedback promoted. Although most of these genes positively correlated with theanine contents in the roots, *CsPUT1*and *CsAAP9* had negative correlation on Dec. 12, Mar. 1, and Mar. 23, suggesting CsPUT1 and CsAAP had a negative role in theanine transport.

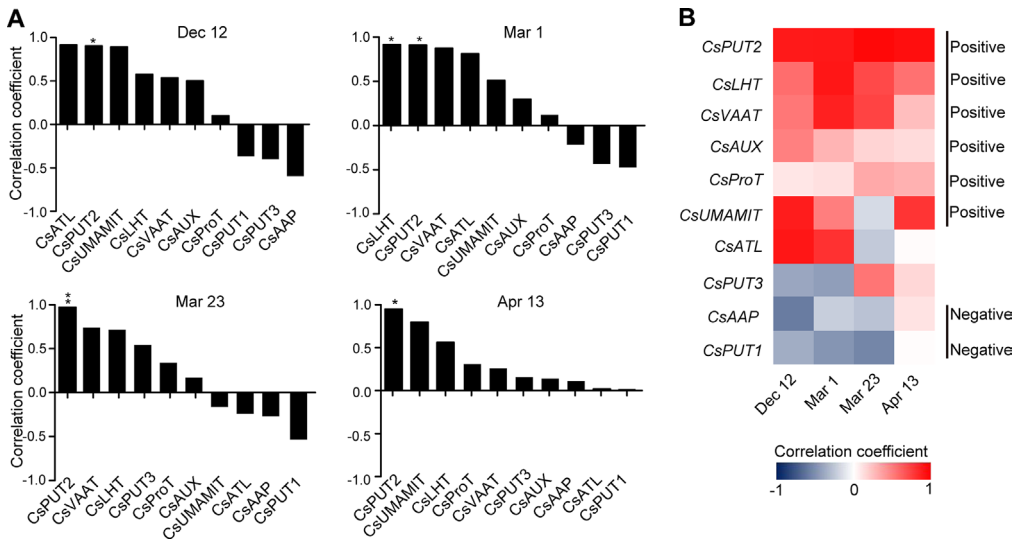


FIGURE 9 | Correlation coefficient between the expression of amino acid transporter genes and the theanine contents in the roots of five representative cultivars at each time point. **(A)** Correlation coefficients at each time points. Asterisks represent statistical significance determined by Student's t-test (*p 0.05, **p 0.01). **(B)** Heat map of the correlation coefficients.

CsLHT Can Transport Theanine

Theanine content in xylem sap increases in spring (Ruan et al., 2012), suggesting there is root-derived theanine transport to the shoot *via* the xylem. To explore the molecular mechanism of theanine root-to-shoot transport, it is necessary to identify what theanine transporter mediated this process. Given that *CsPUT*, *CsLHT*, and *CsVAAT* expression was positively correlated with theanine content in the roots (**Figure 9B**), CsPUT, CsLHT, and CsVAAT were candidates of theanine transporters. As LHT-family amino acid transporters have a high affinity for neutral and acidic amino acids and have been well studied in plants (Tegeder and Ward, 2012), we then examined whether CsLHT can transport theanine.

The yeast 22Δ10Δα strain is an amino acid uptake mutant lacking an amino acid uptaking capacity due to the mutations of 10 amino acid transporter genes (Besnard et al., 2016). To test the theanine transport activity of CsLHT, we expressed *CsLHT* in 22Δ10Δα with an empty vector pYES2 as a negative control and wild-type yeast strain 223344c as a positive control. Under the feeding of 2 or 5 mM theanine, we measured contents of theanine accumulated in these yeast strains. We found wild-type yeast accumulated the highest levels of theanine under both 2 and 5 mM theanine feeding (**Figures 10A**, **B**); *CsLHT*-expressing yeast cells only accumulated theanine under 5 mM theanine feeding. No theanine accumulation, however, was detected in the negative control strain. These results indicated that CsLHT can transport theanine under a higher theanine concentration.

DISCUSSION

Theanine Accumulation Pattern in Root and Bud of Tea Plant in Winter and Spring

As one of the most important components of quality in green tea, theanine and its biosynthesis has been studied intently in tea plants since it was discovered by Yajiro Sakato in 1949 (Ashihara, 2015; Mu et al., 2015). These studies indicated that theanine accumulation was dynamically regulated by seasons (Konishi and Takahashi, 1969; Takeo, 1981; Janet et al., 2015), light (Yang et al., 2012; Deng et al., 2013; Chen et al., 2017; Ji et al., 2018; Sano et al., 2018), nutrient levels (Takeo, 1981; Ruan et al., 2007; Ruan et al., 2010; Ruan et al., 2012; Huang et al., 2018; Kc et al., 2018; Zhu et al., 2019), hormones (Li et al., 2016a), developmental stages (Feldheim et al., 1986; Li et al., 2016b; Liu et al., 2017), and stress (Deng et al., 2012; Wang et al., 2016). Theanine accumulation is also highly tissue-and cultivar-dependent (Fang et al., 2017; Liu et al., 2017; Chen et al., 2018a; Chen et al., 2018b). Theanine accumulation, therefore, is dynamic and highly regulated.

Most of the studies on theanine accumulation under various conditions were performed in one cultivar. In addition, when examining the effects of seasons, comparisons were made between spring, summer, and autumn. Furthermore, because new shoots or tender leaves are the harvested targets for tea, most of the studies focused on theanine accumulation in leaves. However, how theanine accumulation in roots and new shoots changes, during winter and spring, in various cultivars remains to be observed.

In this study, we clearly observed that theanine content in the roots of 13 cultivars slightly deceased or kept stable from Dec. 12 to Mar. 1 and then increased from Mar. 1 to Mar. 23, followed by an obvious decrease on Apr. 13 (**Figure 2**). This is slightly different from the Takeo's report in 1979. He observed that the theanine content in roots slightly increased from Jan. to Feb. and then continuously decreased from Feb. to May; he subsequently concluded that theanine was mainly accumulated in winter. Our results, however, suggested that theanine content in roots is highest in late of Mar. or early Apr. and then decreases from the middle of Apr. This inconsistency may be caused by geographical factors or the origin area of the cultivars (Lee et al.,

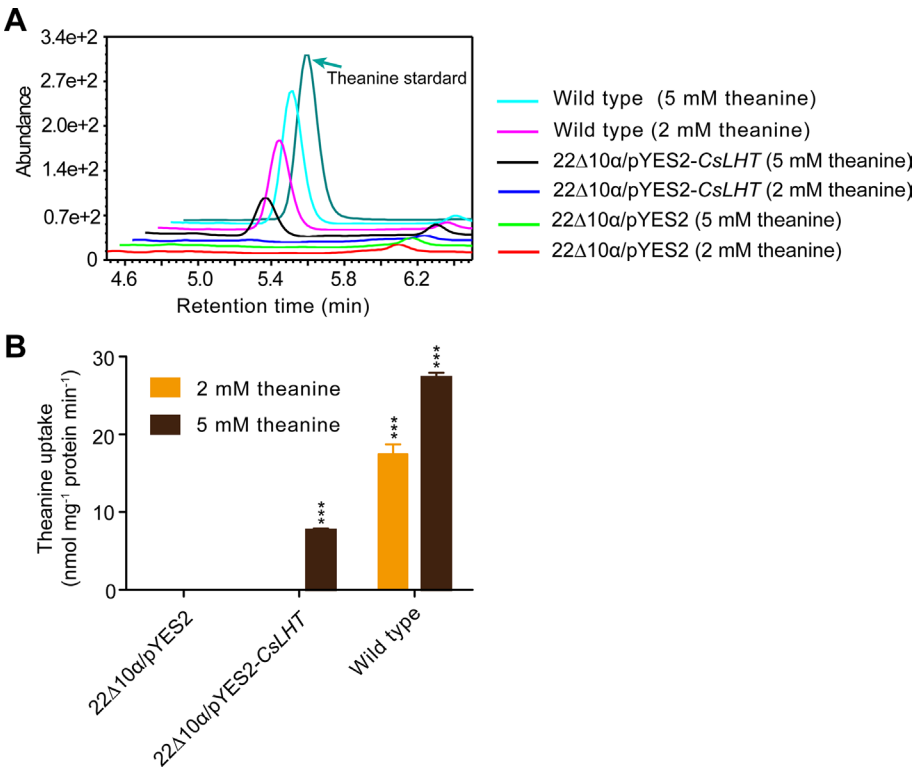


FIGURE 10 | CsLHT transported theanine into yeast cells. **(A)** HPLC identification theanine in the yeast mutant strain 22Δ10α mediated by CsLHT. Yeast transformed with empty vector was used as a negative control and wild type yeast strain (23344c) as a positive control. **(B)** Determination of the theanine contents in 22Δ10α/pYES2, 22Δ10α/pYES2-*CsLHT* and wild-type yeast cells. Data represent means ± SD of three independent biological replicates. Asterisks represent statistical significance determined by Student's t-test (***p 0.01).

2010; Chen et al., 2018a). In addition, our results also indicated that the theanine levels in roots are likely controlled by genetic background; however, the changing pattern is mainly regulated by seasonal factors (**Figure 2**).

Different from that in roots, theanine content in buds was low on Dec. 12 and started to increase on Mar. 1 (**Figure 5**). Theanine content reached the highest in most of the cultivars on Mar. 23 and decreased on Apr. 13. But, in some cultivars, theanine content kept increasing on Apr. 13. Further analyses indicated the different changing patterns of theanine content on Mar. 23 and Apr. 13 were associated with bud sprout time and developmental stages. Therefore, theanine content in buds is regulated by genetic background, seasonal factors, and developmental stages. Here, it is also noteworthy that theanine content decreased quickly in April, and this is highly associated with the decline in green tea quality made from the new buds.

Regulation of the Expression of Theanine Biosynthesis and Amino Acid Transporter Genes

Many previous studies showed that the expression of theanine biosynthesis pathway genes was not always positively correlated with theanine content (Ruan et al., 2010; Fang et al., 2017; Kc et al., 2018; Zhu et al., 2019). Kc et al. found the expression of most theanine biosynthesis pathway genes was negatively correlated with theanine content in leaves (Kc et al., 2018). These results led us to explore the regulation of the expression of theanine biosynthesis and transport pathway genes in response to dynamic theanine accumulation in roots and buds.

In this study, we analyzed the correlation between the expression of theanine biosynthesis pathway genes and theanine content in roots and buds at four time points. These results demonstrated that the expression of theanine biosynthesis pathway genes is generally feedback correlated with theanine content in both roots and buds of tea plants (**Figures 4** and **7**). These results suggested that the expression of theanine biosynthesis genes is generally feedback regulated at transcription level by theanine levels. This is consistent with the regulation of amino acid biosynthesis genes in model plants (Pratelli and Pilot, 2014).

Although the expression of genes in theanine-synthesis pathways is generally feedback regulated in both roots and buds, the feedback regulation in buds is more profound than in roots. In the 14 genes examined, eight genes were feedback regulated in buds; in contrast, the number was six in the roots (**Figures 4** and **7**). More importantly, four genes were always positively correlated with theanine contents in the roots; however, no gene that was always positively correlated was identified in buds.

The molecular mechanisms of theanine transport are largely unknown in tea plants. In this study, we found the expression

of most amino acid transporter genes examined was positively correlated with theanine content in the roots. These results implied that amino acid transporter genes are induced by theanine accumulation. This result also provided candidate genes for identifying theanine transporters.

Putative Key Genes Involved in the Regulation of the Dynamic Biosynthesis

In this study, we observed that the expression of genes in the same family correlated differently with theanine contents (**Figures 4**, **7**, **9**). For example, *CsGDH3* showed continuously positive correlation with theanine content in the roots; *CsGDH4*, however, was always negatively correlated with theanine content in the roots. Similar correlation results were also observed by other reports (Li et al., 2016b; Liu et al., 2017; Zhu et al., 2019). These results indicated that genes in the same family were differently regulated and also suggested varied roles for these genes in theanine biosynthesis and transport.

In addition to this, the correlation degrees were also varied for different genes in theanine biosynthesis pathways and amino acid transporters (**Figures 4**, **7** and **9**). These results suggested *CsGDH3*, *CsGOGAT1*, *CsADC1*, and *CsADC2* have important roles in the positive regulation of theanine biosynthesis in roots by providing glutamate and ethylamine, two direct substrates of theanine biosynthesis.

CsLHT May Mediate Theanine Transport in Tea Plants

The high correlation of *CsPUT2*, *CsLHT*, and *CsVAAT* with theanine content in roots suggested these amino acid transporters have important roles in theanine transport. We further showed that CsLHT can transport theanine in yeast under 5 mM theanine feeding. This result supports the argument that CsLHT is a low affinity transporter for theanine. Considering that theanine content in roots is very high (~60 mg/g dry weight, ~150 mM/kg fresh weight), it is reasonable to have low affinity transport to mediate theanine transport. We will further biochemically characterize CsLHT in terms of its affinity for theanine and its function in mediating theanine root-to-shoot transport in the future. It is also interesting to test the theanine transport activity of CsPUT and CsVAAT.

Taken together study first uncovered the dynamic accumulation pattern of theanine in the roots and buds of tea plants during winter and spring. Next, the expression of theanine biosynthesis and transport genes in response to dynamic theanine accumulation was investigated in the roots and buds of 13 tea plant cultivars at four time points in winter and spring. Finally, a theanine transporter was identified in tea plants. These findings provided fundamental bases for further revealing the regulation mechanisms of theanine biosynthesis and transport in tea plants.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

ZZ, XW, and CW conceived the study and designed the experiments. FL, CD, TY, JM, and SZ carried out the experiments. FL, CD, TY, and ZZ analyzed the data. ZZ, XW, and FL wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01397/full#supplementary-material

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Plant-Climate Interaction Effects: Changes in the Relative Distribution and Concentration of the Volatile Tea Leaf Metabolome in 2014–2016

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Climatic conditions affect the chemical composition of edible crops, which can impact flavor, nutrition and overall consumer preferences. To understand these effects, we sampled tea (*Camellia sinensis* (L.) Kuntze) grown in different environmental conditions. Using a target/nontarget data analysis approach, we detected 564 metabolites from tea grown at two elevations in spring and summer over 3 years in two major tea-producing areas of China. Principal component analysis and partial least squares-discriminant analysis show seasonal, elevational, and yearly differences in tea from Yunnan and Fujian provinces. Independent of location, higher concentrations of compounds with aromas characteristic of farmers' perceptions of high-quality tea were found in spring and high elevation teas. Yunnan teas were distinct from Fujian teas, but the effects of elevation and season were different for the two locations. Elevation was the largest source of metabolite variation in Yunnan yet had no effect in Fujian. In contrast seasonal differences were strong in both locations. Importantly, the year-to-year variation in chemistry at both locations emphasizes the importance of doing multi-year studies, and further highlights the challenge farmers face when trying to produce teas with specific flavor/health (metabolite) profiles.

Keywords: climate change, plant-climate interactions, season, elevation, tea quality, metabolomics, gas chromatography/mass spectrometry

INTRODUCTION

Greenhouse gasses accumulating in the atmosphere cause variability in local climates including prolonged heatwaves, droughts, heavy rains, flooding, cold, and frost, all of which damage crop production (IPCC, 2014; USGCRP, 2017; Fussel et al., 2018). The effect of extreme weather and climate variability on yield has been extensively studied for many crops (Kang et al., 2009; Lobell and Gourdji, 2012; Kurukulasuriya and Rosenthal, 2013). Most studies focus on one climate variable at a time; for example, temperature increases of up to 4°C are expected to decrease rice (Asia),

wheat (India), and maize (US and Africa) yields by 20%–60% (NRC, 2011). Equally important to climate effects on yields is understanding how plant–climate interactions affect crop quality, including the secondary metabolites that contribute to the sensory and nutritional properties of plant materials. Changes in crop quality have been shown to influence consumer preference, acceptance, and, ultimately demand (Ahmed and Stepp, 2016). As attribution science (the science of attributing specific adverse weather conditions to climate change) advances (Otto, 2016; Imada et al., 2018; Knutson et al., 2018), understanding how plant–climate interactions influences human and natural systems is critical in developing long-term, sustainable agro-ecosystems.

Tea (*Camellia sinensis* (L.) Kuntze) is a long-lived crop grown in subtropical regions that are vulnerable to climate variability and is predicted to be severely impacted by climate change (IPCC, 2014; Han et al., 2018). The arrival of the East Asian Monsoon rains, an extreme weather event, is occurring earlier, lasting longer and shortening the spring harvest for high quality tea (Ahmed et al., 2014; Kowalsick et al., 2014; Boehm et al., 2016). Since tea plants are mostly grown in mountainous areas, elevational differences can also affect tea quality: higher quality generally occurs at higher elevation (Han et al., 2017; Kfoury et al., 2018b). Therefore, it is of great interest to understand the effect of season and elevation over multiple years in order to develop strategies for sustaining high quality tea in the era of climate change (FAO, 2015; Han et al., 2017; Han et al., 2018). Our previous work on tea in Yunnan Province, China reveals striking changes in the distribution and concentration of metabolites in response to differences in precipitation and temperature within a 1-year period (Ahmed et al., 2014; Kowalsick et al., 2014; Robbat Jr et al., 2017; Kfoury et al., 2018a; Kfoury et al., 2018b).

Tea has a complex secondary metabolite profile comprised of hundreds of volatile and non-volatile compounds that contribute to quality by impacting flavor, nutritional, and health attributes (Ahmed and Stepp, 2012). Of the ~450 volatile metabolites detected in any given tea sample, our previous research found that two-thirds increased or decreased in concentration; most by more than 50%, more than 100 by 100%, and some by 1,000% due to differences in spring and summer rainfall (~0 vs. 400 mm) and temperature (22°C vs. 28°C) as well as elevational differences in temperature (5°C) (Kowalsick et al., 2014; Kfoury et al., 2018b). We found a greater number of metabolites with higher concentrations in the spring (cooler temperature/no rain) and high elevation (cooler temperature) teas that exhibited sweet, floral, honey-like aromas compared to hay, grassy, earthy notes in the summer (warmer temperature/monsoon rain) and low elevation (warmer temperature) teas. These results are consistent with farmer sensory perceptions of high- and low-quality tea (Ahmed and Stepp, 2012; Ahmed et al., 2014; Han et al., 2017). High elevation teas also contained more and higher concentrations of volatile metabolites that possess analgesic, antianxiety, antibacterial, anticancer, antidepressant, antifungal, anti-inflammatory, antioxidant, antistress, and cardioprotective properties (Kfoury et al., 2018b). This finding is extremely important since time and location of harvest can affect the medicinal properties of tea extracts used in clinical trials (Tsao et al., 2009; Wang et al., 2014; Yan et al., 2017; Balsan et al., 2019).

Less dramatic, although equally important, are the differences in catechin (polyphenolic antioxidants) and methylxanthine (stimulants) concentrations. Although these metabolites were higher in spring tea, the monsoon rains induced higher total phenolic concentrations and antioxidant potential in summer tea (Ahmed et al., 2014). Also, the low elevation tea contained higher concentrations of caffeine, epicatechin gallate, gallocatechin, and catechin, while other catechins and methylxanthines were indifferent to elevation (Kfoury et al., 2018b). Importantly, many tea famers experience up to a 50% decrease in revenue for summer and low elevation tea most likely due to an increase in unpleasant aromatics and compounds associated with bitterness and astringency (Ahmed et al., 2014; Han et al., 2017).

Clearly, both elevation and season have important effects on the chemistry and quality of tea. Whether these differences hold true across years is understudied. Here, we performed a multi-year study to explore the effects of these drivers on tea quality. Specifically, we study the variation of tea quality based on the differences in metabolite distribution and concentration in tea due to seasonal and elevation differences over a 3-year period in Yunnan and Fujian Provinces, China. To understand how changes in climate will affect plant volatiles, it is necessary to obtain the total, detectable metabolome. Here, we employed a targeted/untargeted data analysis approach to analyze gas chromatography/mass spectrometry (GC/MS) data of tea extracted by stir bar sorptive extraction.

MATERIALS AND METHODS

Sample Collection

Tea leaves were collected from four communities located at high and low elevations in two major tea-producing provinces of China during the spring and summer harvest seasons from 2014–2016. This includes one high (1651 m) and one low (1162 m) elevation tea-producing community in Menghai County in Yunnan Province, which grow the large-leaf variety of tea (*Camellia sinensis* var. *assamica*). The spring sampling occurred in March, with summer sampling in June. The other tea-producing communities are in Anxi County in Fujian Province and included one high (690 m) and one low (112 m) elevation site, which grow the small-leaf variety (*Camellia sinensis* var. *sinensis*). The spring sampling occurred in May, with the summer sampling in July. Samples consisted of the terminal bud plus two adjacent leaves from five plants in three plots within three tea farms in each community, collected over three consecutive days. Since results of a previous study showed no significant plot-to-plot differences on the same farm (Ahmed et al., 2014), samples from each plot were pooled and homogenized to produce daily samples (n = 3) and treated as independent replicates within each sampling period at each site. A microwave oven was used in the field to stop enzymatic oxidation (Ahmed et al., 2014; Kowalsick et al., 2014). The dried leaves were shipped to Tufts University, where they were stored at −20°C until analyzed.

Sample Preparation

Aqueous infusions were prepared by brewing 3 g of tea in 30 ml of deionized water at 90°C and cooling to room temperature for 30

min in a closed container. 10ml aliquots were syringe filtered (0.45-µm polytetrafluoroethylene, Fisher Scientific, Pittsburgh, PA) into 10ml Teflon-sealed vials. Organics sorbed into a 0.5-mm thick × 10-mm long polydimethylsiloxane stir bar (Gerstel, Mülheiman der Ruhr, Germany) stirred at 1,200 rpm for 1 h. Stir bars were removed from the vials, rinsed with deionized water, dried with a lint-free wipe, and placed into glass desorption tubes for analysis.

GC/MS Analysis

Analyses were performed on an Agilent (Santa Clara, CA) 6890/5975 GC/MS equipped with a MultiPurpose Sampler (Gerstel). The thermal desorption unit (TDU, Gerstel) provided splitless transfer of the sample from the stir bar into a programmable temperature vaporization inlet (CIS, Gerstel). The TDU was heated from 40°C (0.70 min) to 275°C (3 min) at 600°C/min under 50 ml/min of helium. After 0.1 min, the CIS, operating in solvent vent mode, was heated from −100°C to 275°C (5 min) at 12°C/s. The GC column (30 m × 250 µm × 0.25 µm RXI-5MS, Restek, Bellefonte, PA) was heated from 40°C (1 min) to 280°C at 5°C/min with 1.2 ml/min of constant helium flow. The MS operated in full scan mode between 40 and 350 m/z, with an electron impact ionization energy of 70 eV. The ion source and quadrupole temperatures were 230°C and 150°C, respectively. A standard mixture of C_7-C_{30} *n*-alkanes (Sigma-Aldrich, St. Louis, MO) was used to calculate the retention index of each compound. Concentration differences were calculated as relative peak area differences for each compound using naphthalene-d^8 (Restek) as an internal standard. A total of 300 reference standards were purchased from Sigma-Aldrich, Fisher Scientific, Alfa Aesar (Ward Hill, MA), TCI (Tokyo, Japan), Acros Organics (Pittsburgh, PA) and MP Biomedicals (Santa Ana, CA) to confirm compound identity.

Data Analysis

Ion Analytics (Gerstel) data analysis software was used to analyze the samples based on a target/nontarget approach as previously described in (Robbat Jr et al., 2017; Kfoury et al., 2018a).

Statistical Analysis

Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed on autoscaled (mean-centered and unit-variance scaled) data using MetaboAnalyst 4.0 (Chong et al., 2018). Permutational multivariate analysis of variance (PERMANOVA) was conducted using 999 permutations using the *vegan* package in R (R, 2014; Oksanen et al., 2018). PCA was used to visualize group differences with confirmation made by PERMANOVA. Because current implementations of PLS-DA cannot account for factorial experimental designs, PLS-DA was used only to identify important metabolites for differentiating levels of one variable (e.g., elevation) across all levels of other variables (e.g., across all years and seasons). The quality of the PLS-DA model is described by R^2 and Q^2 values (Eriksson et al., 2006). R^2 measures the degree of fit of data to the model. A seven-fold cross validation was used to produce Q^2, which measures the predictive power of the model. A feature of PLS-DA is the ability to summarize predictor variable importance across all predictive components with a variable importance in projection (VIP) score (Chong and Jun, 2005). Metabolites with a VIP > 1.0 and statistically different among levels of a response variable (Mann-Whitney test, $p < 0.05$) were considered important discriminators. Metabolite diversity was calculated as a Simpson's diversity index (1-D) and the effects of season, elevation year, and their interactions on metabolite diversity were assessed using an ANOVA ($p < 0.05$).

Climate Data

Daily maximum and minimum temperatures were obtained from the Climate Prediction Center's Global Daily Temperature dataset with each grid cell spanning 0.5° latitude × 0.5° longitude (CPC, 2018). Daily precipitation totals were obtained from the Integrated Multi-satellitE Retrievals (IMERG) for Global Precipitation Measurement (GPM) dataset (Huffman et al., 2014), with a spatial resolution of 0.1° latitude × 0.1° longitude. Values are derived from passive microwave sensors from the GPM constellation and ground-based rain gauge observations. Since the IMERG data were not available prior to March 12, 2014, some spring 2014 days are not included in the analysis. The coordinates for each site and corresponding data were imported into a Geographic Information System. Temperature and precipitation values at each site were extracted from corresponding grid cells.

RESULTS

Yunnan teas, including the ones used in this study, were used to create a Yunnan-specific database of ~600 compounds. The database served as the initial list of target compounds when we analyzed tea from Fujian based on our targeted/untargeted workflow. For example, spectral deconvolution of the total ion current chromatogram (TIC, **Figure 1A**) of high elevation, spring tea yielded 444 target compounds (**Figure 1B**), where each colored peak indicates a single compound. Subtraction of the mass spectra for these target compounds revealed 32 compounds (nontargets) specific to this sample (**Figure 1C**). By adding these compounds to the database, they become target compounds for the next sample. Subtraction of their mass spectra in subsequent samples reveals new metabolites that are specific to that sample. Based on this approach, we detected 518 volatile metabolites, 58 of them unique to plants grown in Fujian from 2014–2016. Similarly, 506 metabolites were detected, 46 unique, in plants grown in Yunnan over the same time. It should be noted that none of the compounds detected, including unknowns, are known pesticides in the NIST and Wiley databases.

Although Yunnan and Fujian plants produced 460 common compounds (~90%), differences in their concentration as well as unique metabolites yielded a PCA score plot, **Figure 2**, that separated the samples by location (and/or variety), which we expected due to differences in terroir and farmer practices. Interestingly, the monoterpenes are negatively correlated with PC1 and, therefore, more concentrated in Yunnan tea. Whereas, the sesquiterpenes correlate positively with PC1 and are higher in concentration in Fujian tea. Because of the inherent differences

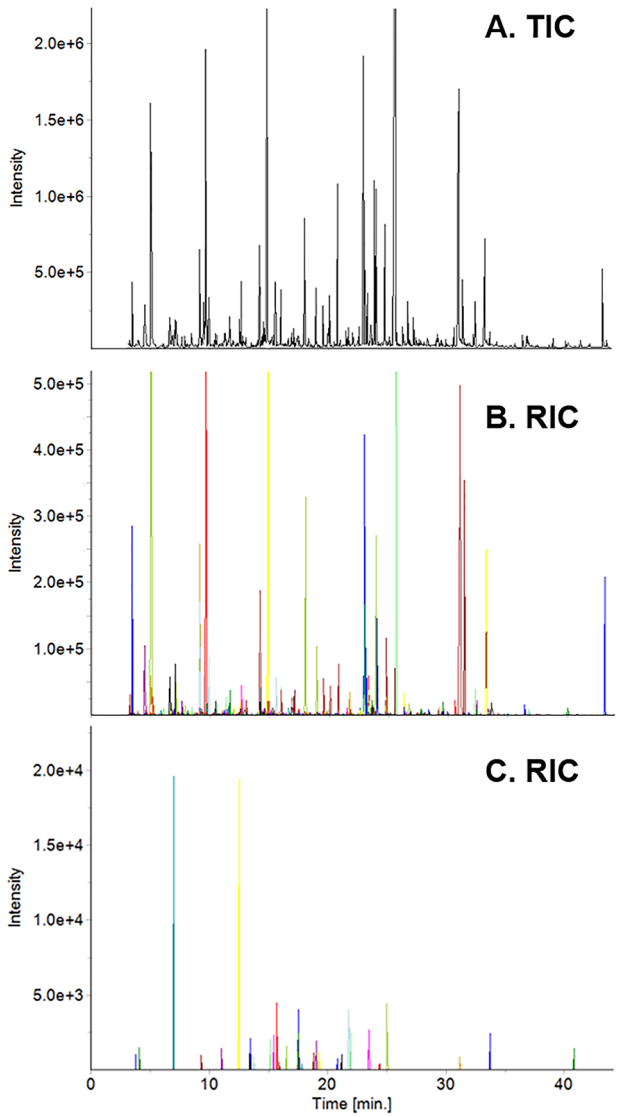

FIGURE 1 | Total ion current (TIC) chromatogram of high elevation spring tea from Fujian **(A)** and reconstructed ion current (RIC) chromatograms of target **(B)** and non-target **(C)** compounds. Each colored peak in the RIC corresponds to a specific compound in the sample.

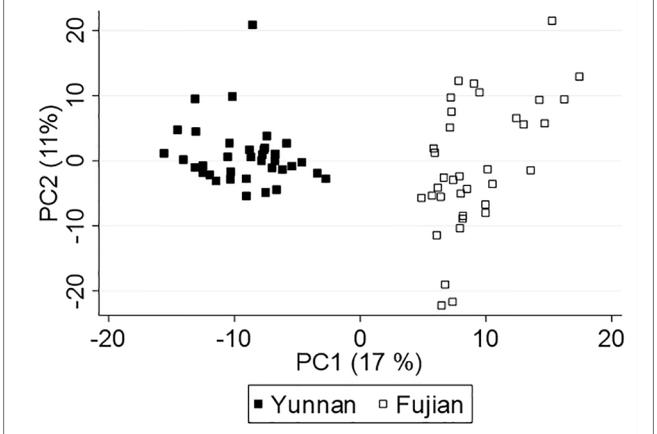

FIGURE 2 | Principal component analysis (PCA) score plot of Yunnan and Fujian tea.

in tea leaf chemistry, we analyzed the data from each province separately to understand the effect of climate on plant metabolites.

Plant-Climate Interactions: Yunnan Tea

Table 1 lists the 10-day cumulative rainfall and average temperature prior to each harvest from 2014–2016. We selected this time period based on previous studies, where differences in metabolite chemistry were observed 5 days after the onset of the East Asian Monsoon rains (Ahmed et al., 2014; Kowalsick et al., 2014). Spring rainfall was ≤ 0.1 mm and summer 58 ± 14 mm, with the seasonal difference in temperature ~3.5 ± 1°C. Although the two elevational sites, from which the samples were harvested, fall within the same latitude/longitude grid cell for temperature, we estimated a 3°C difference between the high (cooler) and low (warmer) elevations using the adiabatic lapse rate (NOAA, 2017).

Figure 3 shows the PCA score plots of PC1 vs. PC2 (A) and PC1 vs. PC3 (B), which explain 43% of the variation in metabolite chemistry. PCA separated the metabolite profiles by elevation (circles vs. triangles) on PC1 (except for 2014), season (open vs. closed shapes) on PC2, and year, 2014 (red) from 2015/2016 (black/blue), on PC3. The separations were confirmed by three-way PERMANOVA showing a significant main effect of elevation ($F_{(1,24)}$ = 34.57, p = 0.001), season ($F_{(1,24)}$ = 11.23, p = 0.001), and year ($F_{(2,24)}$ = 13.05, p = 0.001). PERMANOVA also revealed significant interactive effects between year and elevation ($F_{(2,24)}$ = 13.29, p = 0.001) and year and season ($F_{(2,24)}$ = 4.01, p = 0.010), but not season and elevation ($F_{(2,24)}$ = 0.78, p = 0.425). These interactive effects indicate that there is a different elevational and seasonal effect in at least one of the 3 years. As seen in the score plot (**Figure 3A**), 2014 samples do not separate by elevation along PC1, unlike 2015 and 2016 samples. In addition, 2015 samples separate differently by season than 2014/2016 samples along PC2.

Metabolite diversity was described by the Simpson's diversity index (Simpson, 1949), which accounts for the number of compounds and their relative abundances in the samples. An index value of 0 means complete dominance of one compound and 1, complete evenness of all compounds. As the number and evenness of compounds increases, namely more compounds with similar relative peak areas, so does the diversity index. Metabolite diversity index ranged from 0.81 to 0.95 and was significantly affected by elevation ($F_{(1,24)}$ = 10.85, p = 0.003), season ($F_{(1,24)}$ = 30.42, p < 0.001), and year ($F_{(2,24)}$ = 19.60, p < 0.001). There were significant interactions between season and year ($F_{(2,24)}$ = 7.42, p = 0.003) and between elevation and year ($F_{(2,24)}$ = 4.94, p = 0.016), but not between season and elevation ($F_{(2,24)}$ = 0.27, p = 0.609). These results match the PERMANOVA for the metabolite relative peak area data. In general, metabolite diversity was greatest in low elevation sites and summer, meaning these plants produced a greater number of compounds having similar metabolite concentrations in response to the conditions studied.

TABLE 1 | 10-day cumulative rainfall and average temperature prior to each harvest (Huffman et al., 2014; CPC, 2018).

Yunnan	Elevation (m)	10-day period	Rain (mm)	Temp (°C)	Fujian	Elevation (m)	10-day period	Rain (mm)	Temp (°C)
2014	1651	March 8–17	0.0	22.9±0.8	2014	690	May 1–10	113.2	18.7 ± 2.4
		May 31–June 9	72.3	28.1±1.1			July 21–30	92.3	26.6 ± 1.4
	1162	March 6–15	0.0	22.4 ± 0.7		112	April 21–30	36.0	20.8 ± 2.0
		May 29 – June 7	40.1	28.5 ± 0.9			July 18–27	100.2	28.6 ± 1.8
2015	1651	March 7–16	0.0	23.7 ± 0.4	2015	690	May 1–10	89.1	21.4 ± 2.1
		June 6–15	69.2	26.7 ± 1.0			July 20–29	156.6	24.8 ± 1.4
	1162	March 5–14	0.0	23.6 ± 0.4		112	April 21–30	43.3	21.9 ± 2.4
		June 4–13	69.4	27.2 ± 1.0			July 17–26	140.6	26.7 ± 1.4
2016	1651	March 12–21	0.1	24.7 ± 0.7	2016	690	April 25–May 4	64.4	21.5 ± 2.0
		June 12–21	48.3	25.8 ± 0.9			July 17–26	9.3	27.2 ± 1.8
	1162	March 10–19	0.1	24.4 ± 0.7		112	April 21–30	68.6	22.2 ± 2.1
		June 10–19	47.3	25.8 ± 0.9			July 15–24	10.6	29.0 ± 1.1

Rainfall data prior to March 12, 2014 was not available and could not be included in the analysis.

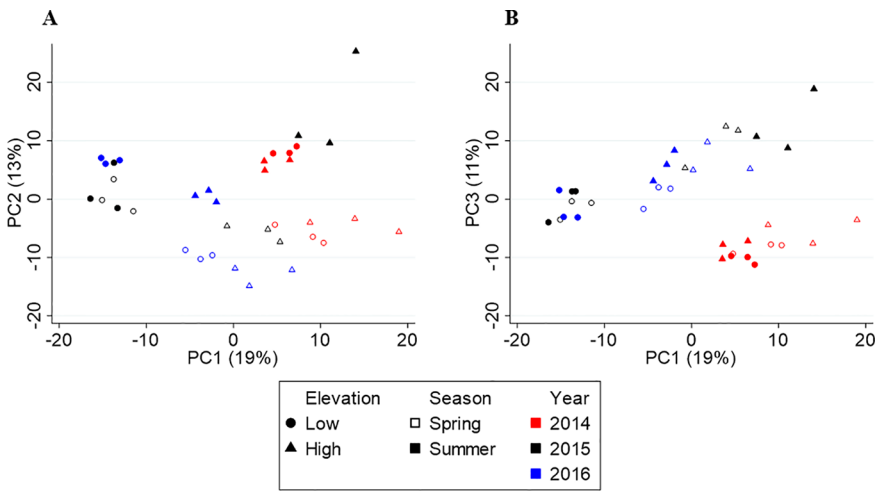

FIGURE 3 | Principal component analysis (PCA) score plots of Yunnan tea. **(A)** PC1 vs. PC2. **(B)** PC1 vs. PC3.

We used PLS-DA to identify metabolites that largely vary between elevations, seasons, and years, independently. The models for elevation (R^2 = 0.867, Q^2 = 0.659), season (R^2 = 0.884, Q^2 = 0.736), and year (R^2 = 0.854, Q^2 = 0.782) had high explanatory and predictive power. We identified 138 metabolites that discriminated high from low elevation (**Supplementary Table S1**), 129 metabolites that differentiated spring from summer (**Supplementary Table S2**), and 155 metabolites that separated years (**Supplementary Table S3**), where the main separation effect is between 2014 and 2015/2016. Some metabolites are affected by more than one climate variable resulting in 353 discriminator compounds.

Plant-Climate Interactions: Fujian Tea

Table 1 also lists the climate data for Fujian Province. In contrast to Yunnan, a larger temperature increase occurs from spring to summer (~6.1 ± 1°C), but rainfall patterns were erratic from year to year. The high and low elevation sites are ~32 km apart from each other and can therefore be distinguished by latitude/longitude climate grids.

Figure 4 shows the score plots of PC1 vs. PC2 (A) and PC1 vs. PC3 (B). Like Yunnan, the first three axes explain 43% of the variation in the data. The score plot revealed metabolite profiles separated by year (red, black, and blue) on PC2, but no clear seasonal or elevational separation was found. The three-way PERMANOVA confirmed the yearly separation ($F_{(2,24)}$ = 7.61, p = 0.001) and lack of elevational separation ($F_{(1,24)}$ = 1.96, p = 0.134), but revealed a significant seasonal separation ($F_{(1,24)}$ = 3.79, p = 0.024). Upon further inspection, a seasonal separation (open vs. closed shapes) along PC4 was found (**Supplementary Figure S1**). There was also a significant interaction between year and season (F = 4.345, df = 2, p = 0.004) but not year and elevation (F = 1.686, df = 2, p = 0.144) or season and elevation (F = 1.470, df = 1, p = 0.205).

Metabolite diversity in Fujian varied significantly by year ($F_{(2,24)}$ = 5.10, p = 0.014) and season ($F_{(1,24)}$ = 4.32, p = 0.049), but not elevation ($F_{(1,24)}$ = 0.003, p = 0.953). None of the two-way or three-way interactions were significant (all p > 0.1). Metabolite diversity was highest in 2014, which had the largest temperature difference between spring and summer (~8°C), and higher in summer compared to spring.

PLS-DA was used to identify metabolites that largely vary between seasons and years, independently. The models for season (R^2 = 0.913, Q^2 = 0.711) and year (R^2 = 0.901, Q^2 = 0.817) had high explanatory and predictive power. We identified 101 metabolites

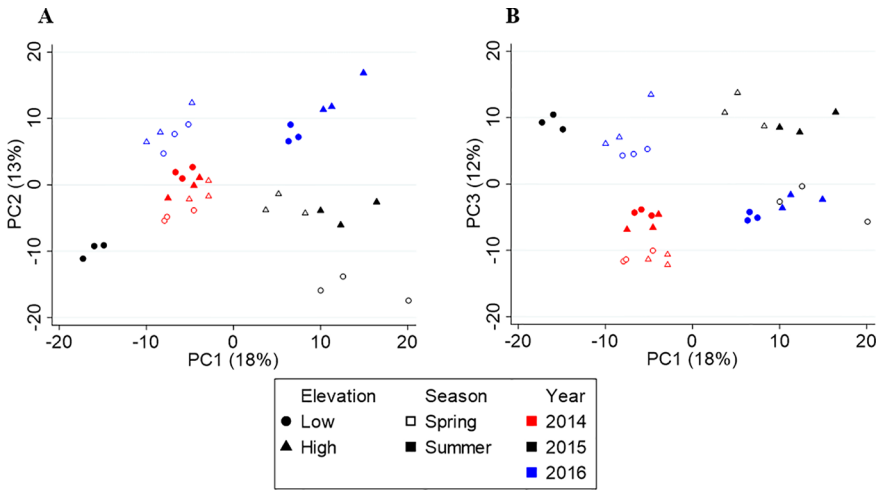

FIGURE 4 | Principal component analysis (PCA) score plots of Fujian tea. **(A)** PC1 vs. PC2. **(B)** PC1 vs. PC3.

that differentiated spring from summer (**Supplementary Table S4**), and 133 metabolites that separated years (**Supplementary Table S5**). Of the 518 metabolites detected in Fujian teas, 226 discriminate the seasonal and/or yearly differences.

DISCUSSION

Elevational Variation

While the elevation difference (~500 m) is the same in the two provinces, it was the largest source of metabolite variation in Yunnan yet had no effect in Fujian. The Yunnan sites are ~1,000 m higher in elevation, meaning the Fujian sites are ~6°C warmer than even the low elevation site in Yunnan (NOAA, 2017). The much warmer temperatures at the Fujian sites could account for the lack of elevational variation seen. In addition, while we classified the site located at 690 m in Fujian as high elevation based on being 500 m higher than the other Fujian site and the elevations found in the area, an elevation of 690 m is not generally regarded as high elevation within the tea industry for high-quality tea.

In Yunnan, four compounds (43, 165, 168, 193—all unknowns) were unique to high elevation samples and four compounds (75, verbenone, 4-methyldecane, 4-ethylbenzaldehyde) were unique to low elevation samples. These compounds could be important biomarkers for distinguishing high and low elevation teas and if the unknowns prove to be of sensory or health importance, identification is necessary. In addition, high elevation teas contain greater concentrations of compounds that have been described as sweet, floral, and fruity (Perflavory, 2015), such as *Z*-jasmone, maltol, methyl 4-methylbenzoate, and bergamal. In contrast, the low elevation teas contain higher concentrations of green, earthy, waxy, and camphoraceous compounds such as 2-phenyl-2-propanol, nonanal, dodecanal, and verbenone (Perflavory, 2015). These results correspond with farmer perceptions that high elevation teas are generally of higher quality due to the sweet, floral, honey-like, and fruity aromatics (Owuor et al., 1990; Ahmed and Stepp, 2012; Han et al., 2017).

In contrast to previous work on Yunnan tea where only discriminating compounds with higher concentrations in high elevation tea had reported health benefits (Kfoury et al., 2018b), in this study compounds with positive correlations to both high and low elevation teas have reported health-beneficial properties (**Supplementary Table S1**). Nutraceutical compounds that distinguish high from low elevation tea include (*E*)-caryophyllene (anticancer, antidepressant, anti-inflammatory), isoeugenol (antibacterial, antioxidant), *epi*-α-cadinol (antibacterial, anti-inflammatory), (*E*)-nerolidol (antianxiety, antimalarial, anticancer), and α-pinene (antiviral, analgesic) (Astani et al., 2010; Tung et al., 2010; Russo, 2011; Guimarães et al., 2013; Bahi et al., 2014; Fidyt et al., 2016; Guerrini et al., 2016; Marteau et al., 2016; Shan et al., 2016; Resende et al., 2017). Health beneficial compounds that differentiate low from how elevation tea exhibit antibacterial (verbenone, decanal, undecanal, dodecanal), antifungal (nonanal), and antiseptic (2-phenoxyethanol) properties (Buhrer et al., 2002; Battinelli et al., 2006; Fujita et al., 2015; Iscan, 2017). Although the volatile metabolites represent a small fraction of the total tea mass, findings indicate that volatile tea extracts have proven health benefits (Takahashi et al., 2014; Li et al., 2017). However, further studies are needed to determine if the compounds found here are present in adequate concentrations to provide these purported health benefits.

Seasonal Variation

Although rainfall and temperature generally increase from spring to summer in both Yunnan and Fujian, Yunnan experiences a striking increase in rain and smaller changes in temperature compared to Fujian, which has a larger temperature increase and erratic rainfall from year to year. The leaves from both provinces had significant chemical variations due to seasonal

changes. Interestingly, nine compounds (N-ethylsuccinimide, isomenthone, (*E*)-β-ocimene, menthone, 160, flouranthene, 1-ethyl-1H-pyrrole, 76, 1-ethyl-1H-pyrrole-2-carboxaldehyde) were discriminators of spring tea and five compounds (36, 167, 181, 224, 2-phenoxyethanol) discriminate summer tea in both Yunnan and Fujian (**Supplementary Tables S2** and **S4**). In addition, 1-ethyl-1H-pyrrole was unique to spring tea in both locations and could be a key spring tea biomarker, independent of location, and variety.

Regardless of the inherent differences between the provinces, more compounds that have been described as sweet, floral, honey-like, and fruity are discriminators of spring tea (Perflavory, 2015). These include (*E*)-β-ocimene, 2,2,6-trimethylcyclohexanone, (*Z*)-jasmone, phenylethyl acetate, and 5-methylfurfural. On the other hand, 1-octen-3-ol, (2*E*,4*E*)-heptadienal, 2-pentylfuran, α-copaene, and *cis*-calamenene are higher in concentration in summer tea and are characterized as green, herbal, earthy, and woody (**Supplementary Tables S2** and **S4**). In addition, compounds significant to both seasons have reported health benefits (**Supplementary Tables S2** and **S4**). Spring-associated compounds include menthone (antibacterial, anti-inflammatory), eucalyptol (antibacterial, cardioprotective), indole (antibacterial, antifungal), and coumarin (anti-inflammatory, anticancer) (Egan et al., 1990; Muroi and Kubo, 1993; Nidiry and Babu, 2005; Santos et al., 2011; Shan et al., 2016; Iscan, 2017). Summer-associated compounds include (*E*)-β-ionone (anticancer, antibacterial), borneol (anti-inflammatory, analgesic), methyl anthranilate (antifungal), and quinoline (antimalarial, anticonvulsant, anticancer) (Muroi and Kubo, 1993; Nidiry and Babu, 2005; Oz et al., 2015; Anjali and Singh, 2016; Furtado Kelly et al., 2016).

Interestingly, in Yunnan, many of the discriminating metabolites are in both spring and high elevation or in the summer and low elevation teas. For example, spring and high elevation teas contain significantly higher amounts of 2-hydroxy-5-methylacetophenone, isoeugenol, 4-methylbenzaldehyde, and norfuraneol that are described as sweet, fruity, floral, honey-like compounds (Perflavory, 2015). In contrast, summer and low elevation teas have significantly higher amounts of 2,6-dimethyl-3,7-octadiene-2,6-diol, 2-phenoxyethanol, octanal, and nonanal that are described as herbal, green, fatty, and metallic (Perflavory, 2015). These findings agree with farmers' perceptions that high elevation and spring teas are higher in aromatic quality possessing sweet, fruity, floral, honey-like characteristics (Ahmed and Stepp, 2012; Ahmed et al., 2014; Han et al., 2017). Most likely, these metabolites are induced by temperature since spring/high elevations are cooler than summer/low elevations.

Yearly Variation and Interactive Effects

In Yunnan, both PCA and PLS-DA show the yearly separation is between 2014 and 2015/2016. Based on the climate data, 2014 experienced the coolest spring and warmest summer temperatures compared to 2015 and 2016. Interestingly, many of the higher concentration compounds in the 2014 tea were also higher in concentration in either spring or summer tea. For example, compounds such as (*E*)-β-ocimene, (*Z*)-herboxide, (3*Z*)-hexenyl acetate, and safranal were higher in concentration in 2014 and spring teas and are described as sweet, herbal, fruity aromas (Perflavory, 2015). Compounds such as (4*Z*)-heptenal, pyridine, and 2-methyldecane were higher in concentration in 2014 and summer teas and are characterized as fatty, green, and fishy (Perflavory, 2015). These compounds are likely being induced by the cooler spring or warmer summer temperatures. In contrast, compounds higher in concentration in 2015/2016 are not affected by seasonal variations such as 4-ethyl-2-methoxyphenol (smoky, phenolic), catechol (no aroma), 4-methyl-3-penten-2-one (sweet, earthy), and γ-heptalactone (sweet, nutty) (Perflavory, 2015), which are likely not influenced by temperature changes. Another indication that 2014 differed from 2015/2016 is that 2014 samples do not separate by elevation, which is confirmed by the interactive effect between year and elevation. Compounds such as hexanoic acid, 2-nonanone, decanal, (*E*)-herboxide, 4-methylbenzaldehyde, and 2-methoxy-4-vinylphenol exhibit no change in concentration between elevations in 2014, but are significantly higher in concentration at one elevation or the other in 2015/2016 tea.

Also, 2014 and 2016 samples separate similarly by season whereas 2015 samples do not follow the same pattern along PC2, which is confirmed by the interactive effect between year and season. While several compounds exhibit the opposite change in concentration from spring to summer, others show a more or less enhanced concentration change in 2015 compared to 2014/2016. For example, (*E*)-β-ocimene, methyl salicylate, and 7-methoxycoumarin exhibit opposite concentration changes from spring to summer in 2015 compared to 2014/2016. In addition, hexanol, isomenthone, and camphor have a greater concentration difference between spring and summer whereas 2*E*-hexenal, benzenacetonitrile and 4-methyl-3-penten-2-one have a smaller difference in 2015. The climate data do not provide any indication as to what is causing these interactive effects. However, (*E*)-β-ocimene, methyl salicylate, 2*E*-hexenal, and 4-methyl-3-penten-2-one are induced by herbivory in tea, so these year-to-year differences could reflect differences in the timing of pest outbreaks (Cai et al., 2014; Kfoury et al., 2017; Scott et al., 2019).

Year-to-year variations account for the greatest source of variation in Fujian and is likely due to the inconsistent rainfall patterns seen from year-to-year (**Table 1**). The significant interactive effect between year and season confirms this finding, not found between year and elevation or season and elevation. In 2016, the plants experienced an extremely dry summer, which is the reverse of the previous two years. As a result, many metabolite concentrations increased/decreased in the opposite direction from 2014 and 2015. For example, safranal (sweet, herbal), norfuraneol (sweet, caramel), cyclohexanone (minty), o-xylene (geranium), isoeugenol (floral, clove), and geranyl acetone (floral, fruity) are higher in concentration in spring tea in 2014 and 2015, but higher in summer tea in 2016. Similarly, 2-ethylhexanol (green, oily), camphor (camphor, medicinal), methyl hexanoate (fruity, fatty), biphenyl (floral, green), and 5-ethyl-2(5H)-furanone (no aroma) are higher in concentration in 2014/2015 summer, compared to the 2016 spring tea. It is likely that the changes in concentration

of these and many other metabolites that behave similarly are the result of changes in rainfall.

CONCLUSION

In this work, we demonstrated that our target/nontarget approach provides efficient and comprehensive analysis of a complex, natural product, namely, tea. Findings show the differences in season, elevation and year cause significant and interactive alterations in tea chemistry. Independent of location, cooler temperatures in the spring and at high elevation concomitant with lower rainfall, results in higher concentrations of compounds with aromas characteristic consistent with farmers' perceptions of high-quality tea. Although many of the metabolites identified have reported health-beneficial properties studies are needed to assess efficacy and identify unknowns that are discriminator compounds that might also contribute to quality. Given the interactive effects found between years and season/elevation, future studies should be cautious in drawing conclusions based on only 1 year of sampling and more multi-year studies should be conducted to assist farmers with the challenge of producing a consistently high quality product. More natural-human systems investigations are needed to assess plant response under ever changing environmental conditions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AR, CO, SA, SC, TG, and JS acquired funding and conceptualized the project. DX, CL, SA, and WH supported with in-field sample collections and logistics. CM acquired and analyzed the climate data. NK acquired and analyzed the metabolite data. NK and ES performed statistical analysis. NK, AR, ES, and CO wrote the manuscript draft. All authors contributed to manuscript revision and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01518/full#supplementary-material

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Glucosyltransferase CsUGT78A14 Regulates Flavonols Accumulation and Reactive Oxygen Species Scavenging in Response to Cold Stress in *Camellia sinensis*

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Glycosyltransferases (UGTs) play diverse roles in cellular metabolism by altering regulatory metabolites activities. However, the physiological roles of most members of UGTs in crops in response to abiotic stresses are unknown. We have identified a novel glycosyltransferase CsUGT78A14 in tea crops, an important economic crops, whose expression is strongly induced by cold stress. Biochemical analyses confirmed that CsUGT78A14-1 showed the highest activity toward kaempferol and is involved in the biosynthesis of kaempferol-diglucoside, whereas the product of CsUGT78A14-2, which differs from CsUGT78A14-1 by a single amino acid, was identified as 3-O-glucoside. The accumulation of kaempferol monoglucosides and diglucosides was consistent with the expression levels of CsUGT78A14 in response to cold stress, as well as in different tissues and genotypes of tea plants. Down-regulation of *CsUGT78A14* resulted in reduced accumulation of flavonols, reactive oxygen species (ROS) scavenging capacity and finally reduced tea plant stress tolerance under cold stress. The antioxidant capacity of flavonols aglycon was enhanced by glucosylation catalyzed by CsUGT78A14. The results demonstrate that CsUGT78A14 plays a critical role in cold stress by increasing flavonols accumulation and ROS scavenging capacity, providing novel insights into the biological role of UGTs and flavonoids in plants.

Keywords: *Camellia sinensis*, tea plant, glucosyltransferase, reactive oxygen species scavenging capacity, flavonoids, cold tolerance

INTRODUCTION

Low temperatures, including chilling and/or freezing temperatures, are one of the most important environmental factors that adversely affect plant growth and agricultural productivity (Chinnusamy et al., 2007). Some plants can enhance their freezing tolerance after exposure to low but non-freezing temperatures for a period of time (Thomashow, 1999). As one of the most important economic

crops, tea plant (*Camellia sinensis*) is widely cultivated in almost 30 countries (Chen et al., 2007). Although tea plant can be grown in various regions, low temperatures are still one of the most important factors that limit its distribution mainly in tropical and subtropical climates because of the thermophilic nature of tea plants (Wang et al., 2012). Therefore, it is essential to understand the physiological response of tea plants exposed in cold stress and explore approaches to improve tea plants cold tolerance.

Plant growth and development can be severely effected by environmental stresses, including cold stress. Exposure of plants to low temperatures can cause an accumulation of reactive oxygen species (ROS) (Manthey et al., 2009), which are recognized as a common risk from abiotic stress and highly reactive and toxic, and affect many cellular functions (Gill and Tuteja, 2010). A series of protective mechanisms are triggered when plants sense the cold temperature (Iba, 2002; Wang et al., 2013). It is well known that the induction of the antioxidant capacity for ROS scavenging is important to protect plants in stresses, including cold stress (Ning et al., 2010; You et al., 2014; Hazman et al., 2015; Li et al., 2017). The accumulation of flavonoids can be induced by biotic and abiotic environmental stresses in plants (Dixon and Paiva, 1995). Flavonoids are among the most bioactive plant secondary metabolites, and are regarded as no enzymatic defense components because of their ROS scavenging capacity (Bolwer et al., 1992). Therefore, flavonoids are important for plants to protect themselves under environmental stresses (Winkel-Shirley, 2002). Over-accumulation of antioxidant flavonoids leads to an enhancement of drought tolerance in *Arabidopsis* (Nakabayashi et al., 2014). However, the biological role of flavonoid glycosides is not clear as they are considered to be less effective anti-oxidants in comparison with the corresponding aglycones (Vogt and Jones, 2000; Gachon et al., 2005). Therefore, the precise role(s) of glycosylation in abiotic stresses is still very difficult to understand until now.

In tea plant, flavonoids are the predominant secondary metabolites, mainly in the form of glycosides. Glycosylation, mediated by diphosphate-dependent glycosyltransferases (UGTs), renders the flavonoids more water soluble and less toxic, and also enable their transport (Bowles et al., 2006; Song et al., 2018). Recently, 132 UGTs were identified in a transcriptome database of the tea plant. The function of most of them were unknown, except four UGTs (CsUGT78A14, CsUGT78A15, and CsUGT82A22, CsUGT73A20) which exhibited catalytic activity toward phenolic acids and flavonoid (Cui et al., 2016; Zhao et al., 2017), and three UGTs involved in the glycosylation of aroma (Ohgami et al., 2015; Jing et al., 2019; Song et al., 2018). Numerous studies have indicated that some UGTs are involved in the regulation of plant growth and development in responses to biotic and abiotic stresses (Tognetti et al., 2010; von Saint Paul et al., 2011; Liu et al., 2015; Palareti et al., 2016), and improved freezing tolerance in *Arabidopsis* (Schulz et al., 2016). However, little is known about the physiological roles of most members of the plant UGTs. The response mechanisms of plants to environmental changes and how these glycoconjugates can contribute to plant protection is still a major challenge.

In this study, the *C. sinensis* UGT gene *UGT78A14* was identified as a gene involved in the regulation of plant cold stress tolerance. The expression of *UGT78A14* was strongly induced by cold stress. *In vitro* assays showed that CsUGT78A14-1 and -2 could catalyze the glucosylation of flavonols and the main products were identified as kaempferol-diglucoside, and 3-O-glucoside, respectively. Down-regulation of *UGT78A14* in the tea plant resulted in reduced accumulation of flavonol glycosides, ROS scavenging capacity and finally reduced tea plant cold stress tolerance. The antioxidant and ROS scavenging capacity of the flavonols was greatly enhanced by glucosylation catalyzed by UGT78A14-1 compared to the corresponding free aglycons. These results provide novel insights into the biological role of flavonoid glycosides in plants, and deepen our knowledge of the response mechanisms of flavonol glycoconjugates to stress in plants.

MATERIALS AND METHODS

Plant Materials

Tea plant samples were collected from the tea plant cultivar and Germplasm Resource Garden of Anhui Agricultural University (Guohe Town) and were immediately frozen in liquid nitrogen. The tea plant samples from *C. sinensis* var. *sinensis* cv. 'Shuchazao,' 'Mingxuan213,' 'Zhenghedabai,' 'Longjingchangye,' 'Mingshanbaihao,' 'Yingshuang,' 'Longjing43,' and 'Fuzao2' were used for metabolites and transcripts analyses. All samples were stored at −80°C until use.

Chemicals and Reagents

All biochemicals including kaempferol, quercetin, myricetin, uridine diphosphate glucose (UDP)-glucose, UDP-galactose, and UDP-glucuronic acid were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were obtained from Sigma or Aladdin (Shanghai, China), unless otherwise noted.

Cold Stress Treatment

For metabolites and transcripts analysis, 1-year-old tea plants were grown at 80% relative humidity and 16 h/8 h light/dark condition. The tea plants were first treated at 4°C, and leaves were collected after 6 h short-term cold stimulus (CS) and 7 days long-term chilling acclimation (CA). Then, the plants were transferred to 0°C for an additional 7 days long-term freezing acclimation (FA). After that, the plants were moved to control conditions (25°C) for 7 days long-term de-acclimation (DA) according to a previous study (Li et al., 2019). The control plants and *UGT78A14*-silenced tea plants were exposed to −5°C for 3 h for cold stress treatment. At least three experimental replicates were conducted for the treatment and control. Young tissues with one bud and two leaves were harvest and were used to determine of antioxidant activity and ROS content.

Ribonucleic Acid Isolation and Complementary Deoxyribonucleic Acid Synthesis

Total RNA from leaves of Shuchazao was isolated using RNAisomatefor Plant Tissue (Takara, Dalian, China) and RNAiso Plus (Takara, Dalian, China). The cDNA was synthesized by reverse transcription from total RNA using PrimeScriptRT Master Mix (Takara, Dalian, China).

Quantitative Real-Time Polymerase Chain Reaction Analysis

Real-time PCR was performed according to our published protocols (Song et al., 2015b; Jing et al., 2018) with gene-specific primers (**Table S1**). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal reference gene. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All qRT-PCR in this study were performed in three biological replicates, and each of which consisted of three technical replicates.

Expression Vector pGEX-4T1-UGTs

The full-length *UGT*s were amplified by PCR from the cDNA of tea plants leaves (primers as shown in **Table S1**). The full-length coding sequences were amplified using proofreading Phusion DNA polymerase according to a published protocol (Jing et al., 2019). The amplified full-length sequences were digested with BamHI and SalI, the resulting gene fragments were cloned into pGEX-4T1 vector, and the recombinant plasmids were subsequently transformed into Trans1T1-competent cells.

Heterologous Protein Expression and Purification

Expression constructs harboring the pGEX-4T1-UGTs and control plasmids were all transformed into *Escherichia coli* strain BL21 (DE3) pLysS cells (Jing et al., 2019). Protein expression was induced by adding 1 mM (final concentration) isopropyl-ß-D-thio-galactopyranoside. The culture was incubated at 16°C overnight. The next day, the proteins were purified by GST bind resin (Jing et al., 2019). Protein concentration was determined by a photometric method (Bradford, 1976).

Enzymatic Activity Assay

Each reaction mixture (5 μl in total) contained 50 mM Tris-HCl buffer (pH 7.5, 10% glycerol, and 10 mM 2-mercaptoethanol), 250 mM UDP-glucose, alcohol substrates, and purified protein (0.5–1 μg per reaction) was used for the initial screening according to Jing et al., (2019) with some motifications. The reaction mixture was incubated for 30 min at 30°C, the reaction was stopped by adding reaction solution of UDP-Glo™ assay reagent (Sheikh et al., 2017). Three biological replicates were carried out. The best reaction temperature and pH was tested according to Jing et al., (2019). For the determination of the kinetic parameters of CsUGT78A14, the sugar donor was fixed at 100 μM, and at least seven different substrate concentrations covering the range from 1 to 500 μM were used at the optimized conditions.

Identification of Products by Liquid Chromatography–Mass Spectrometry

Reaction mixtures contained 5 mM UDP-glucose, 200 μM substrate, and the purified protein (1 to 2 μg) were incubated at 30°C for 2–3 h, the reaction were extracted with 200 μl ethyl acetate for two times. Ethyl acetate was vaporized and the residue was dissolved in 50 μl methanol/water (1:1, v/v) for products identification by liquid chromatography–mass spectrometry (LC–MS) (Jing et al., 2019). Products were identified by comparison of their retention time and MS spectra with those from literature or reference material.

Gene Suppression of *CsUGT78A14* in Tea Using Candidate Antisense Oligonucleotides

Candidate antisense oligonucleotides (AsODN) with complementarity to the segment of the target gene were selected using Soligo software (Ding and Lawrence, 2003) with *CsUGT78A14* as input sequence (**Table S1**). AsODNs were synthesized by General Biosystems Company. To silence the genes, tea leaves were grown in Eppendorf tubes (2 ml) containing 40 μM AsODN-CsUGT78A14 solution for 24 h, the sense oligonucleotides (sODN) were used as control. To silence CsUGT78A14 in the tea leaves attached to the whole tea plant, 1 ml 20 μM AsODN-CsUGT78A14 solution were injected into the tea seeding. Five experimental replicates were conducted for the treatment and control. After 12, 24, and 48 h incubation, the leaves were exposed to −5°C for 3 h, and then harvested and kept at −80°C prior to analysis (Liu et al., 2018).

Metabolite Analysis

The materials collected in this study were ground and kept at −80°C prior to analysis. For metabolites analysis, 50 mg samples were extracted with 1 ml 75% (v/v) methanol for two time; 3 μg ml^{-1} chlorophenylalanine solution was added as internal standard. The metabolites were extracted and sonicated for 20 min at 4°C. After that, the mixture was centrifuged at 12,000 rpm for 10 min under 4°C. The supernatants were used for glycoside analysis by LC–MS. Five experimental replicates were conducted metabolites analysis.

Determination of Fv/Fm

Twenty four hours after the CsUGT78A14 was silenced in tea in the seeding or tea leaves using AsODNs, and tea leaves or seedings were kept in −5°C temperature for 3 h and then recover for 3 h in 25°C conditions. Net photosynthetic rate and maximum photochemical efficiency and of PSII (Fv/Fm) were measured (Li et al., 2018c). Tea plants without cold

treatment were used as controls. All Fv/Fm determination were performed at least in three biological replicates, and each of which consisted of five technical replicates.

Diaminobenzidine and Nitrobluetetrazolium Staining

The superoxide radical and H2O2 were detected using histochemical staining with nitrotetrazolium blue chloride (NBT) and diaminobenzidine (DAB) as described (Romero-puertas et al., 2004). For both staining methods, the seedlings were decolorized in 90% ethanol, followed by 100% ethanol, and then take photographs (Li et al., 2018a). Three biological replicates were carried out.

Determination of Antioxidant Activity and Reactive Oxygen Species Content

The total antioxidant activity was determined using the ferric reducing ability of plasma (FRAP) method with a FRAP reagent kit (Beyotime, Shanghai, China), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method (Beyotime, Shanghai, China), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described previously (Tohge et al., 2005b). At least three biological replicates were carried out.

RESULTS

Expression Patterns of *CsUGT78A14* During Cold Acclimation and Deacclimation

Multi-omics data deposited on the Tea Plant Information Archive (TPIA) showed that TEA007509 was strongly responsive to low temperatures (Li et al., 2019 and Wang et al., 2013). To validate this, 1-year old tea plants were grown under the cold stress condition and transcript levels were measured by real-time qRT-PCR (**Figure 1A**). When the plant were treated with cold stress, the expression levels of TEA007509 in the leaves were increased by approximately 40-fold and 180-fold after 6 h (CS) and 7 days (CA), respectively, when compared with the control (**Figure 1A**). The expression levels continue induced when plants were transferred to 7-day FA treatment at 0°C. It should be noted that the mRNA expression level of TEA007509 was significantly down-regulated when the plants was de-acclimated for 7 days (**Figure 1A**). The gene was assigned to CsUGT78A14 by the UGT Nomenclature Committee (Mackenzie et al., 1997). This observation suggest that CsUGT78A14 clearly responded to cold stress and thus investigated its relevance in cold stress resistance of tea plant.

UGT78A14-1/-2 Specifically Catalyze the Glucosylation of Kaempferol

To verify the function of CsUGT78A14, we first characterized the enzymatic activities of its encoded proteins. Two alleles of CsUGT78A14 (assigned to CsUGT78A14-1 and -2) were obtained from *C. sinensis* var. *sinensis* cv. Shuchazao. These UGTs were expressed in *E. coli* BL21 and the purified protein was verified by SDS–PAGE (**Figure S1**).

The enzymatic activities of these two proteins were tested with kaempferol, quercetin, and myricetin as selected substrates. Both CsUGT78A14-1 and -2 could use kaempferol and quercetin as a substrate (**Figure 1B**) when UDP-glucose was used as donor substrate. The enzymatic activities of CsUGT78A14-1 and -2 were further tested with 53 substrates selected from a wide range of chemical classes (**Figure S3**) and three donor sugars (**Figure S4**). Both CsUGT78A14-1 and CsUGT78A14-2 showed a similar substrate tolerance and preferred kaempferol and UDP-glucose as acceptor and donor substrates, respectively (**Figure 1B**). Low but detectable activity was measured toward quercetin (40% of that of kaempferol). However, the activity toward plant metabolites such as, myricetin, vanillic acid, vanillin, ferulic acid, gallic acid was negligible (**Figure S3**).

A Diglucoside is the Main Product of UGT78A14-1 but -2 Only Forms Monoglucosides

The formation of glycosides was confirmed by LC-MS of enzyme assays containing UDP-glucose, acceptor substrate, and purified CsUGT78A14 protein. Interestingly, at least two product peaks were detected when using kaempferol and quercetin as acceptor substrates, indicated that CsUGT78A14 performed multiposition glycosylation on flavonols. The above-mentioned products were identified by retention time, parent ions (H adducts), and daughter ion spectra (MS/MS) in comparison with standards (Zhao et al., 2017). The main products of CsUGT78A14-1 was identified as a diglucoside by comparison of its parent and daughter ion spectra with a reference compound (**Figure 1D**) (Zhao et al., 2017), in addition to two monoglucoside products, identified as kaempferol-3-glucoside, and -7-glucoside (**Figure 1C**), when kaempferol was used as substrate. However, CsUGT78A14-2 only formed a trace amount of the diglucoside (**Figure 1C**) and one monoglucoside, which was identified as kaempferol-3-glucoside. Glucosides formed by CsUGT78A14-1 and -2 from quercetin was lower but the product profiles were identical to that of kaempferol (**Figure S5**). The kinetic parameters of recombinant CsUGT78A14-1 and CsUGT78A14-2 were also compared (**Figures S6–S8** and **Table 1**). These data indicated that the main product of UGT78A14-1 was a diglucoside, whereas the major product of UGT78A14-2, which differs from UGT78A14-1 in a single amino acid, was identified as 3-O-glucoside.

Accumulation of Flavonol Glycosides and *UGT78A14* Transcripts in Different Tissues, Genotypes, and in Response to Cold Stress

We also assessed the relative levels of kaempferol and quercetin glycosides in leaves of eight different genotypes of the tea plant. Kaempferol glycosides were more abundant in *C. sinensis* var.

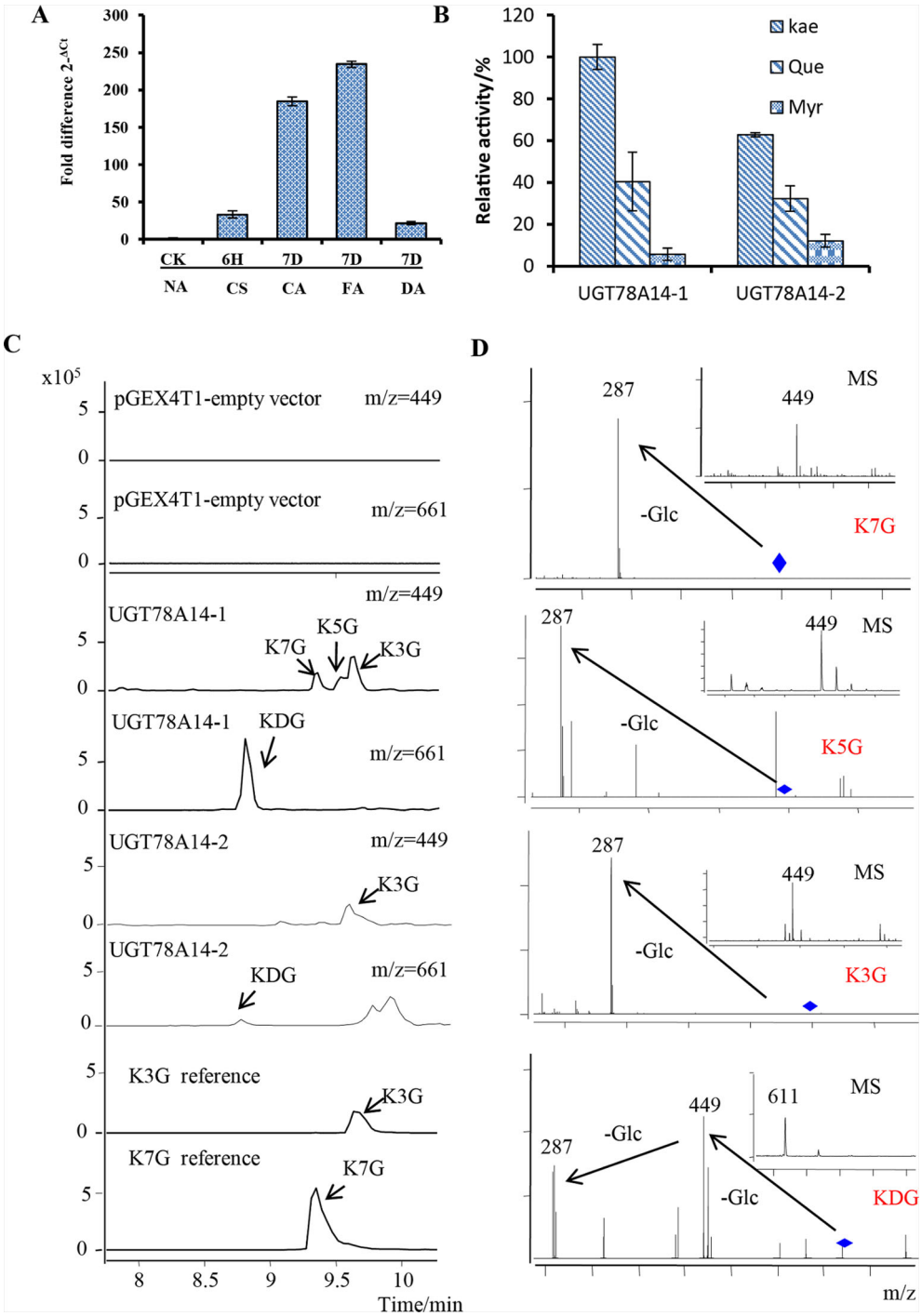

FIGURE 1 | *CsUGT78A14* expression during cold stress and enzymatic analysis of its encoded protein. Expression patterns of CsUGT78A14 during cold acclimation and de-acclimation **(A)**. Enzymatic analysis of the recombinant UGT proteins encoded by CsUGT78A14-1, and CsUGT78A14-2 with kaempferol, quercetin, and myricetin as substrates **(B)**. Liquid chromatography–mass spectrometry analysis of enzymatically formed products by CsUGT78A14-1, CsUGT78A14-2, and empty vector control **(C)**. Mass spectra of formed products by CsUGT78A14-1, CsUGT78A14-2 **(D)**. Data are presented as mean ± SE of at least three biological replicates.NA: tea plants were grown at room temperature. CS, cold stimulus, tea plants were grown at 4°C for 6 h. CA, chilling acclimation, tea plants were grown at 4°C for 7 days. FA, freezing acclimation, tea plants were first treated at 4°C for 7 days, and then treated at 0°C for an additional 7 days. DA, de-acclimation, tea plants were first treated at 4°C for 7 days, then at 0°C for additional 7 days, and moved to normal condition 25°C for 7 days. K3G, kaempferol-3-O-glucoside; K5G, kaempferol-5-O-glucoside; K7G, kaempferol-7-O-glucoside; KDG, kaempferol-diglucoside.

TABLE 1 | Kinetic parameters of recombinant CsUGT78A14-1 and CsUGT78A14-2 proteins.

	Substrate	Km μM	Vmax nKat·mg^{-1}	Kcat/K_M s^{-1} mM^{-1}
UGT78A14-1	Kaempferol			
	Gal	27.1603	0.2155	0.411556
	Glc	27.0785	0.389	0.745146
	GA	47.7616	0.11933	0.129595
	Quercetin			
	Gal	4.9994	0.2008	2.083349
	Glc	9.3001	0.2917	1.626916
	GA	76.5363	1.0417	0.705978
CsUGT78A14-2	Kaempferol			
	Gal	64.5678	0.8616	0.692159
	Glc	8.1793	0.2369	1.502329
	GA	7.0651	0.2152	1.579939
	Quercetin			
	Gal	102.0854	6.4588	3.281742
	Glc	6.0176	0.2188	1.885994
	GA	6.0787	0.2229	1.902022

sinensis cv. 'Mingxuan213' (MX213), cv. 'Zhenghedabai' (ZHDB), and cv. 'Longjingchangye' (LJCY) than in cv. 'Yingshaung' (YS) and cv. 'Fuzao2' (FZ2) (**Figure 2B**), consistent with the *UGT78A14* transcript levels in these genotypes. The relatively higher abundance of kaempferol glycosides and *UGT78A14* transcripts in different tea plant genotypes agreed with the UGT products observed in the *in vitro* assays. Further, consistent with the higher abundance of kaempferol in the first leaves, *UGT78A14* transcripts were also more abundant in the first leaves (**Figure 2C**). In contract, first leaves showed a very low content of quercetin glycosides. This analysis also indicated that kaempferol is more likely an *in planta* substrate of UGT78A14 than quercetin.

It should be noted that the amount of a putative kaempferol diglucoside (*m/z* 611 in positive mode) increased gradually at 4° C, and was strongly induced by five-fold when plants were transferred to 0°C for 7 more days. The concentration of this putative kaempferol diglucoside was lowered to the level of control when the plant was brought to room temperature for 7 days (**Figure 2D**). The correlation of *CsUGT78A14* transcript accumulation with the accumulation of flavonol glycosides, especially kaempferol glycosides (**Figure 2D**), showed that CsUGT78A14 plays a role in the production of the flavonol glycosides in response to cold stress in tea plant.

Suppression of *UGT78A14* Reduces Flavonols Accumulation in Tea Plant

To obtain insight into the physiological roles of CsUGT78A14 in the tea plant, the expression level of *CsUGT78A14* was transiently suppressed in *C. sinensis* leaves by gene-specific antisense oligodeoxynucleotide suppression according to Zhao et al. (2019). Tea plants treated with sense oligodeoxynucleotide were used as control. The expression level of *CsUGT78A14* in tea leaves treated with AsODN_CsUGT78A14 was significantly reduced compared with the control (**Figure 3A**), which indicated that the AsODN method is effective for this gene in the tea plant.

The content of flavonol glycosides in *CsUGT78A14*-silenced tea leaves with or without a cold stress was determined and compared to study the hypothesis that *CsUGT78A14* could affect cold tolerance by glycosylation of flavonoids. As expected, the content of glycosides in *CsUGT78A14*-silenced tea plants was reduced (**Figure 4B**). LC-MS analysis confirmed that *CsUGT78A14*-silenced tea leaves produced significantly ($P < 0.05$) lower levels of kaempferol monoglucosides (3-glucoside and 7-glucoside) and diglucoside when compared with that in the controls (**Figure 4B**).

The concentration of total flavonols was also reduced in *CsUGT78A14*-silenced tea leaves (**Figure 4C**). To explore why the concentration of flavonoids was reduced we examined the expression levels of flavonoid synthesis-related genes *CHS*, *CHI*, *F3H*, and *FLS* in controls and *UGT78A14*-silenced tea leaves. Interestingly, the transcription of all these genes in *UGT78A14*-silenced tea plants was obviously reduced, especially under cold stress condition (**Figure 4D**). This suggested that the down-regulation of *UGT78A14* can lead to decreased expression levels of flavonoid-related genes *via* feedback inhibition of the structural genes (Yin et al., 2012), which is consistent with the observed reduction of the flavonoid content mentioned above. Therefore, *UGT78A14* appears to play a key role in modulating the formation of flavonoids and their glycosides in the tea plant.

Suppression of *UGT78A14* Reduces Cold Tolerance in the Tea Plant

To further explore the role of *CsUGT78A14-1* for cold tolerance in the tea plant, Fv/Fm value was measured after to analyze the status of the photosystem II (Li et al., 2015). Purple-blue color (**Figure 4A**), together with quantitative analysis of Fv/Fm values revealed that exposure to cold stress led to the significantly damage of photosystem II (**Figures 4B**, **C**). Fv/Fm values in *CsUGT78A14*-1-silenced tea leaves (AsODN) in both cut leaves (**Figures 4B**, **C**) and leaves attached to the whole plant (**Figure 4D**) were all significantly reduced compared to the values measured for the control leaves under cold stress. The concentration of flavonoid glycosides was negatively correlated with the stress-induced damage in the tea plant, which indicated that *CsUGT78A14-1* responds to low temperatures by glycosylation of flavonoid.

CsUGT78A14 Involved in the Regulation of Flavonoid Biosynthesis and Reactive Oxygen Species Scavenging Activity in the Tea Plant

Flavonoids can act as a potent scavenger of free radicals and superoxide radicals (Del et al., 2008). Thus, the ROS levels were determined when the expression of *CsUGT78A14* was suppressed. The control plants and *UGT78A14*-silenced tea

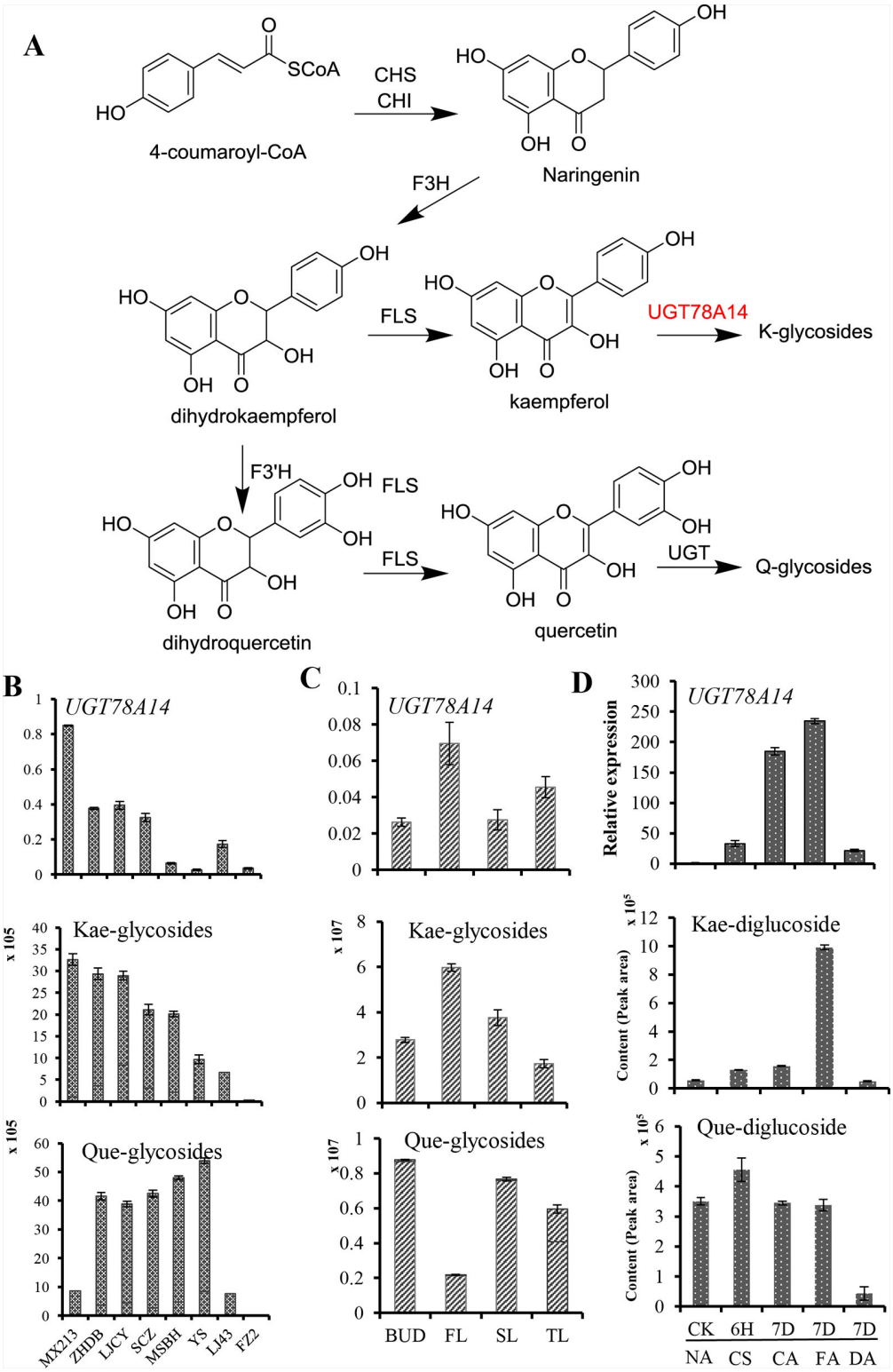

FIGURE 2 | Flavonol glycosides accumulation and CsUGT78A14 transcripts in response to cold stress, and in different tissues and genotypes of tea plant.The biosynthesis pathway of kaempferol and quercetin glycosides in the tea plant **(A)**. Relative expression levels of CsUGT78A14 and accumulation of flavonoid glycosides in response to cold stress **(B)** in three different genotypes of tea plant **(C)**, as well as in different leaves (FL first; SL second; TL third) and one bud of the tea plant **(D)**. For cold stress conditions please refer to **Figure 1**. Data are presented as mean ± SE of at least three biological replicates.

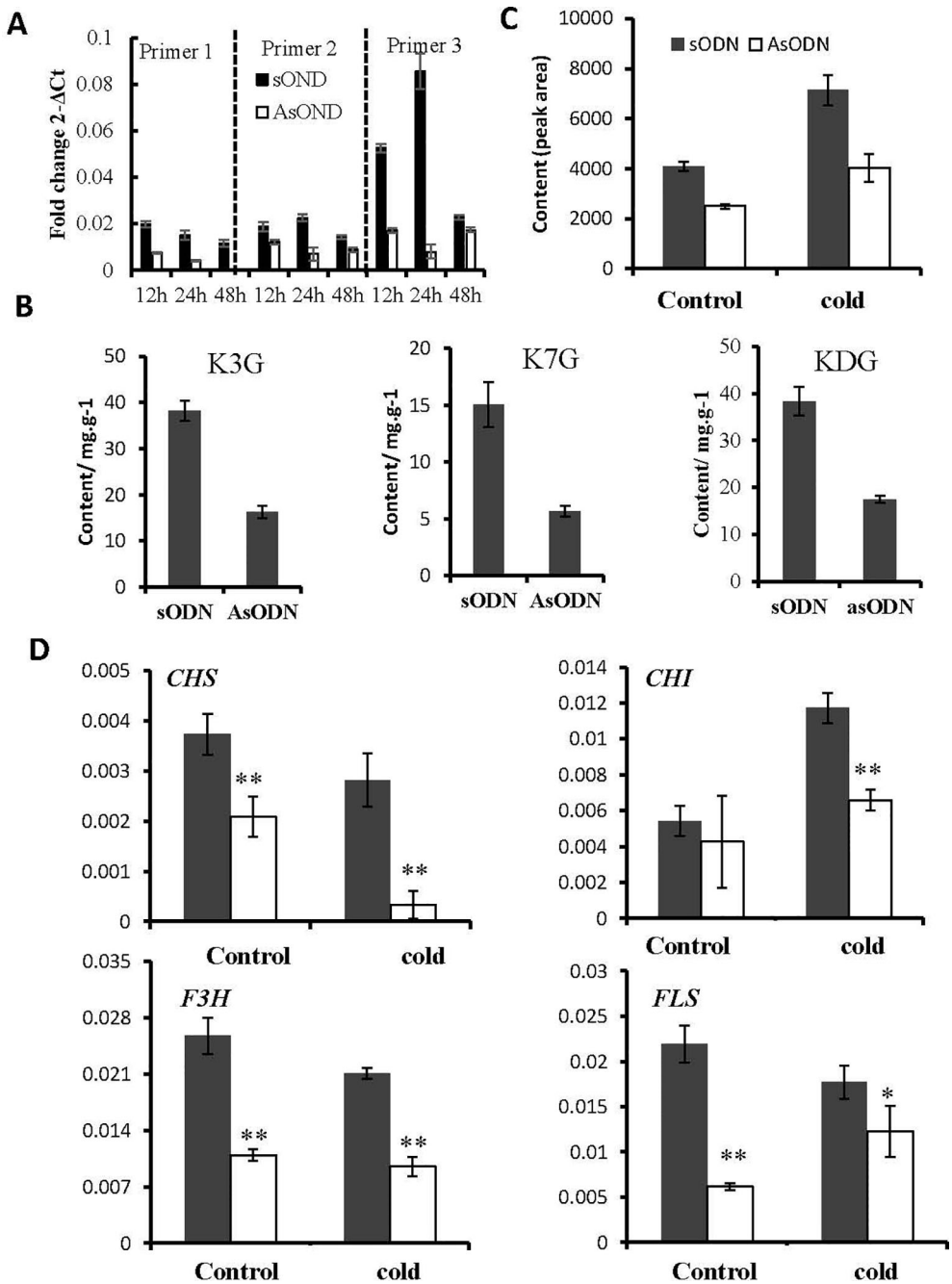

FIGURE 3 | Suppression of CsUGT78A14 reduces flavonols accumulation and flavonoid-related genes expression in tea plant under normal and cold stress conditions. Quantitative PCR analysis of CsUGT78A14 expression in the control and *CsUGT78A14*-silenced tea leaves **(A)**. Liquid chromatography–mass spectrometry analyses of kaempferol glucosides in the control and *CsUGT78A14*–silenced tea leaves **(B)**. Flavonoid accumulation **(C)** and flavonoid-related genes expression **(D)** in the control and *CsUGT78A14*–silenced tea leaves. For all extractions and evaluations, at least three biological replicates were performed. Asterisks indicate significant differences relative to the control (Student's t-test:*$P < 0.05$; **$P < 0.01$).

plants were exposed to −5°C for 3 h. DAB and NBT staining was performed for detecting H_2O_2 and superoxide, respectively (**Figure 4E**). The *CsUGT78A14*-silenced leaves exhibited deeper and broader staining than the control leaves.

In addition, total flavonoids were extracted and subjected to FRAP and DPPH assays to test the antioxidant activity in UGT78A14-silenced plants and compared with that in the control plants (**Figures 4F**, **H**). The results showed that the

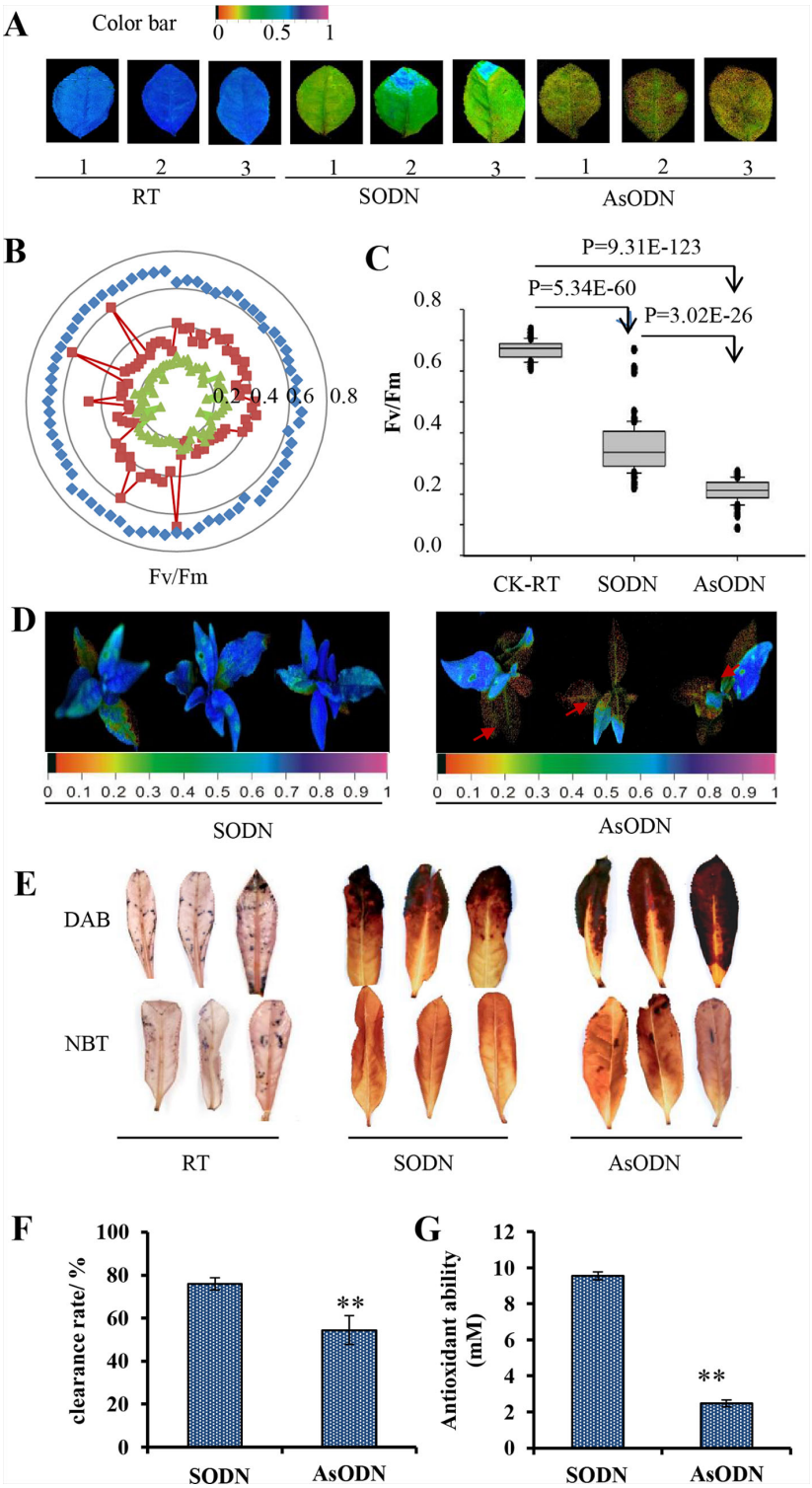

FIGURE 4 | Suppression of CsUGT78A14 reduces cold tolerance in tea plant. Pseudo color image **(A)**, radar plot **(B)**, and statistical analysis **(C)** of Fv/Fm in the control and *CsUGT78A14*–silenced tea leaves under normal and cold stress conditions. Each data point presents the mean of at least three biological replicates, each of which consisted of five technical replicates. Rseudo color image in the control and CsUGT78A14–silenced tea leaves under normal and cold stress conditions **(D)**. Nitrotetrazolium blue chloride and diaminobenzidine staining of the control and *CsUGT78A14*–silenced tea leaves under normal and cold stress conditions **(E)**. Antioxidant activity analysis of flavonoids extracted from the control and *CsUGT78A14*–silenced tea leaves measured by 2,2-diphenyl-1-picrylhydrazyl **(F)** and ferric reducing ability of plasma method **(G)**.

CsUGT78A14-silenced plants have significantly lower antioxidant capacities than those of the control, suggesting that CsUGT78A14 is involved in ROS scavenging.

CsUGT78A14 Regulates the Reactive Oxygen Species Scavenging Capacity of Flavonoid

As kaempferol can act as a potent scavenger of free radicals and superoxide radicals (Del et al., 2008), we inferred that the glycosylation process of kaempferol probably plays a key role in response to cold stress rather than only change the accumulation of flavonoids. To test our hypothesis, we compared the antioxidant and ROS scavenging activity of glycosides and their aglycones. As the products of our enzyme is too complex, to compared the antioxidant and ROS scavenger activity of glycosides formed by the enzymes and its aglycone, here, the CsUGT78A14-1 protein and empty vector protein were incubated with kaempferol and quercetin. Both kaempferol and quercetin glycosides were formed from the reaction with CsUGT78A14-1, whereas glycosides were not present in the reaction with the control protein. The ROS scavenging activity of both reaction products from CsUGT78A14-1 and control protein was compared. The ROS scavenging capacity of kaempferol and quercetin incubated with CsUGT78A14-1 was significantly enhanced compared to the control when analyzed by the DPPH, FRAP, and ABTS method (**Figure 5**). These results demonstrate that the glucosylation process catalyzed by CsUGT78A14 significantly promoted the ROS scavenging activity of the substrates compared to their corresponding free aglycons.

DISCUSSION

Products Comparison Formed by CsUGT78A14-1 and -2

Recent analysis showed that CsUGT78A14-2 is responsible for the biosynthesis of flavonol 3-O-glucoside *in vitro* (Cui et al., 2016) but its physiological role in the tea plant remained unknown. CsUGT78A14-1 differs from CsUGT78A14-2 in only one amino acid and can produce both kaempferol 3- and 7-O-glucoside.

Interestingly, CsUGT78A14-1 was also able to form kaempferol 3,7-diglucoside, which was identified by its retention index, MS and MS2 data in comparison with an authentic reference (Zhao et al., 2017). The amount of diglucoside produced by CsUGT78A14-1 was higher than that of the monoglucosides. The data indicated that Ala at 438 position of CsUGT78A14-1 plays a key role for the formation of the diglucoside *in vitro* and is located near the GSS motif, which was recently postulated as an important differentiation criterion between mono- and disaccharide-forming GTs (Huang et al., 2018; **Figure S3**).

The *in vitro* activity of UGTs can not fully reflect the *in planta* function of the enzyme, as the products formation will be affected by substrate availability (Song et al., 2015a). In this study, the *in vivo* activity of CsUGT78A14 was further studied by AsODNs-mediated silencing in the tea plant. Down-regulation of *CsUGT78A14* in the tea plant resulted in a reduced accumulation of kaempferol and quercetin monoglucosides and di-glucosides under cold stress (**Figure 3B**). Both *in vitro* and *in vivo* data confirmed that CsUGT78A14 was responsible for the biosynthesis of both flavonol monoglucosides and diglucosides in the tea plant, which have been rarely reported in plants until now.

CsUGT78A14 Affects Cold Stress Tolerance in the Tea Plant

As cold stress affects both yield and quality of tea (Wang et al., 2017), it is urgent to develop strategies to improve cold tolerance of the tea plants (Wang et al., 2013; Yin et al., 2016). Flavonoids are a representative group of secondary metabolites and can be induced by both biotic and abiotic environmental stresses (Dixon and Paiva, 1995). Ectopic expression of *UGT76E11* enhances abiotic stress tolerance in *Arabidopsis* by increasing flavonoid accumulation (Li et al., 2018a). The *Arabidopsis* UGT79B2 and UGT79B3, identified as anthocyanin rhamnosyltransferases, contribute to cold, salt, and drought stress tolerance *via* modulating anthocyanin accumulation (Li et al., 2017). In this study, CsUGT78A14 was strongly induced in response to cold stress, promoter analysis also confirmed CsUGT78A14 contain lots of stress-responsive element (**Table S2** and **Figure S9**). The amount of flavonoid glycosides were increased when the plants were treated at cold stress and the amount of flavonoid glycosides decreased when the plants were de-acclimated (**Figure 1**). This suggested that flavonoids and their glycosides also play a key role for cold stress tolerance in the tea plant.

Photosynthesis is sensitive to temperature fluctuations (Mondal et al., 2004; Wang et al., 2012). As an essential chlorophyll fluorescence parameter, Fv/Fm is sensitive to cold stress (Liang et al., 2007). Fv/Fm was significantly decreased in cold stress in this study (**Figure 6**), consistent with previous reports which showed that cold stress reduces Fv/Fm but promotes oxidative stress in tomato plants (Zhou et al., 2012). Fv/Fm values was significantly decreased by about 50% when exposed to cold stress (**Figures 4B**, **C**). Fv/Fm values in *CsUGT78A14*-silenced tea leaves were significantly reduced compared to the control leaves under cold stress. DAB and NBT staining showed that CsUGT78A14-silenced leaves exhibited deeper and broader staining than the wild-type leaves (**Figure 4D**). Taken together, our data showed that CsUGT78A14 plays a role in the regulation of hydrogen peroxide (H_2O_2) and superoxide accumulation. The reduction in Fv/Fm values might be due to the excessive accumulation of ROS under cold stress, which led to the induction of lipid peroxidation (Augspurger, 2013; Li et al., 2018b). The data presented here demonstrate that CsUGT78A14 plays a key role in the regulation of ROS scavenging capacity of flavonoids in *C. sinensis*.

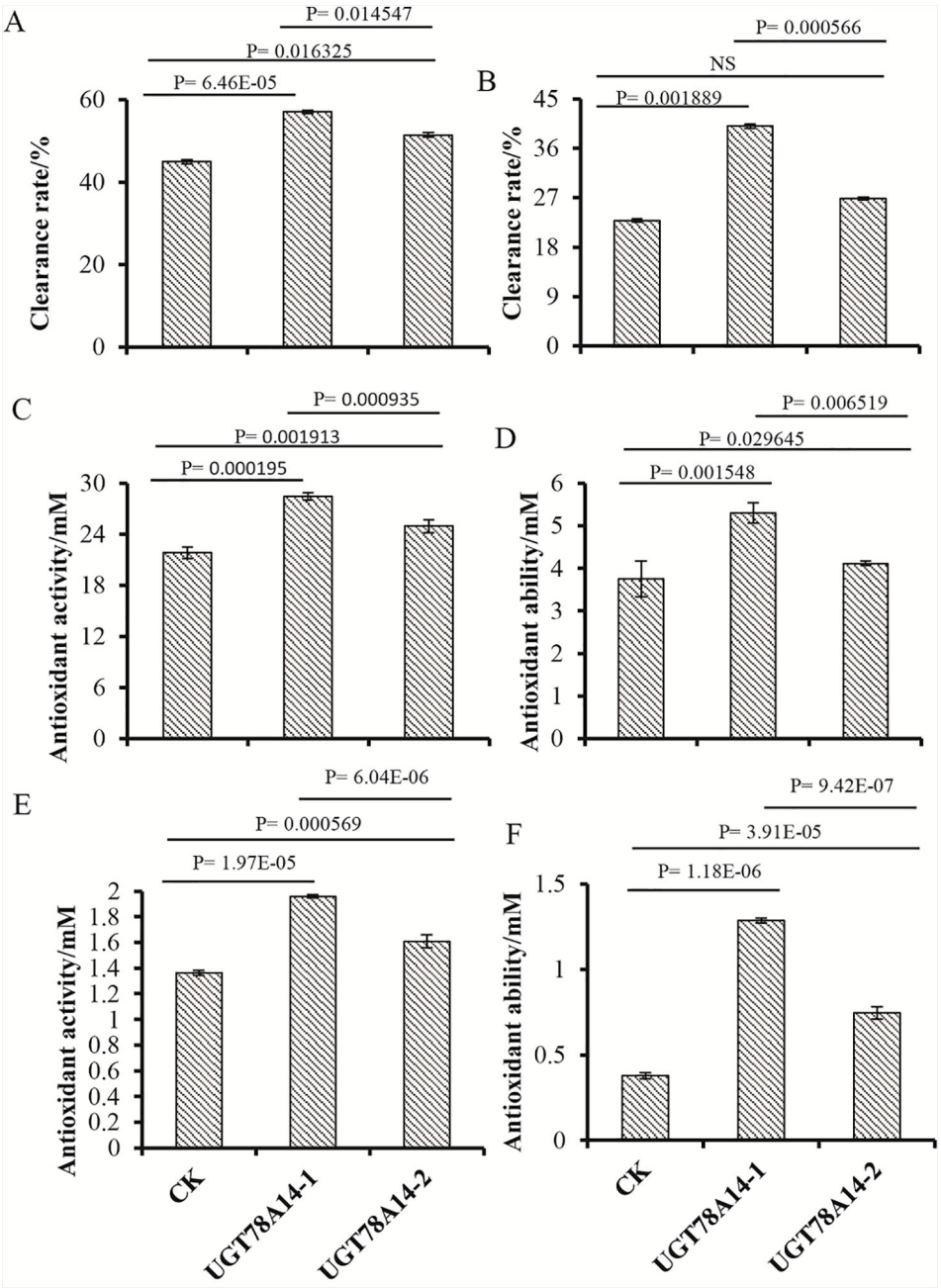

FIGURE 5 | CsUGT78A14 confer flavonoids enhanced reactive oxygen species (ROS) scavenging capacity. ROS scavenging activity of reaction products formed by CsUGT78A14-1 and control was compared. Kaempferol **(A**, **C**, **E)** and quercetin **(B**, **D**, **F)** were used as substrates. ROS scavenger capacity of kaempferol and quercetin incubated with CsUGT78A14-1 was significantly enhanced compared to the control by 2,2-diphenyl-1-picrylhydrazyl **(A**, **B)**, ferric reducing ability of plasma **(C**, **D)**, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method **(E**, **F)**. Three experimental replicates were conducted for the ROS scavenging capacity analysis. NS, P=0.05141.

CsUGT78A14 Regulates Both Flavonols Accumulation and Reactive Oxygen Species Scavenging in Tea Plants

Plant glycosyltransferases play diverse roles in the activity modification of metabolites which might involved in the regulation of ROS homeostasis (Mittler et al., 2004). Recently, flavonols were demonstrated can act as antioxidants and reduce the production of ROS (Watkins et al., 2014). Some reports have discussed the relationship between flavonoid and abiotic stresses, but the function of flavonoids and their glycosides remained unclear.

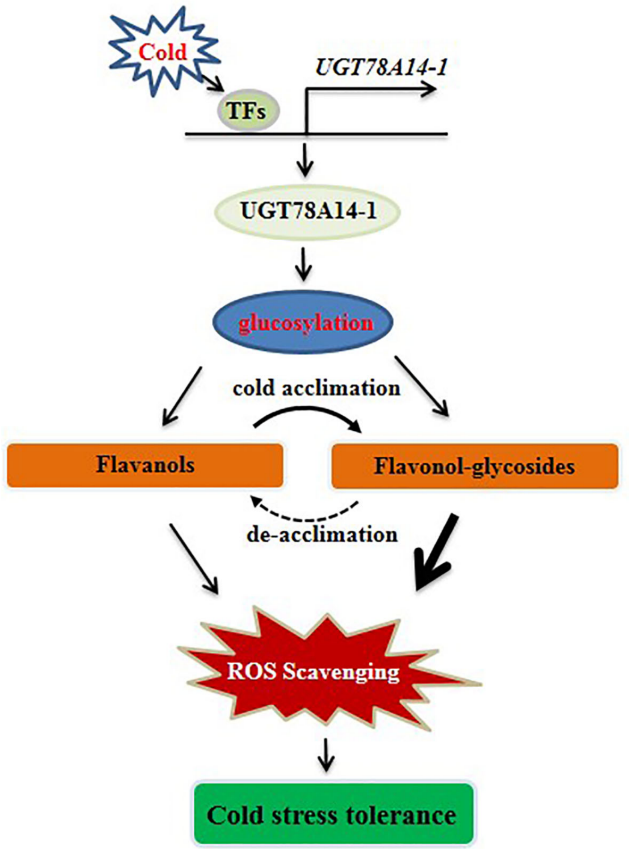

FIGURE 6 | Working model of UGT78A14 involved in cold stress response in tea plant. The line thickness represents contribution ability. Different from most of the previous studies, it should be highlighted that the antioxidant capacity of flavonols aglycon was greatly enhanced by glucosylation catalyzed by CsUGT78A14-1, indicating the glycosylated products play a critical role in abiotic stresses rather than a storage forms.

ROS scavengers ability of flavonoids are related to the number and arrangement of their hydroxyl groups (Del et al., 2008). The hydroxyl group(s) on the aglycone is important for scavenging DPPH, and the sugar moieties do not necessary (Tohge et al., 2005a; Seyoum et al., 2006). The flavonoid aglycones have been suggested as a stronger antioxidants compared with that of their glycosides because the free hydroxyl groups play an important role in ROS scavenging (Rice-Evans et al., 1996). For instance, loss of function of UGT73B1/B2/B3, which was confirmed involved in flavonoids glycosylation, led to improvement of oxidative stress tolerance whereas UGT73B2 un-regulation increased the plants sensitivity to ROS (Chae et al., 2006). Water stress led to the accumulation of quercetin 3-O-glucosides and decreased antioxidant enzyme activities, suggested that the glycosides could act as ROS scavengers (Fini et al., 2012). Glycosyltransferases ugt73b3 and ugt73b5 mutants exhibited an accumulation of ROS in *Arabidopsis thaliana* (Simon et al., 2014). The biological role of the flavonoid glycosides is far away to be understand as they are less effective antioxidants than the corresponding aglycones (Vogt and Jones, 2000; Gachon et al., 2005) but they are accumulate during cold stress.

In the current study, the DPPH and FRAP assays showed that kaempferol glycosides exhibit a much higher antioxidant capacity compared to the control, suggesting that the modified flavonoid glycosides detected in this system have radical scavenging activities, and the glucosylation catalyzed by CsUGT78A14-1 play an important role in redox homeostasis. A very recent study showed that the antioxidative progress flavonoids can be altered by the environments, in solution quercetin glucosides showed a higher antioxidant activity than quercetin (Zheng et al., 2017), which is consistent with our current study. Therefore, the enhancement of ROS scavenging capacity of flavonoids catalyzed by CsUGT78A14 contributes at least in part the mechanism which increases cold stress resistance in the tea plant (**Figure 6**).

The antioxidant and ROS scavenging activity was further examined in *CsUGT78A14-1*-silenced tea leaves and we found that they showed a lower antioxidant capacity and reduced total flavonols compared to the control tea leaves (**Figures 4E**, **F**), consistent with the down-regulation of flavonoid synthesis-related genes *CHS*, *CHI*, *F3H,* and *FLS*. It is believed that when CsUGT78A14 was silenced, less substrates will be consumed by CsUGT78A14, which in turn inhibit the expression of the upstream enzyme genes and reduce the biosynthesis of flavonols. This result is consistent with our observation that down-regulation of CsUGT78A14 also alters the transcript levels of the upstream enzyme genes. Taken together, our results confirmed CsUGT78A14 could regulate both flavonols accumulation and ROS scavenging in response to cold stress in tea plants.

CONCLUSION

CsUGT78A14 plays a critical role in cold stress by increasing flavonols accumulation and ROS scavenging capacity. These results not only enable the use of CsUGT78A14 in tea plant improvement, both to enhance cold stress tolerance and to increase flavonoid accumulation, but also provide novel insights to the underlying mechanism of the interaction of UGTs with cold stress tolerance in plant.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CS, WS, and MZ conceptualized the initial study. MZ, JJ, TG, NZ, and TJ were involved in the experimental layout. MZ, JJ, TG, TJ, JW, and QB performed the lab experiments. MZ and QB performed the RNA extraction and transcriptome analyses. CS,

WS, and MZ drafted the initial article; all authors discussed the results, reviewed the article, and approved the final article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01675/full#supplementary-material

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UV-B Induces Distinct Transcriptional Re-programing in UVR8-Signal Transduction, Flavonoid, and Terpenoids Pathways in *Camellia sinensis*

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Plants are known to respond to Ultraviolet-B radiation (UV-B: 280–320 nm) by generating phenolic metabolites which absorbs UV-B light. Phenolics are extraordinarily abundant in *Camellia sinensis* leaves and are considered, together with pleasant volatile terpenoids, as primary flavor determinants in tea beverages. In this study, we focused on the effects of UV-B exposure (at 35 μW cm^{-2} for 0, 0.5, 2, and 8 h) on tea transcriptional and metabolic alterations, specifically related to tea flavor metabolite production. Out of 34,737 unigenes, a total of 18,081 differentially expressed genes (DEGs) due to UV-B treatments were identified. Additionally, the phenylpropanoid pathway was found as one of the most significantly UV-B affected top 20 KEGG pathways while flavonoid and monoterpenoid pathway-related genes were enhanced at 0.5 h. In the UVR8-signal transduction pathway, *UVR8* was suppressed at both short and long exposure of UV-B with genes downstream differentially expressed. Divergent expression of *MYB4* at different treatments could have differentially altered structural and regulatory genes upstream of flavonoid biosynthesis pathways. Suppression of *MYB4-1&3* at 0.5 h could have led to the up-regulation of structural *CCOAOMT-1&2, HST-1&2, DFR-4, ANR-2*, and *LAR-1&3* genes resulting in accumulation of specialized metabolites at a shorter duration of UV-B exposure. Specialized metabolite profiling revealed the correlated alterations in the abundances of catechins and some volatile terpenoids in all the treatments with significant accumulation of specialized metabolites at 0.5 h treatment. A significant increase in specialized metabolites at 0.5 h treatment and no significant alteration observed at longer UVB treatment suggested that shorter exposure to UV-B led to different display in gene expression and accumulation of specialized metabolites in tea shoots in response to UV-B stress. Taken together, our results indicated that the UV-B treatment applied in this study differentially altered the UVR8-signal transduction, flavonoid and terpenoid pathways at transcriptional and metabolic levels in tea plants. Our results show strong potential for UV-B application in flavor improvement in tea at the industrial level.

Keywords: ultraviolet radiation, UVR8 photoreceptor, abiotic stress, phenolics, HY5, terpenoids metabolism, climate change, flavonoid accumulation

INTRODUCTION

Tea is a widely consumed non-alcoholic beverage, made from tender tea leaves (*Camellia sinensis*) (Banerjee, 1992), which contain substantial volatiles and extraordinarily high amount of polyphenols (16–30%, dry weight) (Graham, 1992) such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG). These specialized compounds are crucial for tea brisk, umami taste, pleasant scent, and anticancer, antibacterial, and immunostimulant effects (Kumazawa, 2006; Yu et al., 2014; Han et al., 2016).

Metabolic characteristics of tea plants might be evolutionally developed and associated with the environments through the course of evolution. *C. sinensis* is native to southwest Asia and is grown at high altitudes (Meegahakumbura et al., 2018), receiving high fluence rates of UV-B radiation (280–320 nm) compared to plant species grown in low altitude regions (Madronich et al., 1995; Casati et al., 2006). The impact of UV-B on plant growth and development is largely dependent on irradiation dose and plant species (Casati and Walbot, 2003; Jenkins, 2009; Pontin et al., 2010). UV-B can regulate gene expression both by UV-B specific and non-specific pathways (Jenkins, 2009). High fluence of UV-B may activate both UV-B specific and non-specific pathways leading to the generation of reactive oxygen species (ROS), DNA and protein damage, inhibition of photosynthetic reactions and protein synthesis (Jansen et al., 1998; Frohnmeyer and Staiger, 2003), and consequently leading to a wide array of changes at molecular and morphological levels in plants (Mackerness et al., 1999; Brown et al., 2005; Meegahakumbura et al., 2018). Ambient UV-B at low fluence rates has regulatory effects on plant growth and development, biochemical composition (Boccalandro, 2001; Suesslin and Frohnmeyer, 2003) and affect the expression of a wide range of genes (Casati and Walbot, 2003; Casati and Walbot, 2004; Jenkins, 2009). These UV-B specific alterations in plants are mediated by the signaling transduction module of UV RESISTANCE LOCUS8 (UVR8) and COP1 (Rizzini et al., 2011; Jenkins, 2017), which contains multiple transcription factors such as ELONGATED HYPOCOTYL5 (HY5) (Binkert et al., 2014), and MYB family members (Stracke et al., 2009). UV-B induced metabolomic changes (including enhanced flavonoids, anthocyanins, and many other phenolics) have been reported in some plant species (Takshak and Agrawal, 2016). Correspondingly, UV-B enhanced transcriptional alteration has also been reported in multiple structural and regulatory genes in phenylpropanoid/flavonoid pathways such as *PHENYLALANINE AMMONIA-LYASE* (*PAl*), *CHALCONE SYNTHASE* (*CHS*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *FLAVANONE-3-HYDROXYLASE* (*F3H*), *MYB12* and *MYB111* (Brown et al., 2005; Stracke et al., 2009) and other transcription factors of the regulatory MYB/bHLH/WD40 (MBW) complexes, which regulate phenylpropanoid biosynthesis (Xu et al., 2015; Li et al., 2017). Interestingly, phenolic metabolites including diverse flavonoids, which are deposited in plant epidermal tissues (Greenberg et al., 1996; Rozema et al., 1997), absorbing light in the wavelength ranges 250–270 nm and 330–350 nm (Kumar et al., 2018) with an absorption peak around 300 nm (Gábor, 2013), function as a UV-absorbing sunscreen and, in turn, offer protection to plants from UV-B damage (Li et al., 1993; Brown et al., 2005; Liu et al., 2015). A recent study applying UV-B treatment to tea plants revealed their distinct responses compared to some other plants (Liu L. et al., 2018). For example, only EGCG was induced, while the abundance of other catechins remains unchanged. In addition, the genes (*UVR-8*, *COP1*, *HY5*) in the UV-B signaling transduction module are not consistently UV-B up-regulated (Liu L. et al., 2018) as found in many other plant species (Rizzini et al., 2011; Jenkins, 2017).

Pleasant and characteristic aroma of teas is highly demanded and considered as a key indicator for good quality of teas. Tea aroma compounds are commonly generated from various metabolite precursors during the manufacturing process (Ho et al., 2015). In tea leaves biosynthesis of volatile terpenoids, which impart teas with floral scent, is an essential prerequisite for the accumulation of volatile terpenoid conjugates and volatile release after conjugate hydrolysis. In plants, diverse terpenoid compounds are known synthesized through the condensation of the terpenoid building blocks dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) generated from the mevalonate (MVA) and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathways, respectively (Hemmerlin et al., 2012). Biosynthesis of different terpenoids is catalyzed using prenyl diphosphate precursors by different terpene synthases *(TPS)* (Chen et al., 2011). Recently a bifunctional gene generating two transcripts coding a linalool synthase and a nerolidol synthase, respectively was reported in tea and the transcript for linalool synthase alone was induced by methyl jasmonic acid (Liu G.F. et al., 2018). Additionally, a few *TPS* genes such as nerolidol synthase genes were also characterized in tea plants (Zhou et al., 2017; Liu G.F. et al., 2018). Moreover, supplemental UV-B radiation to harvested tea shoots for 2 h in the manufacturing process can enhance the abundance of many odorants including linalool in the resulting tea (Jang et al., 2010). In addition, some *TPS* genes were found being activated by UV-B irradiation in peach (Liu et al., 2017). Therefore, it would be interesting to find out whether different time duration (0, 0.5, 2, and 8 h) of UV-B irradiation, rather than stress conditions of the excised leaves, can affect the expression of *TPS* genes in the intact tea leaves.

Specialized metabolites synthesis in plants is affected by many endogenous and exogenous factors; however, transcriptional regulation plays a key role and thus becomes a subject for better understanding the molecular mechanisms underlying metabolic flux regulation (Patra et al., 2013). It is well established that tea leaves are rich in UV-B absorbing polyphenolics, such as catechins (Graham, 1992). Hence, it would be interesting to investigate whether UV-B induced transcriptional responses of tea plants are distinct from those of other plant species with low polyphenolic abundance, with an emphasis on those genes related to the flavonoid pathways for a better understanding of the UV-B transcriptional regulation of polyphenolic production.

This study was designed to clarify UV-B induced alterations in gene transcription and metabolites related to tea flavor determinants (catechins and volatiles) (Zheng et al., 2008; Jang et al., 2010) for theoretical understanding of transcriptional regulation of flavor determinant formation and practical

improvement of green tea beverage flavor. Thus UV-B radiation was supplemented to tea plants with different exposure durations and transcriptional and metabolic profiling was performed with an emphasis on UVR8-signal transduction, flavonoid, and volatile terpenoid pathways to determine a potential transcriptional control of tea flavor-determinant production. Our findings open a window for future studies on the physiological and ecological functions of volatiles terpenoids in response to environmental-related stress in the era of climate change.

MATERIALS AND METHODS

Plant Materials and UV Treatments

One-year-old potted plants of *Camellia sinensis* cv. "Longjing-43" were planted in the tea farm of Anhui Agricultural University located at 31°55′ 42.8″ N, 117°12′ 09.1″ E, Hefei, China. For UV-B treatments, an effective dose rate at ≅35 μW cm^{-2} irradiances at 311 nm normalized UV-B was provided according to Jang et al. (2010) for four different exposure durations (0, 0.5, 2, and 8 h). For each exposure treatment with biological triplicates, 10 plants were applied for each replicate. The UV-B radiation was generated using a TL 100W/01 tube (311 and 313 nm spectrum peaks, Philips, Germany) in laboratory condition under the constant white light (100 μmol·m^{-2}·s^{-1}) delivered by LEDs (Tops 10 Power Pure White Led OSW4XAHAE1E) 2.7 m) above the plants. The UV-B tube was placed 40 cm above the plants and the irradiation area was 0.5*1.8 m as described earlier (Jang et al., 2010). The group set as a control (0 h) was placed under white light and exposed to the same UV-B tube but covered with a 120 μm clear polyester plastic filter (Hangzhou Phillis Filter Tech Co., Ltd., Hangzhou, China), which absorbed more than 95% of UV-B without affecting photosynthetically active radiation (PAR). After UV-B exposure, two or three shoot tips with two folded leaves were collected from each treated or control plant and immediately frozen in liquid nitrogen and maintained at –80°C for RNA extraction and metabolite analysis. Totally, 12 samples were obtained.

RNA-Seq Library Construction and Illumina Sequencing

Plant samples subjected to different durations of UV-B exposure (0, 0.5, 2, and 8 h) were used to extract total RNA using RNAprep pure Plant Kit (TianGen Biotech., Ltd., Beijing, China). The RNA integrity was examined using both agarose gel electrophoresis and a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). For RNA-seq analysis, equal amounts of RNA extract was obtained from all the 12 samples pooled together and sent to Shanghai OE Biotech (Shanghai, China) for cDNA library construction and sequencing to generate transcriptomic data for this study. Enrichment of mRNA, fragmentation, the addition of adapters, size selection, PCR amplification, and RNA-Seq was performed by staff at Shanghai OE Biotech (Shanghai, China). mRNA was first enriched from 20 μg total RNA using magnetic beads with Oligo (dT) (Invitrogen, Beijing, China) and then fragmented, followed by cDNA synthesis using these short fragments as templates and random primers (Takara). Then, the double-stranded cDNA fragments were ligated to adapters using T4 DNA ligase (Invitrogen, United States). The two final cDNA libraries were constructed after PCR enrichment of these ligated products. Multiplexed RNA-Seq libraries were sequenced using the Illumina HiSeqTM 2000 platform (Illumina, Inc., Shanghai, China). Moreover, *De novo* assembly of RNA-Seq reads and functional annotation of the unigenes were also conducted using the transcriptome assembler Trinity (Grabherr et al., 2011). Unigene sequences obtained from the UV-B treated and non-treated control were aligned using BLASTX against the databases of GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes). All sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession number PRJNA597433).

RNA-Seq Data Analysis

For the identification of high-quality sequencing data and clean reads, quality control was performed on the raw reads. Filtered clean reads from each of the 12 samples were independently aligned to the reference transcripts of tea genome (Wei et al., 2018) with the help of Bowtie2 software (Langmead et al., 2009) and used to approximate the abundance of gene transcripts using the RSEM method (Li and Dewey, 2014) measured as fragments per kilobase of transcript per million fragments sequenced (FPKM) (Trapnell et al., 2010). Based on method described by Audic and Claverie (1997), differentially expressed genes (DEGs) were identified. False discovery rate (FDR) of >0.001 and an absolute value of FPKM fold-change of >2 as the thresholds were used to examine the significance DEGs. Wilcoxon signed-rank test was used to analyze the difference in gene expression among the groups and to evaluate the influence of UV-B on the transcriptional level of genes in tea shoots. P-value < 0.05 was considered statistically significant (**Supplementary Figure S3**), and a heat map of expression values was generated with the help of the T-MeV 4.9.0 software (Howe et al., 2011). Annotation analyses of DEGs were performed using WEGO software (Ye et al., 2018) for GO term functional classification, and pathway enrichment analysis of DEGs was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

HPLC Analysis for Catechins and Caffeine

Tea samples used in this study were freeze-dried for catechin and caffeine analysis. The sample (0.5 g) was placed into a 100 mL vial with 20 mL boiled water in a 90°C water bath for 5 min, and then was filtered and quantified according to Han et al. (2016) with some modifications. In brief, an HPLC (600 Controller, 2489 UV/Visible Detector; Waters, Milford, MA, United States) equipped with a C18 column (5 μm × 4.6 mm × 250 mm; Phenomenex, Torrance, CA, United States) was used. The compounds were quantified (mg/g DW) using calibration curves determined from authentic standards as described before (Feng et al., 2014). Data were statistically analyzed using one-way

analysis of variance (*t*-tests, $p < 0.05$). Chemical analyses had three biological replicates.

GC-MS Analysis for Terpenoid Volatiles

Gas chromatography (Agilent 7697A)/mass spectrometry (Agilent 7890A) (GC/MS) and a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm; Agilent) were used in this study. Chemicals were identified by comparing the retention time and mass spectrum either with those of authentic standards or NIST mass spectral library version 17. Compounds were quantified based on the calibration curves established using a series of diluted solutions prepared with authentic standards (Han et al., 2016). The concentrations of the volatiles were expressed as relative content in percentage. Data were also statistically analyzed using one-way analysis of variance (*t*-tests, $p < 0.05$). The repeatability of the analytical method was evaluated using triplicates of independently prepared samples as for catechin analysis.

Quantitative Real-Time PCR Assay

For studying the UV-induced transcriptional alterations in tea plants, the qPCR analysis was performed on a CFX96 platform (Bio-Rad, www.bio-rad.com/) and the Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Expression of annotated genes in UVR8-signal transduction, phenylpropanoids, volatile terpenoids pathway and *TPS* genes in the UV-B treated tea shoots for 0, 0.5, 2 and 8 h treatment were quantified using gene-specific primers (**Supplementary Table S2**). QPCR program was computerized as follows: 95°C for 10 min; 40 cycles of 95°C for 10 s, annealing at 58°C for 15 s, and elongation at 72°C for 34 s; followed by a heat dissociation protocol from 55°C to 95°C. PCR reaction efficiencies for all test genes were over 90% and their transcript levels were normalized using the reference gene 18s RNA, then calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Dissociation curves for each amplicon were evaluated to verify the specificity of each amplification reaction. All the qPCR analyses were carried out using three biological replicates each containing technical triplicate and the data were subjected to analysis using SPSS 17.0 statistical package.

TABLE 1 | Summary of RNA-Seq statistical assembly.

Assembly				
Total raw reads	**Total clean reads**	**Total clean bases (G)**	**Q30%**	**GC%**
662,300,000	644,490,000	97.5064	95.55%	45.02%
Annotation				
	GO	**DEGS**	**KEGG**	**DEGS**
Treatments	Unigenes		Unigenes	
0.5 hr	23888	1929	10849	755
2 hr		3495		1570
8 hr		6883		3449

RESULTS

RNA-Seq Analysis Reveals Re-programing of Global Transcription in the Tea Shoots in Response to UVB Exposure

RNA-seq analysis from all tea samples resulted in 91.03G clean data with 6.89∼8.07G for each sample and the Q30 base distribution ranged from 95.06 to 95.94%. The rate of the alignment between sequencing reads and the reference genome (Wei et al., 2018) fell in a range of 92.14∼94.17%. Further, a total of 23,888 unigenes and 12,307 differentially expressed genes (DEGs) were identified based on method described by Audic and Claverie (1997), differentially expressed genes (DEGs) were identified (**Supplementary Figure S3**). False discovery rate (FDR) of >0.001 and an absolute value of FPKM fold-change of >2 as the thresholds were used to examine the significance DEGs (**Table 1** and **Supplementary Table S1**). All DEGs were visualized in a Venn diagram for differential transcriptional alterations due to different UV-B exposure durations. The highest number of DEGs was observed at 8 h treatment (8 h vs. 0h) while the lowest at 0.5 h (0.5 h vs. 0 h) treatment. Sixty up-regulated and twenty-seven down-regluated DEGs were found common to all the UV-B treated groups (**Figure 1A**). Numerous pathways of KEGG associated with DEGs were UV-B affected. Among the affected KEGG pathways categorized into cellular processes, environmental information processes, genetic information processes and metabolism, the majority of DEGs were associated with metabolism processes. Biosynthesis of other specialized metabolites was mostly affected after glycan biosynthesis and metabolism at 0.5 h as compared to other pathways at the same exposure time. Metabolism of terpenoids and polyketides were affected by all the treatments with significant effects at 8 h treatment (**Figure 1B**).

Among UV-B induced top 20 affected KGG pathways, six pathways were affected by all the three treatments, including amino acid biosynthesis and protein processing in endoplasmic reticulum (**Figure 2A**). Phenylpropanoid biosynthesis was noted in addition to plant hormone signal transduction, carbon metabolism, photosynthesis which were among the eight pathways affected by the two treatments only (**Figure 2B**). Monoterpenoid biosynthesis together with Rap1 and Ras signaling pathways was noted among the 11 affected pathways unique to 0.5 h treatment. In addition, upregulated examples for UV-B irradiated shoots include 8 and 4 genes involved in flavonoid and monoterpenoid pathways, respectively at 0.5 h (**Supplementary Table S5**). Ribosome was the most affected pathway unique to 8 h treatment among other 7 pathways including citrate cycle. We observed the least number of pathways unique to 2 h treatment with perixisome and terpenoid backbone biosynthesis as the most affected (**Figure 2C**). As expected, some stress-related pathways were also UV-B affected such as response to oxidative stress, UV-B signaling transduction, and stimuli to light, bacteria, fungi, and multiple hormones (**Supplementary Figure S1** and **Supplementary Table S4**).

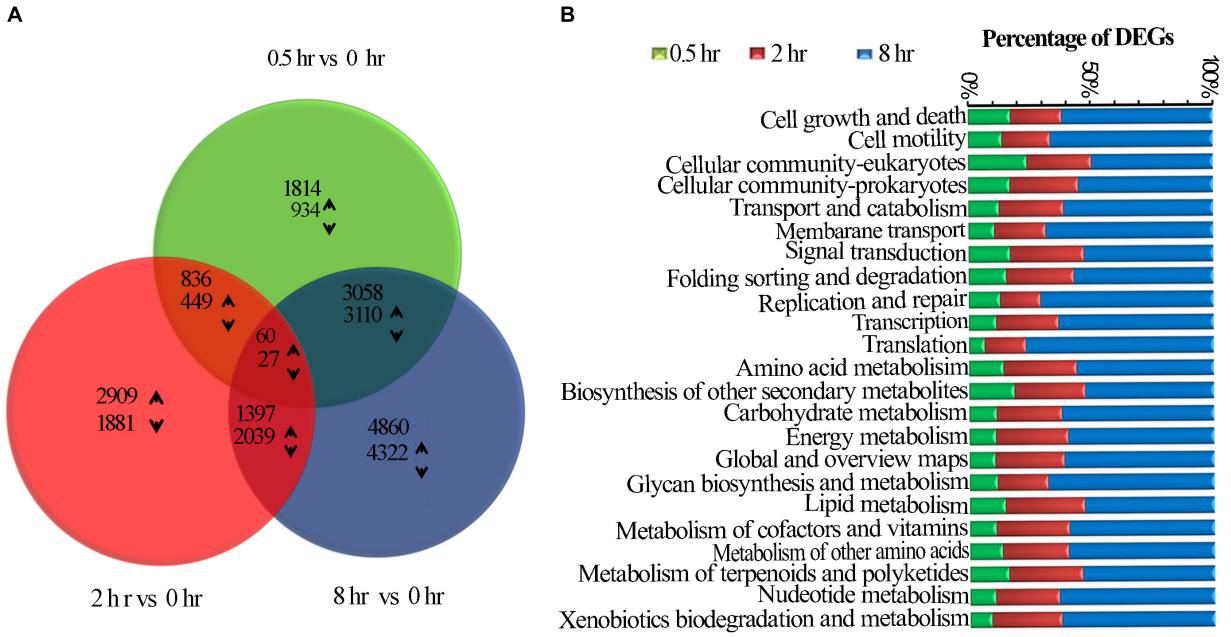

FIGURE 1 | UVB induced transcriptomic changes in tea shoots. **(A)** Venn diagram showing the statistics of the number of common and unique differentially expressed genes between different comparison groups (0.5 h Vs. 0 h, 2 h Vs. 0 h, and 8 h Vs. 0 h (control) treatments. The numbers of differentially expressed transcripts are provided as down-regulated and up-regulated Using arrows. **(B)** The number of all the DEGs in the entire KEGG pathway affected in the three treatments.

It is interesting to note that some pathways such as "Glycine, serine and threonine metabolism," "Phenylalanine, tyrosine and tryptophan," "Biosynthesis of amino acids," Ribosomes and "Protein processing in endoplasmic reticulum" were significantly affected when a longer exposure duration (for 8 h) was applied, while other pathways such as "Plant hormone signal transduction, Phenylpropanoid biosynthesis" were affected when exposed for 2 h, and 0.5 h of UV-B exposure whiles others such as "Photosynthesis, Methane metabolism," and "Plant hormone signal transduction", were more pronouncedly affected for a longer exposure period (**Figures 2A–C** and **Supplementary Table S3**).

Differentially Expressed Genes in UVR8-Mediated Signal Transduction Pathway

UVR8 is a UV-B receptor and mediates UV-B induced plant responses through signal transduction pathway (Kliebenstein et al., 2002). To understand roles of *UVR8* and its signal transduction pathway in tea plants in response to UVB exposure at different time periods, transcriptional alteration of the genes in this pathway were examined in this study. We identified only one UVR8 transcript in our transcriptomic data even though 10 more UVR8 transcripts were retrieved from tea genome after performing a local blast. Our data showed that in all the UV-B treated tea plants, *UVR8* transcription levels were down-regulated (**Figure 3**). Transcription levels of *HY5*, *MYB4*, *bHLH62*, and *MYB12,* transcriptional factors regulating the downstream structural genes, were all affected differently at different exposure times (**Figure 3A**). Here, we observed *HY5* was down-regulated at 0.5 h and up-regulated at 2 and 8 h treatments. Four *MYB4* (*MYB4-1, MYB4-2, MYB4-3,* and *MYB4-4*) were observed to be altered differently through all the treatments while *MYB12* and *bHLH* 62 were up-regulated at 8 h treatment (**Figure 3B**). Transcript abundance of 8 selected genes in this pathway was validated using real-time qPCR analysis. Consistent UV-B responses at different exposure time were observed between the RT-qPCR analysis and RNA-Seq gene expression (**Figure 3B**). UVR8 transduction pathways responded to UVB exposure leading to suppression of *UVR8* gene and differential expression of genes downstream UV8. Further work need to be done to find out the role of other 10 *UVR8* genes in tea plant.

UV-B Induced Differential Expression of Genes in the Flavonoid Biosynthesis Pathway

It has been widely reported that UV-B irradiation induces flavonoids and anthocyanins accumulation which acts as UV-B protectant in different plant species. The tea plant is classified as extraordinary plant species due to its enormous accumulation of flavonoids (Punyasiri et al., 2004). We examined the effect of different time exposure of UV-B on tea shoots using transcriptomic data (**Figure 4**). Thirty transcripts were differentially affected in this pathway with 11 down-regulated and 19 up-regulated. We observed up-regulation of transcripts *4CL2-1, 4CL2-3* were up-regulated at 2 and 8 h treatment while *4CL2-2* and *4CL2-4* were upregulated at 8 and 2 h treatment,

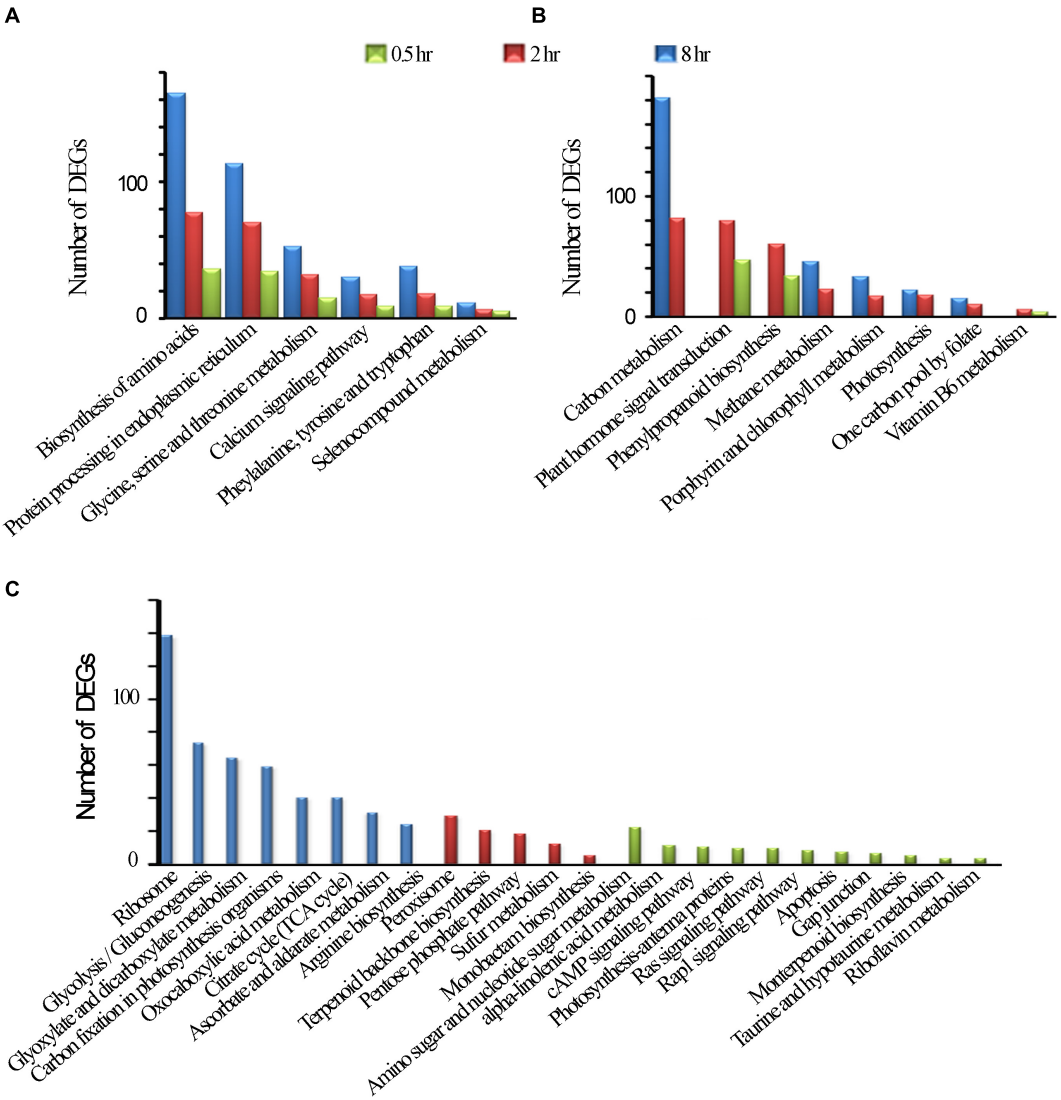

FIGURE 2 | KEGG Enrichment top 20. **(A)** common DEGs in all the three treatments. **(B)** Common DEGs to the two treatments. **(C)** Unique DEGs to each of the three treatments.

respectively. *DFR-4, ANR-2, CHI, FSLI, ANS, LAR-1, LAR-2*, and *LAR-3* at 2 and 8 h exposure periods. *CCOAOMT-1, CCOAOMT-2, HST-1, HST-2, ANR-2, DFR-4, LAR-1* and *LAR-3* were up-regulated at 0.5 h exposure consistent with observation made earlier in flavonoid synthesis pathway. *CHS-1, CHS-2, CHS-3, DFR-1, DFR-2-DFR-3, ANR-1, F3′5′H-1, F3′5 ′H-2*, and *F3′H* were all down-regulated at 8 h exposure time. Moreover, divergent expression patterns of some genes in phenylpropanoid pathway including transcriptional factor *MYB4* were also noted (**Figure 4A** and **Supplementary Table S5**). *MYB4-1* and *MYB4-3* were all down-regulated by all the three treatments while *MYB4-2* was up-regulated in all the treatments. It was interesting to note suppression of most structural genes at 8 h treatment but no changes were observed in 0.5 and 2 h treatment. Up regulation of *MYB4-2* at 8 h treatment could have suppressed the structural genes as observed at 8 h treatment. *MYB4-4* was up-regulated at 0.5 and 2 h treatment, but such an enhancement did not exert any detectable regulatory effects on its target genes except for *F3′5′H-1*, in flavonoid pathway. Down regulation of *MYB4-1* and *MYB4-3* at 8 h treatment could have led to upregulation of structural genes *4CL2-1, 4CL2-2, 4CL2-3, 4CL2-4, DFR-4, ANR-2, CHI, FSLI, ANS, LAR-1, LAR-2*, and *LAR-3* (**Figure 4B**). Analysis of MYB4 protein sequences revealed differences in their structures (**Supplementary Figure S2**). The up-regulation and down-regulation of *MYB4* could be the reason for divergent expression of genes in flavonoid pathway.

To validate the reliability of gene expression analysis in this study, 26 genes with potential roles in flavonoid biosynthesis, were selected for real-time qPCR analysis. Our real-time qPCR data showed that these genes expression patterns, matched the gene expression patterns observed in RNA-Seq analysis (**Figure 4B**).

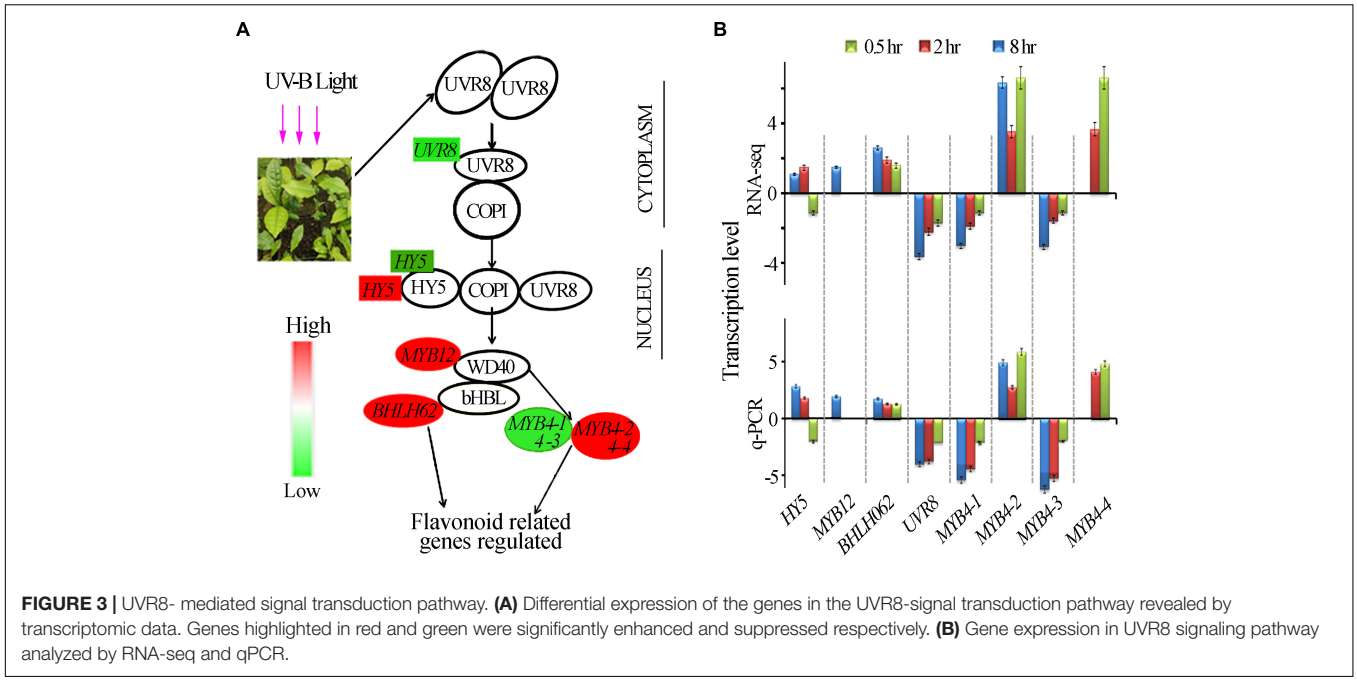

FIGURE 3 | UVR8- mediated signal transduction pathway. **(A)** Differential expression of the genes in the UVR8-signal transduction pathway revealed by transcriptomic data. Genes highlighted in red and green were significantly enhanced and suppressed respectively. **(B)** Gene expression in UVR8 signaling pathway analyzed by RNA-seq and qPCR.

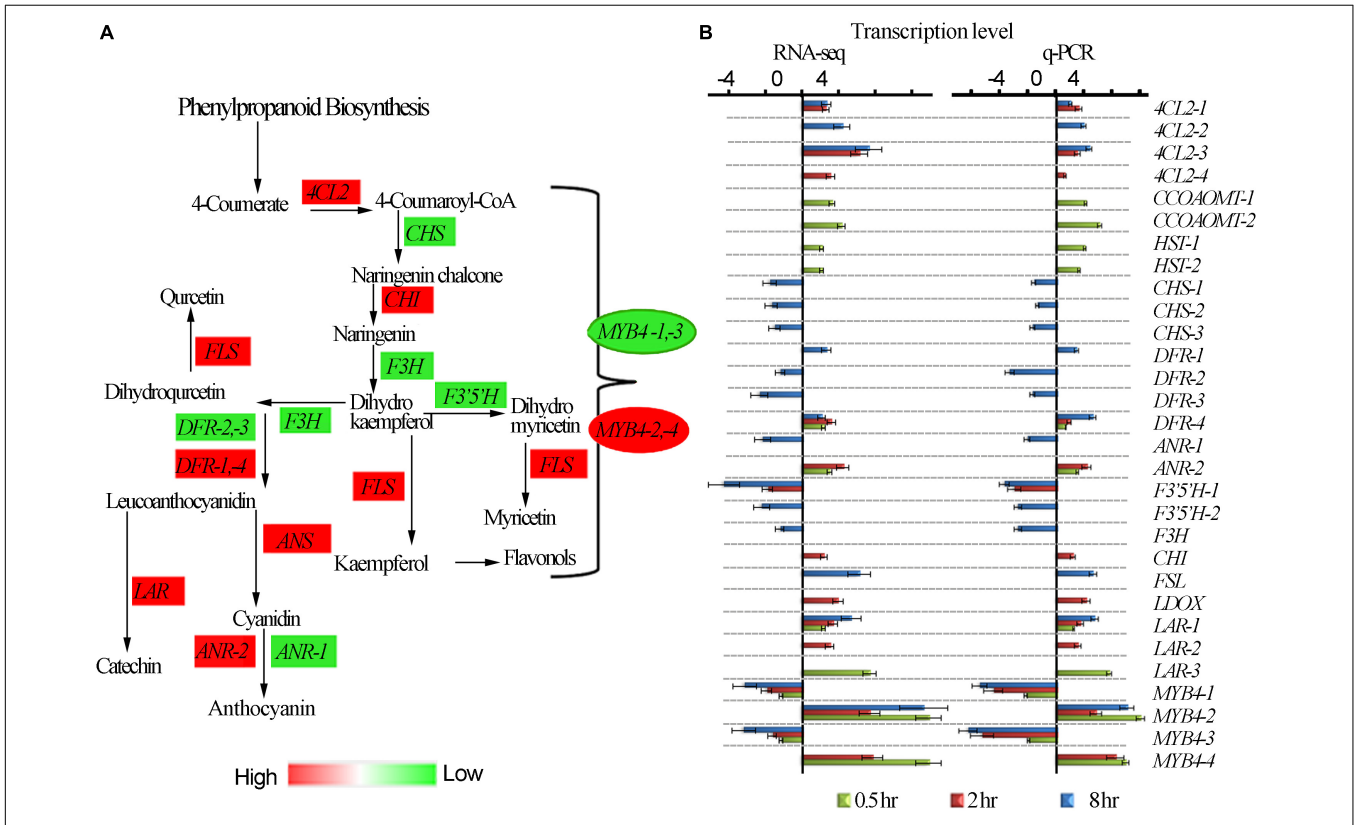

FIGURE 4 | UV-B induced differential expression of flavonoid genes. **(A)** Differential expression of flavonoid pathway genes revealed by transcriptomic data. **(B)** Shows RNA-seq and q-PCR gene expression in pheneylpropanoid pathway. *4CL2*, *4-COUMARATE:COA LIGASE*; *CHS*, *CHALCONE SYNTHASE*; *F3H*, *FLAVANONE 3-HYDROXYLASE*; *F3'H*, *FLAVANOID 3'-HYDROXYLASE*; *H3'5'H*, *FLAVANOID 3',5-HYDROXYLASE*; *DFR*, *DIHYDROFLAVONOL 4-REDUCTASE*; *FLS*, *FLAVONOL SYNTHASE*; *ANR*, *ANTHOCYANIDIN REDUCTASE*; *ANS*, *ANTHOCYANIDIN SYNTHASE; F5H*, *FERULATE 5-HYDROXYLASE. CCOAOMT – CAFFEOYL-COA O-METHYLTRANSFERASE; HST-HYDROXYCINNAMATECOA SHIKIMATE TRANSFERASES; LDOX – LEUCOANTHOCYANIDIN DIOXYGENASE.*

UV-B Induced Differential Expression of Genes in Terpenoid Biosynthesis Pathway

One of the unresolved questions related to terpenoid metabolism is whether light differentially regulates the expression of genes involved in terpenoid biosynthesis (Dolzhenko et al., 2010). To have a better insight into the terpenoid biosynthesis pathway by UV-B light exposure, we exposed tea shoots to UVB to four different exposure periods as described in methodology and evaluated some of the main genes involved in the terpenoid biosynthesis (**Figure 5A**). We compared the expression levels of the genes in diterpenoid, monoterpenoid, and sesquiterpenoid pathways in all the UV-B treatments. Most of genes in diterpenoid biosynthesis were up-regulated across all the UV-B treated tea shoots. The majority of genes were down-regulated in monoterpenoid biosynthesis in 2 and 8 h exposure while upregulation was observed at 0.5 h treatment. Four genes in monoterpenoid biosynthesis pathway annotated as TPS13-1, TPS13-2, (+)-neomentholdehydrogenase1-1 (MNR1-1), (+) neomentholdehydrogenase1-2 (MNR1-2), were up-regulated (**Supplementary Table S5**). In sesquiterpenoid biosynthesis, we observed upregulation of most genes at 2 h followed by 8 h treatment. Significant downregulation of genes was observed in 8 h treatment followed by 2 h treatment (**Figure 5B**). UV-B irradiation induced up-regulation in all the genes in the IPP and DAMPP biosynthesis pathways in almost all the treatments except *HGMR-2* and *PMK-1, DXS* and *DXR* (**Figure 5C**). To validate the reliability of gene expression analysis in this study, 20 genes with potential roles in terpenoid biosynthesis, were selected for real-time qPCR analysis. Our real-time qPCR data showed that these genes expression patterns, matched the gene expression patterns observed in RNA-Seq analysis (**Figure 5B**). Majority of *TPS* genes which are crucial in terpenoid synthesis were not identified in our transcriptomic data. We identified 21*TPS* genes from tea genome for qPCR analysis to evaluate the effect of UVB exposure at different duration. Data from qPCR on *TPS* genes showed significant enhancement at 0.5 h with *TPS* genes annotated as linalool synthase strikingly enhanced (**Figure 5D**).

UV-B Induced Changes in Specialized Metabolite Abundance

In order to understand the impact of the UV-B treatment on flavor metabolite production in tea leaves, the abundances of flavor determinants catechins, caffeine, and terpenoid volatiles were quantified. The results showed that UV-B exposure for 0.5, 2, and 8 h resulted in differential accumulation of catechins. We analyzed catechins and majority of them including GA, ECG, EC, GCG and the most predominant EGCG in tea shoot tips flavor determinants had a statistically significant change at 0.5 h ($p < 0.05$) treatment as compared to 2 and 8 h treatment which showed reduced levels with an exception of GA, C, GCG and EGC which showed statistically significant increased levels in all the comparison groups ($p < 0.05$) (**Figure 6A**). All eight UV-B-upregulated genes (**Supplementary Table S5**) in the flavonoid pathway at 0.5 h treatment could be the reason why flavonoid levels were enhanced at the same period treatment.

In this study, we focused in determining the effect of different time durations of UV-B on terpenoid production. We analyzed the effect of different exposure periods of UVB on accumulation of terpenoids in tea shoots by quantifying different volatiles. We noted differential accumulation of terpenoids in the three treatments in comparison to control (0 h). A statistically significant accumulation of volatile compounds was noted at 0.5 h treatment. The majority of monoterpenoids were significantly decreased at 2 and 8 h treatments except geraniol which was significantly enhanced in all the three treatments. Linalool was significantly enhanced at 0.5 h treatment and decreased at 2 and 8 h treatments. We noted up-regulation of genes in monoterpenoid biosynthesis pathway at 0.5 h treatment (**Supplementary Table S5**), perhaps this could explain the increase in level of volatiles at 0.5 treatment. Other volatiles that were significantly enhanced in terpenoid pathway across the three comparisons were D-limonene, *cis*-linalool oxides, Z-nerol, and neral. The majority of volatiles in the non-terpenoid pathway were enhanced across the three comparison groups (**Figures 6B,C**) with significance enhancement noted at 0.5 h treatment. Our data clearly indicated that specialized metabolites are altered at short exposure (0.5 h) to UV-B.

DISCUSSION

UV-B Induced Changes in UVR8-Mediated Signal Transduction Pathway

UV-B enhanced production of protectant screening phenolic compounds in plants have been well documented (Li et al., 1993; Rozema et al., 1997; Agati et al., 2011; Cañal et al., 2017), tea leaves contain much higher levels of polyphenols (16-30% of dry weight) than many other plants (Graham, 1992; Almajano et al., 2008). Some of these phenolics also function as flavor determinants for teas. UV-B radiation to tea plants may result in distinct responses from many other plant species and affect tea flavor. In this study, our transcriptomic analysis revealed that global transcriptomic alterations occurred in the tea shoots exposed to UV-B with the three different durations, similar to the previous findings (Casati et al., 2011). In addition, UVR8 was found down-regulated in all treatments in our experiments, as reported in maize (Casati et al., 2011) but contrary to the finding in *Arabidopsis* (Cloix et al., 2012; Heijde and Ulm, 2013; Vanhaelewyn et al., 2019). UV-B exposure altered the abundances of many specialized metabolites despite suppression of *UVR8* (Casati et al., 2011). Similar observatios were noted in our study where accumulation of flavonoid and volatile at 0.5 h treatment occurred. The stability of HY5 is enhanced by UVB irradiation leading to the monomerization of receptor *UVR8* which forms UVR8-COP1 complex accumulating in the nucleus enabling the transcription of genes derived from specific pathways responding to UV-B (Qian et al., 2016). *CHALCONE SYNTHASE (CHS), FLAVONOL SYNTHASE (FLS)* and numerous other genes belonging to diverse hormone and metabolic pathways are target genes for *HY5* which are up-regulated by UV-B

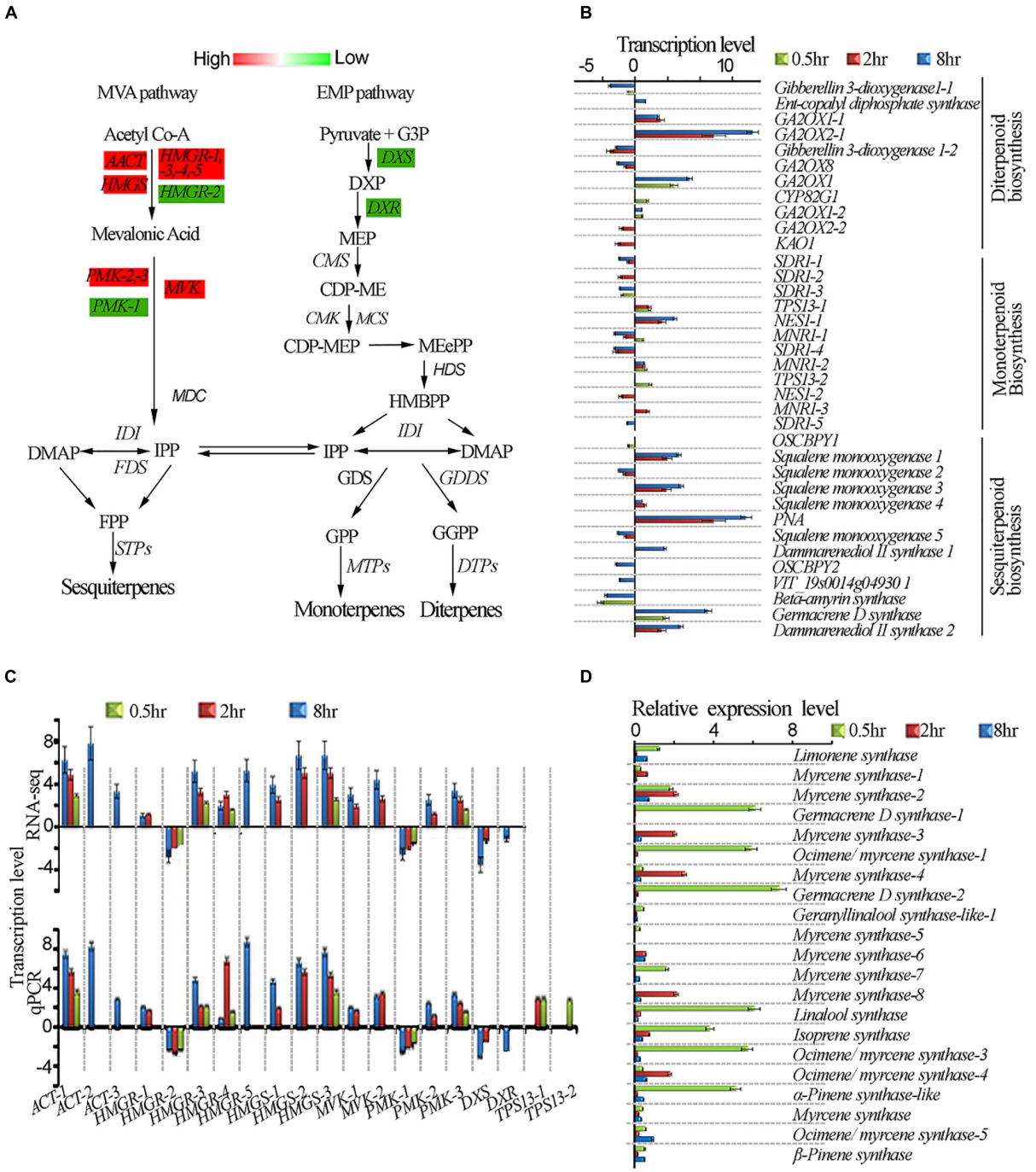

FIGURE 5 | UV-B induced enhancement of terpenoid biosynthesis pathway. **(A)** Differential expression of terpenoid pathway genes revealed by transcriptomic data. **(B)** Shows genes affected in diterpenoid, monoterpenoid and sesquiterpenoid biosynthesis pathways. **(C)** Shows RNA-seq and qPCR gene expression in terpenoid pathway. **(D)** qPCR gene expressions of selected *TPS* genes in terpenoid pathway. *Cs18srRNA* was employed as a reference gene for data normalization using $2^{-\Delta\Delta Ct}$ method. *HMGS*, *3-HYDROXY-3- GLUTARYL COENZYME A SYNTHASE*; *HMGR*, *3-HYDROXY-3-METHYL-GLUTARYL COENZYME A REDUCTASE*; *DXS*, *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE*; *CMK*, *4-CYTIDINE 50-DIPHOSPHO-2-C-METHYL-D-ERYTHRITOL KINASE*; *HDS*, *HYDROXY-2-METHYL-2-(E)-BUTENYL 4-DIPHOSPHATE SYNTHASE*; *GPPS*, *GERANYL PYROPHOSPHATE SYNTHASE*.

(Gangappa and Botto, 2016), resulting in induction of flavonoid accumulation which acts as UV-B protectants (Demkura and Ballaré, 2012). In line with this, we observed the up-regulation of *HY5* at 2 and 8 h treatments in this study and the up-regulation of *FLAVONOL SYNTHASE (FLS)* at 8 h treatment. *CHALCONE SYNTHASE (CHS)* was down-regulated in our

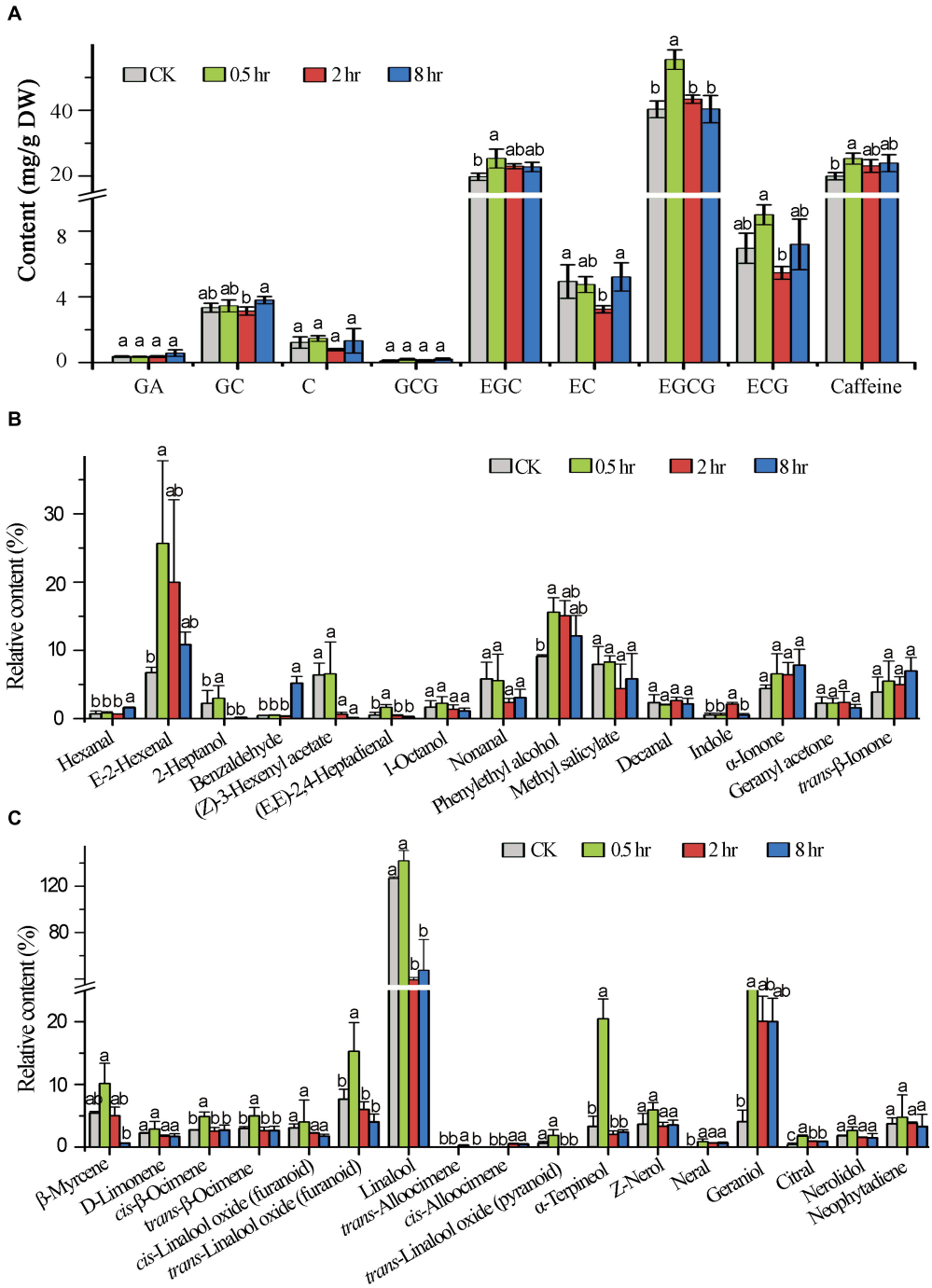

FIGURE 6 | Induced metabolic changes in secondary metabolites biosynthesis pathway. **(A)** The concentration of major catechins in tea samples (0.5, 2, and 8 h) analyzed by HPLC. **(B,C)** Shows Volatile compound analysis GC-MS. Data shown are the average mean ± SE of three replicates (n = 3). Columns with different letters (a, b, ab) for the same compound had significant differences among the different treatments according to one way ANOVA and a fisher's LSD test at $p < 0.05$.

experiments contradicting early studies in other plants. The deferential induction of genes downstream of UVR8 could have been due to direct UV-B and not necessarily the suppressed *UVR8* and COP1 interaction as observed early by Jenkins (2009). We hypothesize the negative response of *UVR8* in tea could be part of feedback to suppress physiological changes stimulated by UV-B exposure due to already extraordinary accumulation of secondary metabolites in tea plants (Almajano et al., 2008; Punyasiri et al., 2004). Further studies need to be carried out to ascertain this hypothesis.

Through the regulatory module of COP1 and UVR8 (Rizzini et al., 2011; Jenkins, 2017), some transcriptional factors such as R2R3-MYB, bHLH, and WD40 (MBW ternary complexes) might be regulated, consequently controlling numerous enzymatic steps involved in the biosynthesis of flavonoid in plants (Mano et al., 2007; Zhao et al., 2013). Previous studies in tea plants have demonstrated the positive and negative regulatory role of R2R3-MYB in regulation of enzymes involved in flavonoid production (Zhao et al., 2013; Xu et al., 2015). *HY5* regulates the expression of *MYB12* as reported earlier which is a specific transcriptional factor for *FLAVONOL SYNTHASE (FLS)* responsible for accumulation of flavonoid under UV-B light exposure (Stracke et al., 2010). Transcription factor *MYB4* has been reported to be induced by UV-light which suppresses *C4H, 4CL, LAR, CHS* and *ANR2* in *A. thaliana, Brassica rapa*, and *C. sinensis* to mediate UV-B dependent anthocyanin and phenylpropanoid synthesis (Schenke et al., 2011; Li et al., 2017). In this study we observed up regulation of *MYB12* at 8 h corresponding to enhancement of *FLAVONOL SYNTHASE (FLS)* and divergent expression of *MYB4* corresponding to divergent alteration of genes in flavonoid synthesis pathway. *bHLH* is another family transcription factor in plants that plays several functions ranging from regulation of floral organ development to flavonoids biosynthesis (Sorensen et al., 2003; Ohno et al., 2011). Recently, transcriptional regulator *CsbHLH* was found to negatively regulates EGCG3"Me biosynthesis which is a major source of *O*-methylated catechin in tea. In this study, *bHLH* 62 was up-regulated at 8 h treatment. In addition, tea *MYB4a* negatively regulates *C4H*, *4CL*, *CHS*, *LAR*, and *ANR2* by physical interaction with target gene promoters (Li et al., 2017). In tobacco transgenics overexpressing tea *MYB4a,* a total of twenty (20) genes related to phenylpropanoid pathways are suppressed (Li et al., 2017), including the majority of UV-B suppressed genes (*CHS-1, CHS-2, CHS-3, DFR-1, DFR-2-DFR-3, ANR-1, F3′5′H-1, F3′5′H-2,* and *F3′H*) found in this study and likely to have been affected by *MYB4-2 and MYB4-4* which were upregulated. *MYB4-1* and *MYB4-3* was suppressed subsequently leading to enhancement of *4CL2-1, 4CL2-2, 4CL2-3, 4CL2-4, DFR-4, ANR-2, CHI, FSLI, ANS, LAR-1, LAR-2,* and *LAR-3,CCOAOMT-1, CCOAOMT-2, HST-1, HST-2, ANR-2, DFR-4, LAR-1* and *LAR-3*. The divergent expression of *MYB4* in this study could be the reason for transcriptional alterations on metabolic and structural genes in tea under UV-B exposure. Taken together these results validate the previous studies on how UV-B affects genes in UVR8-mediated signal transduction pathway altering secondary metabolites biosynthesis. Such transcriptional alterations might collectively be responsible for the UV-B induced metabolic and structural gene transcriptional alterations in tea plants.

UV-B Induced Changes in Flavonoid Biosynthesis Pathway

Since a signaling metabolite must increase quickly in UVB irradiated shoots to induce transcriptome changes in response to stress within a short period (Mackerness, 2000), we predicted high level of specialized metabolites accumulation in treated shoots in 0.5 treatment. Our chemical analysis revealed that the majority of tea catechin including GA, ECG, EC, GCG and the most predominant EGCG in tea shoot tips as flavor determinants were enhanced at 0.5 h treatment compared to 2 and 8 h treatments, which significantly decreased with an exception of C, GCG, and EGC which was significantly enhanced in all the four treatments. There was no significant change at 2 and 8 h treatment compared to control in most of the tested metabolites, suggesting that the initial exposure to UVB led to different display in gene expression in tea shoots. This observation are in line with those made by Hectors et al. (2007) and Zhu et al. (2019). Even though the mechanisms underlying such differential abundance alterations in the tested catechins need further investigation, this finding is largely consistent with a previous report that a low fluence range of UV-B radiation results in decrease in all tested catechins throughout all time intervals (0, 30, 60, and 360 min), except for a slight increase in total catechin abundance detected at the interval of 30 min only in the tea cultivars "Fudingdabai" and "Yulan" (Zheng et al., 2008). Interestingly, the UV-B induced changes in the tested catechins in both cultivars are related to their original catechin abundances: the higher original catechin abundances in tea leaves, the less increase of UV-B induced catechin production after the first 30 min treatment and the more significant decrease of the UV-B induced catechin production after 360 min treatment. Besides, a recently published study indicated that long periods (1 to 16 days, 8 h per day) of a mild UV-B treatment (20 $\mu W/cm^2$) applied to tea plants did not affect the abundance of the majority of flavonoid compounds, except for that of EGCG, kaempferol-7-*O*-glucoside, and GC (Liu L. et al., 2018). However, the detected GC enhancement only after 16 days of UV-B treatment relative to the control plants grown in hydroponic culture might result from a substantial decrease in all tested metabolites in control, not from UV-B treatment. In addition, UV-B irradiation reduces the abundance of anthocyanins in the berries of the red grape cultivar "Cabernet Sauvignon," which generally contain high levels of polyphenolics (Sun et al., 2009). Whereas UV-B irradiation enhances polyphenol production in the berries of "Sauvignon blanc" (Liu et al., 2015), a white grape cultivar usually containing low levels of polyphenols. Taken together, these results suggest that the distinct responses of tea plants after UV-B radiation might result from their constitutively high levels of polyphenols. Such alterations in the flavor determinant metabolites such as catechins might affect tea beverage astringency, bitterness (Chaturvedula and Prakash, 2011; Feng et al., 2014) and biological effects on consumers. Further investigation on the mechanism underlying the difference in UV-B induced metabolite production between tea and some other plants is required.

UV-B Induced Changes in Volatile Terpenoid Pathway

In plants, volatile terpenoids can be synthesized through the mevalonate and methylerythritol phosphate (MEP) pathways (Schwab et al., 2008). Genes in the MVA pathway such as *AACT1* have been reported to be triggered by abiotic stresses in alfalfa (Soto et al., 2011). Studies in *populus trichocarpa* have indicated

that overexpression *PtHMGR* resulted in the increase in content of terpenoids including lycopene, gibberellic acid (GA), ABA, and carotenes indicating its role in terpenoid biosynthesis regulation (Wei et al., 2019). *AACT1 HMGR*, *HMGS*, and *DXS*, *PMK* and *MVK* from either the MVA or MEP pathway are all enhanced by light, jasmonic acid and ethylene (Hemmerlin et al., 2012). All these genes except *HGMR-2*, *PMK-1 DXR and DXS* were found to be enhanced by UV-B treatment in our work. Our qPCR data showed significant enhancement at 0.5 h with *TPS* genes annotated as linalool synthase strikingly enhanced. This could explain the accumulation of linalool at 0.5 h compared to other treatments. Four genes in monoterpenoid biosynthesis pathway annotated as MNR1-1, MNR1-2, TPS13-1, and TPS13-2 were up-regulated at 0.5 h treatment consistent with the UV-B enhanced monoterpenoids. Our data suggested that UV-B differentially affected volatile terpenoid biosynthesis with great potential for tea aroma improvement.

Terpenoids play a key role in the survival of plants as well as beneficial to human health. We analyzed the effect of different exposure periods (0, 0.5, 2, and 8 h) of UVB on accumulation of terpenoids in tea shoots. UV-B induced changes in metabolic and transcriptional profiles of terpenoid volatiles in tea shoot-tips. We examined whether a different time duration of UV-B irradiation alone can affect the expression of *TPS* genes and the production of related terpenoid volatiles in the intact tea leaves, rather than the excised leaves, in which enhancement of numerous volatiles (including linalool and nerolidol) and the expression of the genes, respectively, encoding ß-glucosidase and ß-primeverosidase were reported after UV-B exposure for 2 h (Jang et al., 2010). Our metabolic profiling showed that UV-B could induce the production of several potent monoterpenoid odorants such as linalool and geraniol in the intact tea leaves. We observed differential accumulation of terpenoids in the three treatments compared to control (0 hr). A significant accumulation of volatile compounds was observed at 0.5 h treatment. The majority of monoterpenoids were significantly decreased at 2 and 8 h treatment except geraniol which was significantly enhanced in all the treatments. Linalool was significantly enhanced in 0.5 h treatment and decreased at 2 and 8 h treatment. This is consistent with transcriptomic changes in monoterpenoid biosynthesis pathway in which most of the genes were suppressed at 2 and 8 h treatment and enhanced at 0.5 h treatment. This result is also in agreement with the two previously published reports that multiple volatile terpenoids can be enhanced in grapes and peach due to UV-B treatment (Gil et al., 2012; Liu et al., 2017). Our data suggest that a short period of UV-B exposure for 0.5 h could potentially improve tea aroma. Taken together, these findings suggest that alteration in the expression of genes responsible for terpenoid biosynthesis contributes to metabolic regulation of tea under UV-B irradiation.

CONCLUSION

We have found that UV-B induced distinct transcriptomic alterations in the UVR8-signal transduction, phenylpropanoids and volatile terpenoids pathways in *Camellia sinensis*. Numerous studies on effect of high fluence UV-B on plants including Arabidopsis have indicated that UVR8 is up-regulated influencing signal transduction downstream of UV-B perception through to the induction of UV-B protectants such as flavonoids. This is in contrast with our observation in this study where UVR8 was down regulated in all the treatments. There was no significant increase in specialized metabolites except at 0.5 h where major flavonoids and terpenoids were enhanced. However, despite enhanced flavonoid and terpenoid accumulation at 0.5 hr, we observed divergent expression of genes in flavonoid biosynthesis pathway including transcription factor *MYB4*. Tea is extra ordinarily rich in phenolic compounds, it will be interesting to understand molecular mechanism underlying down regulation of *UVR8* in tea under UV-B exposure without significantly influencing accumulation of flavonoids as observed at 2 and 8 h treatment. The accumulation of special metabolites at shorter period of treatment indicates that metabolite signals are induced in response to stress as a defense mechanism. Stability of metabolites after 2 h duration suggested that the plant had acclimated to stress and displayed different expression profiles to those exposed over shorter duration. The 0.5 h treatment showed accumulation of key flavor determinants in tea which can be practically applied at industrial level in tea industry.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI SRA data IDs: SRR10768500–SRR10768511 and BioProject ID PRJNA597433.

AUTHOR CONTRIBUTIONS

LS and H-CZ performed the experiments, conducted the data analysis, and manuscript draft preparation. Z-XH started the project and prepared the samples for RNA-Seq. SW conceived and designed the study, analyzed the data, and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00234/full#supplementary-material

FIGURE S1 | Shows the effect of different time period exposure of UV-B light on stress inducement.

FIGURE S2 | Gene structure and phylogenetic tree of *MYB4* variants in UVB treated tea shoots.

FIGURE S3 | Statistics of Differential expressed genes among different treated samples.

TABLE S1 | Summary of RNA-seq statistical Assembly.

TABLE S2 | Primers used in this study for qPCR validation of RNA-seq.

TABLE S3 | Top Total Significant KEGG pathways associated with dififfferentially expressed genes (DEGs) induced by different treatments.

TABLE S4 | Selected stress UV-B induced genes in tea shoot tips.

TABLE S5 | Top Up and Downregulated KEGG classifications associated with dififfferentially expressed genes (DEGs) induced by different treatments.

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From Chloroplast Biogenesis to Chlorophyll Accumulation: The Interplay of Light and Hormones on Gene Expression in *Camellia sinensis* cv. Shuchazao Leaves

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Chloroplast development and chlorophyll metabolism have been well described in model plants but not in perennial woody crops. Of particular interest is the interplay between light and hormones under shade conditions. We report that the shade induced accumulation of chlorophylls in *Camellia sinensis* cv. Shuchazao leaves is at least as a result of (a) positive changes in chloroplast development and (b) light/hormonal regulation of genes and transcription factors involved in the chlorophyll biosynthesis pathway. Under shade conditions, leaves developed an abundance of enlarged chloroplasts encapsulating more prominent thylakoid membranes. Four major metabolites in the chlorophyll biosynthesis pathway namely Chl *a*, Chl *b*, DPP, and Mg-Proto IX increased under shade conditions while PBG decreased significantly. Significant changes were found at the transcription level of regulators of chloroplast biogenesis (*GLK1* and *LHCB*), the structural genes in the chlorophyll biosynthesis pathway (*HEMA1*, *CLH1*, *PORA,* and *CAO*) and potential components involved in light signaling (*PHYA*, *CRY1*, *HY5,* and *DELLAs*). Two central signal integrators (*GLK1* and *LHCB)* between the nucleus and chloroplast showed clear responses to shade, suggesting a crucial role of light in regulating chloroplast development in tea leaves. Concurrent with the changes in gene expression, the concentrations of endogenous phytohormones (auxin, cytokinin, and gibberellins) increased significantly in the later stages of shade conditions. Two key integrators involved in the hormone signal pathways, EIN3 and EBF1/2, increased under shade conditions suggesting that shade induced changes to hormone levels may play some role in modulating chlorophyll biosynthesis in the tea leaves. Overall, this data suggests that the light and hormone influence over chloroplast development and chlorophyll biosynthesis in *Camellia* is similar to that of *Arabidopsis*. This study provides new insights into the molecular mechanisms that regulate chlorophyll biosynthesis in response to light and hormones in a commercially important woody plant such as *Camellia*, which may facilitate the breeding of high-chlorophyll tea cultivars for the improvement of sensory features of the green tea product.

Keywords: *Camellia sinensis*, chlorophyll, chloroplast, hormones, light regulation, response to shade

INTRODUCTION

Tea, one of the products made from the processed leaves of tea plants [*Camellia sinensis* (L.) O. Kuntze], has been recognized as the most popular non-alcoholic beverage worldwide (Liu S. et al., 2018) because of the pleasant tastes, fragrances and health benefits (Chacko et al., 2010; Song et al., 2018). The color of the infusion is one of the most important characteristics determining the quality of the product (Kaneko et al., 2006) and been the focus of the tea industry. Green tea makes use of unwilted leaves and buds, and many strategies have been implemented to enhance and improve the color of tea products from the tea plantation to the processing factory. One such strategy is growing plants under shade. This improves the quality of fresh leaves through the enhancement of chlorophyll accumulation and the balancing of secondary metabolites (Song et al., 2012; Wang et al., 2012; Liu L. et al., 2018). The leaves of tea plants grown under shade conditions contain a higher level of chlorophylls (Liu G. F. et al., 2017; Liu L. et al., 2018), which contributes to a bright green color of the product (Wang et al., 2012). In addition to color improvement of the tea infusion, the shaded leaves contain high levels of amino acids but lower catechin content, which also improves the sensory qualities (Wang et al., 2012; Lu et al., 2014). Studies on the effect of shade on the tea plants were initiated decades ago, with major systematic analyses focused on the influence of secondary metabolites, such as catechin and flavonol biosynthesis (Koretskaya and Zaprometov, 1975; Saijo, 1980; Song et al., 2012). However, little attention has been paid to the mechanisms of regulation of chlorophyll biosynthesis by light when referring to the interplay between light and hormones. Under shade conditions when the roles of light and hormones are considered, the molecular mechanisms involved in chlorophyll biosynthesis underlying shading effects become more complex and intricate in this commercially important beverage crop.

In higher plants, chlorophylls are the most abundant tetrapyrroles. They function as photosynthetic pigments to harvest light energy and transfer the absorbed energy to the reaction center in which charge separation occurs (Tanaka and Tanaka, 2007; Tanaka et al., 2011). Chlorophyll is initially biosynthesized from glutamate, which is then converted to 5-aminolevulinic acid (ALA) and further converted to protochlorophyllide (DPP), leading to the "chlorophyll cycle" which refers to the interconversion between chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) (Tanaka and Tanaka, 2007; Tanaka et al., 2011). Chlorophyll metabolism in the model plant *Arabidopsis* has been well described, including all enzymatic pathways, encoding genes and intracellular location of enzymes, as well as the potential transcription factors (TFs) (Tanaka and Tanaka, 2007; Hörtensteiner and Kräutler, 2011; Tanaka et al., 2011; Liu X. et al., 2017). In contrast, reports on the activities of genes and enzymes of the chlorophyll biosynthesis pathway in perennial woody plants are still limited, particularly in relation to the chlorophyll metabolism modulated by light and hormones in *Camellia*.

Light is probably one of the most important environmental factors that regulate the chlorophyll biosynthesis pathway in higher plants (Tanaka and Tanaka, 2007). Red and far-red light sensors (phytochromes, PHYs), blue light receptors (cryptochromes, CRYs) and the circadian clock machinery mediate the light signaling, which modulates the expression of genes involving in chlorophyll metabolism (Tanaka et al., 2011). Two classes of TFs, namely Phytochrome Interacting Factors (PIFs) and Elongated Hypocotyl 5 (HY5), regulate chlorophyll biosynthesis in opposing manners (Chen et al., 2004; Leivar and Quail, 2011). PIFs, which accumulate in dark-grown seedlings and are directly targeted by photo-activated PHYs for degradation, negatively regulate chlorophyll biosynthesis and photosynthetic genes to optimize the greening process of the seedling (Stephenson et al., 2009; Song et al., 2014). Conversely, HY5 regulates nuclear gene transcription and functions as a central downstream factor in light signaling to promote seedling photomorphogenesis (Chen et al., 2004; Xu et al., 2016). While HY5 and PIF proteins act downstream of phytochrome signaling, the transcription factors Golden2-Like (GLK) 1 and GLK2 influence chlorophyll biosynthesis independently of the PHYB signaling pathway (Waters et al., 2009). GLKs strongly up-regulate the expression of genes

Abbreviations: ACC, 1-aminocyclopropane carboxylic acid; AHK, Arabidopsis Histidine Kinase; ALA, 5-aminolevulinic acid; ALAD, ALA dehydratase/PBG synthase; ANOVA, one-way analysis of variance; ARCs, accumulation and replication of chloroplasts; ARR, the type B Arabidopsis response regulators; BR, brassinosteroid; BRM, BRAHMA; CAO, chlide a oxygenase; CBR, Chl *b* reductase; Chl *a*/*b*, chlorophyll *a*/*b*; CHL, magnesium chelatase encoding gene; CHLG, Chl synthase; CHLH, magnesium chelatase; CLH, chlorophyllase; ClpP6, Clp protease proteolytic subunit 6; COP1, Constitutively Photomorphogenic 1; Cpn60, heat shock protein family D (Hsp60) member 1; CPO, coprogen III oxidase; CRYs, cryptochromes; CTK, cytokinin; DELLAs, gibberellic acid-regulated growth-repressing DELLA proteins; DET1, de-etiolated 1; DPP, divinyl protochlorophyllide; DVR, 3, 8-divinyl pchlide a 8-vinyl reductase; EBF1/2, EIN3-binding F-box 1/2; EIN3/EIL1, Ethylene Insensitive 3/EIN3-LIKE 1; ELISA, enzyme-linked immunosorbent assay; ETH, ethylene; EX1/2, EXecuter1/2; FAR, FAR-red-impaired response; FeCh, ferrochelatase; FHY, FAR-RED Elongated HYpocotyl; FLU, FLUorescent in blue light; FPKM, fragment per kilobase of exon model per million mapped reads; FtsH2, filamentation temperature-sensitive H2; FtsZ1/2, tubulin-like protein FtsZ1/2; GA, gibberellins; GC1, giant chloroplast 1; GLK, golden2-like protein; GluRS, Glu-tRNA synthetase; GluTR, Glu-tRNA reductase; GSA-AT, GSA aminotransferase; GUN4, GENOMES UNCOUPLED 4; HCAR, 7-hydroxymethyl Chl *a* reductase; HDA15, histone deacetylase 15; HEMA, Glu-tRNA reductase; HO, heme oxygenase; HSP, heat shock proteins; HY2, phytochromobilin, ferredoxin oxidoreductase; HY5, Elongated HYpocotyl 5; HYH, HY5 Homolog; IAA, Indole-3-Acetic Acid; LHCB, light-harvesting complex II chlorophyll binding protein; LPA2, Low PsII Accumulation 2; LSD, least significant difference; MgCh, magnesium chelatase; MgCY, Mg-Proto IX monomethyl ester cyclase; MgD, Mg-dechelatase; MgMT, S-adenosyl-L-methionine, Mg-Proto IX methyltransferase; Mg-Proto IX, Mg-Protoporphyrin IX; MinD, chloroplast division factor MinD; MSL2, MscS-like/mechanosensitive ion channel protein 2; NOL, Chl *b* reductase encoding gene NOL; PAO, pheophorbide a oxygenase; PAT1, scarecrow-like transcription factor PAT1; PBG, porphobilinogen; PBGD, PBG deaminase/HMB synthase; PDV1/2, ARC5 recruitment factor PDV1/2; PET, photosynthetic electron transport; PHYs, phytochromes; PIF, Phytochrome Interacting Factor; POR, pchlide oxidoreductase; PPH, pheophytinase; PPO, protoporphyrinogen IX oxidase; Proto IX, protoporphyrin IX; PTM, PHD type transcription factor with transmembrane domains; RCCR, red Chl catabolite reductase; RGL1, DELLA protein RGL1; rpoT, NEP protein encoding gene; RT-PCR, quantitative real-time PCR; RVE1, REVEILLE 1; SAL1, chloroplast retrograde signal gene SAL1; sco1, snowy cotyledon 1; SL, strigolactones; STY, serine, threonine and tyrosine; TEM, transmission electron microscope; TFs, transcription factors; THF1, thylakoid formation 1; TOC, translocase of chloroplast; TRX, thioredoxin; UROD, urogen III decarboxylase; Urogen III, uroporphyrinogen III; UROS, urogen III synthase; VIPP1, vesicle inducing plastid protein.

that are involved in chlorophyll biosynthesis, including *Glu-tRNA reductase* (*HEMA1*), *magnesium chelatase* (*CHLH*), *pchlide oxidoreductase* (*PORB*), and *chlide a oxygenase* (*CAO*).

In addition to the influence of light, endogenous phytohormones are also recruited to mediate the metabolism of the chlorophyll biosynthesis pathway (Liu X. et al., 2017). Key components in the light signaling pathway such as HY5 and PIFs, connect light signals to the signaling pathways of multiple phytohormones, including cytokinin (CTK), gibberellin (GA) and auxin (Lau and Deng, 2010). It has been suggested that the two hormones (CTK and GA) serve to regulate HY5 at the protein level and that HY5 promotes photomorphogenesis partly by modulating auxin, GA and abscisic acid signaling (Lau and Deng, 2010). Ethylene (ETH) and its precursor (1-aminocyclopropane-1-carboxylate acid, ACC) play critical roles in leaf senescence, root elongation and protecting cotyledons from photooxidative damage when the seedlings are exposed to light (Liu X. et al., 2017; Han et al., 2019; Lv et al., 2019). Ethylene Insensitive 3/EIN3-Like 1 (EIN3/EIL1) is the master transcription factor in the ethylene signaling pathway (Guo and Ecker, 2004), which markedly inhibits the accumulation of pchlide and directly binds to the promoters of *PORA* and *PORB* to activate their gene expression (Zhong et al., 2014).

The biogenesis of chloroplasts is also subjected to light and hormone regulation (Waters and Langdale, 2009; Pogson et al., 2015). In dicotyledonous seedlings, chloroplast biogenesis can be described as the differentiation from the plastid progenitor to an undeveloped proplastid, a precursor structure that has no photosynthetic capacity, to a mature chloroplast (Kessler and Schnell, 2009; Waters and Langdale, 2009; Pogson et al., 2015). This process is highly light-dependent, differs between species and to date the molecular intricacies have not been fully characterized. Golden2-like proteins (GLK1 and GLK2) are an important class of nuclear transcription factors required for transcription of genes encoding chloroplast proteins, providing the anterograde signal from the nucleus to the chloroplast to trigger the induction of photomorphogenesis (Oh and Montgomery, 2014). Conversely, this forward communication is balanced by a retrograde signal from the chloroplast to the nucleus (Chi et al., 2015). A number of experimental approaches over the past 30 years have confirmed the existence of this retrograde signal and arrangement of integrators, which participate in this signal have been suggested to be light-regulated, such as HY5 (Chi et al., 2015) and the light-harvesting complex II chlorophyll binding protein (LHCB) (Waters and Langdale, 2009).

Evidence from previous studies indicates that the reduction of light exposure enhances chlorophyll accumulation in tea leaves (Song et al., 2012; Wang et al., 2012; Liu L. et al., 2018). However, the molecular mechanisms for chlorophyll biosynthesis are still mysterious, especially when referring to the interplay between light and hormones under shade conditions. It is unclear if the regulatory mechanisms of model plants and perennial woody plants such as the *Camellia* are similar. In recognition of the importance of chlorophyll content in determining green tea characteristics, we investigated the effect of shade conditions on chloroplast development, accumulation of major metabolites and the activities of genes/TFs involved in the chlorophyll biosynthesis pathway, and the potential roles of hormones in shade induced chlorophyll changes in tea leaves. To examine the effect of shade on chloroplast development and chloroplast ultrastructure, we measured the expression of genes/TFs that are involved in chloroplast biogenesis and differentiation. The levels of major metabolites in the chlorophyll biosynthesis pathway, including Chl *a*, Chl *b*, ALA, porphobilinogen (PBG), protoporphyrin IX (Proto IX), uroporphyrinogen III (Urogen III), DPP and Mg-Protoporphyrin IX (Mg-Proto IX) were also determined. Furthermore, we analyzed endogenous phytohormones likely to be involved in chloroplast development, including auxin, CTK, GA, brassinosteroid (BR), strigolactones (SL), and ACC. In addition, genes and TFs involved in chloroplast development and chlorophyll biosynthesis were analyzed at the transcriptional level, with emphasis on genes/TFs which potentially function in the regulation by light and or hormone on chlorophyll biosynthesis, as suggested by previous studies. Overall, this research aimed to investigate the interplay of light and hormones on the regulation of chlorophyll biosynthesis in response to shade conditions in a commercially important tea crop, *C. sinensis* cv. Shuchazao.

MATERIALS AND METHODS

Shade Treatments and Sample Collection

Shade treatments and sample collections were described previously with minor modifications (Liu L. et al., 2018). This study was carried out at Anhui Agricultural University research tea plantation (31°.55′ North, 117°.12′ East; Tea Plant Cultivar and Germplasm Resource Garden in Guohe Town), using 24 years old *C. sinensis* cv. Shuchazao plants propagated from cuttings (Zhang X. et al., 2018). The plants were 1.4 m wide, 1.5 m tall from the soil surface and 0.5 m between plants within the row. 12 rows of plants (50 m long and 1.4 m wide of each row, 2 m between and 0.6 m within row spacing) were selected for the treatments. Treatments consisted of plants exposed to natural unobstructed light (control, C) and plants exposed to 80–90% shade (shade, S; 10–20% light transmitted). The black nylon shade nets (Non-gfeng Company, Hefei, China) were placed about 1.5 m above the tea plants on the12th of April in 2017 at the onset of a new round of budburst. Each treatment was replicated three times and the positions of treatments randomized statistically within 6 rows of plants.

Photosynthetically Active Radiation (PAR) was measured using a Light Scout® Quantum Light Meter (Item#3415F, Spectrum Technology® Inc., United States). For sample collections, tea buds with the first developing leaf were collected for RNA-Seq and RT-PCR analyses at different stages during shade treatments (4h, 1d, 3d, 7d, and 14d). For metabolite and hormone analysis, the first developing leaf was collected at different stages during shade treatments (1d, 3d, 7d, and 14d). For chloroplast ultrastructure observation, the first developing leaf was collected at 21d of shade treatment. All the materials were frozen immediately in the field using liquid nitrogen and stored at −80°C for future analysis.

Chloroplast Ultrastructure

Fresh leaves were excised and immediately dipped in pre-cooled glutaraldehyde PBS solution (5% glutaraldehyde in 0.1 M PBS, pH 7.2, 4°C), assisted by a syringe for better infiltration according to a previous publication (Liu G. F. et al., 2017). Glutaraldehyde-infiltrated leaves were cut into small pieces (about 2 mm × 3 mm) and sectioned on a TCS CM1900 freezing microtome (Leica, Germany). The ultrathin sections were double lead stained and photomicrographed using an HT-7700 transmission electron microscope (TEM; Hitachi, Japan). The preparation of the ultrathin section, staining, and TEM observation was carried out at the Biotechnology Center in Anhui Agricultural University (Hefei, China).

Metabolite and Phytohormone Analysis

Chlorophyll analysis was carried out as described previously (Lu et al., 2019). Fresh leaves (0.1 g) were cut into small pieces and extracted overnight in 10 ml solvent (5% acetone in 95% ethanol, v/v) until the pieces became completely decolored. Absorbance of the extract was measured in an ultraviolet spectrophotometer (U-5100, Hitachi, Japan) at A_{645} and A_{663} and the concentrations chlorophyll *a* and *b* were calculated using the formula: Chl *a* = $12.7A_{663} - 2.69A_{645}$; Chl *b* = $22.9A_{645} - 4.68A_{663}$ (Arnon, 1949).

The concentrations of major metabolites in the chlorophyll biosynthesis pathway and phytohormones, including ALA, PBG, Urogen III, Proto IX, Mg-Proto IX, DPP, CTK, GA, ACC, BR, and SL were determined using enzyme-linked immunosorbent assay (ELISA) kits (mIbio Co. Ltd., Shanghai, China) according to the manufacturer as reported previously (Liu et al., 2019). The principle of the kit is based on the original ELISA method with modifications. Purified plant antibody was used to coat microtiter plate wells to create a solid-phase antibody. Once the selected compound was added into the wells, the compound was combined with the second antibody which was labeled conjugated to a specific enzyme, and then the antibody-antigen-enzyme complex was incubated. The concentration of the ligand was further determined according to the following enzymatic chromogenic reaction. Tea leaves were collected and grounded in liquid nitrogen. Accurately 100 mg of the powder was weighed and extracted in a fixed volume of 0.01 M PBS buffer (pH 7.4; PBS: tissue ratio = 10: 1, v/w) overnight at 4°C. Then the suspension was centrifuged at 10,000 rpm for 10 min and the supernatant was collected for further determination. Forty microliter of the Sample Dilution was added into the well of the Microelisa Stripplate (blank well did not add sample and the HRP-Conjugate reagent, wells for the standard curve followed the same operation), and 10 μl of the supernatant was added into the sample well (final dilution is fivefold), then mixed gently by shaking the plate. Subsequently, 100 μl of the HRP-Conjugate Reagent was added into each well (except the blank well), then the plate was sealed with the Closure Membrane and incubated at 37°C for 60 min. Then the Closure Membrane was removed, the reagent was discarded and the well was washed 5 times by the Wash Solution. After drying in a fume hood for 5 min, 50 μl of the Chromogen Solution A and 50 μl of the Chromogen Solution B were added into each well, then the plate was placed in the dark for 15 min at 37°C and the reaction was stopped by 50 μl of the Stop Solution. The absorbance at 450 nm was detected within 15 min of adding the stop solution and the concentration of the compound was calculated by extrapolation from the standard curve and the concentrations expressed per mass of leaf tissue extracted.

RNA-Seq Analysis

RNA-Seq analysis was conducted as described previously (Liu L. et al., 2018). RNA extraction, library construction and RNA-Seq performed by the Illumina HiSeq2000 were carried out by Wuhan Bosaixi Biotechnology Company (Wuhan, China). Clean reads were combined and separately assembled using the transcriptome assembler Trinity (version r20140717). After removing adaptor sequences, duplication sequences, ambiguous reads and low-quality reads, an average of 6 GB clean data per sample was generated. The final assembly of tea samples had 82322 unigenes (≥500 bp) with an N50 length of 1,206 bp. Functional annotation revealed 57823, 40003, 34066, 11963, and 34972 unigenes with alignments to NR (non-redundant protein database), Swiss-Prot (Annotated protein sequence database), KOG (Clusters of orthologous groups for eukaryotic complete genomes), KEGG (Kyoto encyclopedia of genes and genomes) and GO (Gene ontology) databases, respectively. Selected unigenes were validated according to the tea plant genome in the Tea Plant Information Archive (TPIA) (Wei et al., 2018; Xia et al., 2019)[1].

RT-PCR Analysis

RT-PCR analysis was conducted as previously described with minor modifications (Tai et al., 2015). Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Shanghai, China). TURBO DNA-free™ Kit (Sigma-Aldrich, Shanghai, China) was used to remove traces of genomic DNA. Single-stranded cDNA used for RT-PCR were synthesized using a Prime-Script™ Strand cDNA Synthesis Kit (Takara, Dalian, China). The SYBR green method (Takara, Dalian, China) was used for RT-PCR analysis performed by a CFX96 Touch real-time PCR detection system (Bio-Rad, United States). Tea gene *CsACTIN* (HQ420251.1) was used as an internal housekeeping gene. The relative expression levels of the amplified products were calculated using the $2^{-\Delta\Delta Ct}$ method (Yang and Huang, 2018). The primers for selected unigenes were designed online by NCBI Primer-BLAST (**Supplementary Table S1**)[2].

Statistic Analysis

All data presented in this study were calculated from three biological replicates, including the concentrations of chlorophylls and metabolites, hormones, RNA-Seq and RT-PCR analyses. Statistical analyses were conducted using the Minitab 17.0 statistical software (Minitab Inc., Coventry, United Kingdom). Data were analyzed by one-way analysis of variance (ANOVA) and a Fisher's least significant difference (LSD) test at the 5% level.

[1] http://tpia.teaplant.org

[2] https://www.ncbi.nlm.nih.gov/tools/primer-blast/

RESULTS

Shade Induced Stimulation of Chloroplast Development

Visual changes in leaf color traits were observed after shade treatment (**Figure 1**). An enhancement of dark green color could be observed visually from the second day to 2 weeks of shade. TEM observation further revealed dramatic responses to shade in chloroplast development. When compared to the non-shaded mature leaves of the control treatment, in shaded leaves, chloroplasts were (a) more numerous (8.67 per cell transection compared to 3.25 per cell transection) and (b) more rounded (**Figures 1B,F**). The number of starch granules per cell transaction increased due to the higher number of the chloroplast. Furthermore, the shaded leaves contained more tightly and intensively stained grana stacks, with a much higher number of thylakoids per granum and a higher stacking degree of thylakoids (**Figures 1C,D,G,H**).

Shade Induced Changes to Chlorophyll and Metabolite Concentrations

Consistent with the alteration in leaf color, significant responses to shade conditions were detected in the concentrations of major metabolites in the chlorophyll biosynthesis pathway in tea leaves (**Figure 2**). The concentrations of Chl *a* and Chl *b* were significantly increased in shaded leaves, which were about twofold higher than that in the non-shaded leaves (**Figure 2C**). Simultaneously, a decrease was observed in the ratio of Chl *a*/*b* at different time points during shade treatment (**Figure 2B**). Among the major metabolites involved in the chlorophyll biosynthesis, DPP and Mg-Proto IX showed some increases in response to shade, while a significant decrease was found in the PBG accumulation. Proto IX and Urogen III were also analyzed, but no significant changes were found between the control and shade treatments.

Shade Regulation of Genes Involved in Chloroplast Biogenesis

To investigate the effect of shade on chloroplast development at the transcriptional level, RNA-Seq technology was utilized to analyze gene activities in tea buds from both the control and shade treatments. The representative unigenes were selected from its multiple transcripts according to a comprehensive evaluation of parameters according to the following order: unigenes with best alignments to the reported sequence, the annotated unigenes with the highest fragment per kilobase of exon model per million mapped reads (FPKM), and unigenes differentially expressed.

For chloroplast development, the transcript abundance of 37 annotated unigenes involved in the chloroplast biogenesis was analyzed. These candidate genes included genes involved in nuclear transcription, chloroplast gene transcription, protein import and processing, retrograde signaling and chloroplast division (**Figure 3** and **Supplementary Table S2**). From 4h to 14d of shade, a group of unigenes showed significant increases. GLK1, the known nuclear transcript factor directly required for the transcription of genes encoding chloroplast proteins for chloroplast development (Waters et al., 2009), showed a consistent increase under shade conditions. LHCB was shown to directly interact with snowy cotyledon 2 (SCO2) which suggests a role in loading the vesicles with the photosynthesis-related proteins for transport to the thylakoids (Tanz et al., 2012). A dramatic increase of *LHCB* expression was found throughout the whole period of shade treatment. Conversely, *SCO2* presented a significant decrease in response to shade, especially during the early period of treatment. HY5, a transcription factor that has been reported to mediate light signaling to regulate nuclear gene transcription for chloroplast biogenesis in *Arabidopsis* (Xu et al., 2016), showed a significant decrease in gene expression throughout shade conditions. Similarly, the metalloprotease encoding gene *Filamentation temperature-sensitive H2* (*FtsH2*) showed decreases immediately at 4h and 1d, but no significant response was detected in the later period of shade treatment.

Shade Regulation of Genes Involved in Chlorophyll Metabolism

To explore the effects of shade on the activities of genes involved in chlorophyll metabolism, 38 annotated unigenes encoding 26 major enzymes were annotated from tea transcriptome datasets (**Supplementary Table S3** and **Figure 4**). These candidate genes could be conceptually divided into several groups: 13 genes involved in the "common steps"; 8 genes involved in the "chlorophyll branch"; 7 genes involved in the "chlorophyll cycle"; 6 genes involved in the "chlorophyll breakdown"; and 4 genes involved in the "Heme/bilin branch." Among these, genes involved in the "chlorophyll cycle" significantly increased in transcript level throughout the whole shade period. As shown in **Figure 4**, *chlorophyllase 1* (*CLH1*) and *CAO,* which participates in the conversion between Chl *a* and Chl *b*, showed a significant increase from 3d to 14d of shade. The transcript of *HEMA1* presented a similar response, with a significant increase at the later stage of shade treatment. *PORA* showed a significant early decrease after 4h and the transcript level increased at the later stage of shade treatment. In contrast, *CHLH*, which inserts Mg^{2+} into Proto IX, showed a significant decrease during the early stages (4h to 3d) of shade treatment but the expression level returned to normal after 14d.

Shade Induced Changes to the Concentrations of Phytohormones

To determine the responses to shade on chloroplast development and chlorophyll accumulation in more detail, the concentrations of various phytohormones were analyzed in both the shaded and non-shaded leaves (**Table 1**). Six hormones were analyzed at different stages of shade treatment (1d, 3d, 7d, and 14d). CTK, which was reported to regulate the expression of many chloroplast-related genes (Chiang et al., 2012) and mediate the etioplast-to-chloroplast transition (Cortleven and Schmülling, 2015). CTK showed a significant increase in leaves under shade conditions. At day 1 the concentration had increased to 142% of the control and by days 3–123% of the control. Both GA and auxin were seen to increase in concentration later in the

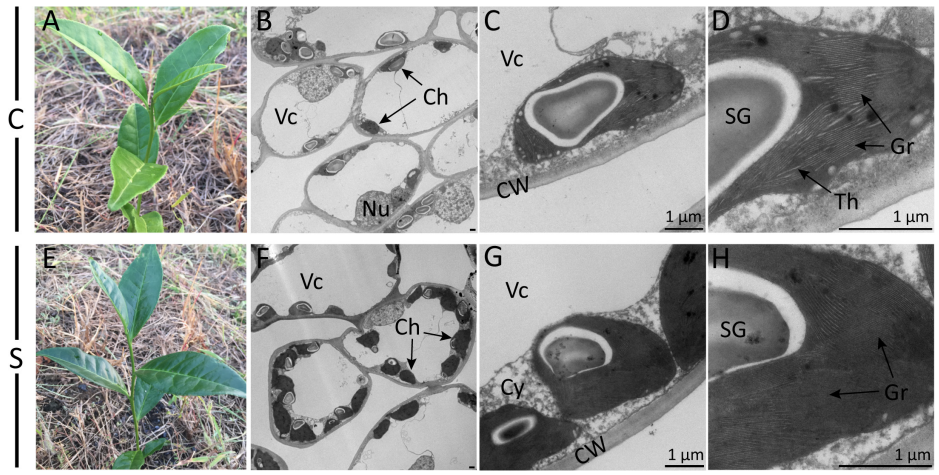

FIGURE 1 | Effect of shade on *camellia* leaves at the gross- and ultrastructural levels. Gross images show the coloration of typical control **(A)** and shaded **(E)** tea shoots. Photomicrographs show the ultrastructure of chloroplasts in both the control treatment **(B–D)** and the shade treatment **(F–H)** collected at 21 days of treatment. Magnifications of the TEM images were captured at ×0.7K **(B,F)**, ×5K **(C,G)** and ×12K **(D,H)** on a HT-7700 transmission electron microscope (Hitachi, Japan). Lead stain. C, control treatment; S, shade treatment; Vc, vacuole; Ch, chloroplast; Nu, nucleus; CW, cell wall; Cy, cytosol; SG, starch granule; Th, thylakoid; Gr, grana. Bars = 1 μm.

shade treatment period. The concentration of GA increased from 0.83 to 1.05 pmol/g after 14 days and increased by 42 and 45% of the control values at 7d and 14d of shade, respectively. It has been reported that ETH, BR and SL were involved in the hormone regulation of chloroplast development and photo-pigmentation accumulation in *Arabidopsis* (Zhong et al., 2009; Luo et al., 2010; Tsuchiya et al., 2010). No significant changes in ETH, BR and SL concentrations were detected and ACC showed only a slight but significant decrease at 3d of shade treatment.

Effect of Shade on the Activities of TFs and Integrators of the Light-Hormone Signal Networks

It has been well known that light and hormones play important roles in regulating chlorophyll accumulation in *Arabidopsis* (Lau and Deng, 2010; Liu X. et al., 2017). The integrators, such as PHYA, CRY1, PIFs, HY5, EIN3, and DELLA proteins, are key transcription regulators in light and/or hormone signaling pathways (Tanaka et al., 2011; Liu X. et al., 2017). Transcripts of integrators and key genes believed to be involved in the regulation by light and/or hormones of chlorophyll biosynthesis were analyzed by both the RNA-Seq and RT-PCR (**Supplementary Tables S4, S5** and **Figure 5**).

PHYA and CRY1 sense far-red/red and blue/UV-A light, respectively, and initiate intracellular transduction to alter the expression of nuclear genes (Chaves et al., 2011; Burgie and Vierstra, 2014). Both *PHYA* and *CRY1* increased significantly in the shaded leaves when compared with the non-shaded leaves. Simultaneously, key integrators *EIN3* and *DELLAs* showed significant increases in response to shade treatment. Especially *DELLAs*, including *DELLA1*, *DELLA3,* and *DELLA4* were found to be shade-induced. The E3 ligases EBF1 and EBF2 were found to function with COP1 in EIN3 degradation and ethylene signal regulation of seedling photomorphogenesis (Shi et al., 2016; Wang et al., 2019). Both *EBF1* and *EBF2* showed a response to shade, *EBF1* expression increasing significantly throughout the shade period while *EBF2* expression increased only at 7d. In contrast, as the important integrator of light-hormone signal networks, *HY5* showed a clear decrease in gene expression throughout shade treatment. *GUN4* has been reported to be light-activated and acts as a putative target of HY5 (Lee et al., 2007) to promote the conversion of ALA to the "chlorophyll branch" (Tanaka et al., 2011). No significant shade response was determined in the expression of *GUN4*. Light signal components, including PIFs (PIF1/3), COP1, Far-red elongated hypocotyls (FHYs), and Fluorescent in blue light (FLU) were detected in both the control and shade treatments, but there were no significant changes.

Correlation Network Between Genes and Metabolites in Response to Shade

To further explore the regulatory mechanism by light and hormones on chloroplast development and chlorophyll biosynthesis, the correlation network among expression of candidate genes/TFs, major metabolites in the chlorophyll biosynthesis pathway and hormones were statistically analyzed (**Figure 6**; Shannon et al., 2003). As shown in **Figure 6A**, the key genes and TFs involved in the chlorophyll biosynthesis were selected and the interaction network was built between genes and key candidate TFs (relevant factor $\geq$ 0.80 was selected for the interaction network) (Zhang S. et al., 2018). Significant correlations were detected between blue/red light receptors (*CRY1* and *PHYA*) and the TFs *DELLAs* (*DELLA3* and *DELLA4*), *EFB1* and *sco1*, which are relevant to light

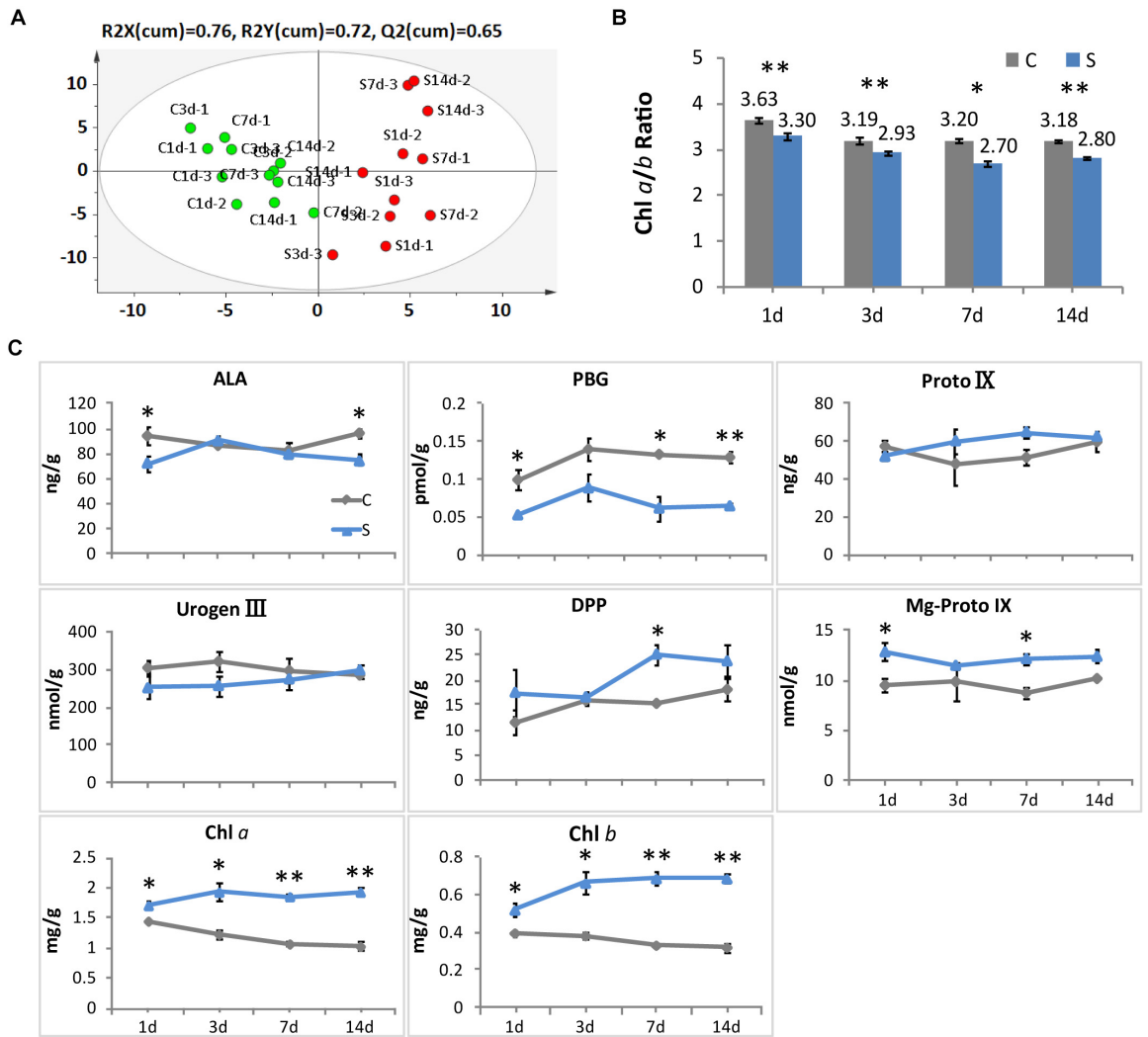

FIGURE 2 | Effect of shade on the concentrations of chlorophylls and the main precursors in tea leaves. **(A)** The OPLS-DA analysis of the main precursors and chlorophylls in tea leaves from the control and shade treatments. **(B)** The ratio of chlorophyll *a* and chlorophyll *b* in tea leaves from the control and shade treatments. **(C)** The accumulation of chlorophylls and main precursors in tea leaves from the control and shade treatments. 4h, 1d, 3d, 7d, and 14d indicate time points during the shade period. ALA, 5-aminolevulinic acid; PBG, porphobilinogen; Proto IX, protoporphyrin IX; Urogen III, uroporphyrinogen III; DPP, divinyl protochlorophyllide; Mg-Proto IX, Mg-Protoporphyrin IX; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*. Data shown are from three independent biological replicates ($n = 3$). *Significant differences comparing the control treatment at each time point according to one-way ANOVA test and a Fisher's LSD at the 5% significance level (*$p \leq 0.05$, **$p \leq 0.01$). OPLS-DA analysis was conducted by SIMCA 13.0 (UMETRICS, https://umetrics.com/).

signal transductions. Correlations were also found among *CRY1/PHYA* and genes (*PORA* and *HEMA1*) and hormones (CTK and GA) involved in chlorophyll biosynthesis. Strong interaction was detected between the expression of *GLK1* and *LHCB*, suggesting a regulation between these genes in tea leaves. Correlations were also detected among *LHCB* and enzyme encoding genes in the chlorophyll biosynthesis pathway, including *HEMA1*, *PORA*, and *CAO*. Furthermore, HY5 has been known to participate in the light and hormone regulation of chlorophyll biosynthesis (Waters and Langdale, 2009; Xu et al., 2016; Liu X. et al., 2017). In the current study, there was a significant association between the expression of HY5, blue/UV-A light receptor CRY1 and the light signal integrators (*DELLA3/4* and *EBF1*). In addition to the interaction between candidate genes and TFs, the interactions between genes/TFs and relative plant hormones with responses to shade were also conducted. A strong interaction between auxin and HEMA1 expression was detected, consistent with previous findings in *Arabidopsis* (Tanaka et al., 2011; Kobayashi et al., 2012). The hormone GA exerted a regulatory effect on *PORA* expression in tea leaves in response to shade treatment. A strong interaction was also detected between CTK and *HY5*, *PHYA*, *CRY1*, *EBF1*, and *DELLA3*, suggesting some regulation by CTK of chlorophyll biosynthesis in tea leaves. The changes in metabolites involved in chlorophyll biosynthesis (DPP, Mg-Proto IX, PBG, Chl *a*, and Chl *b*) were analyzed, and strong

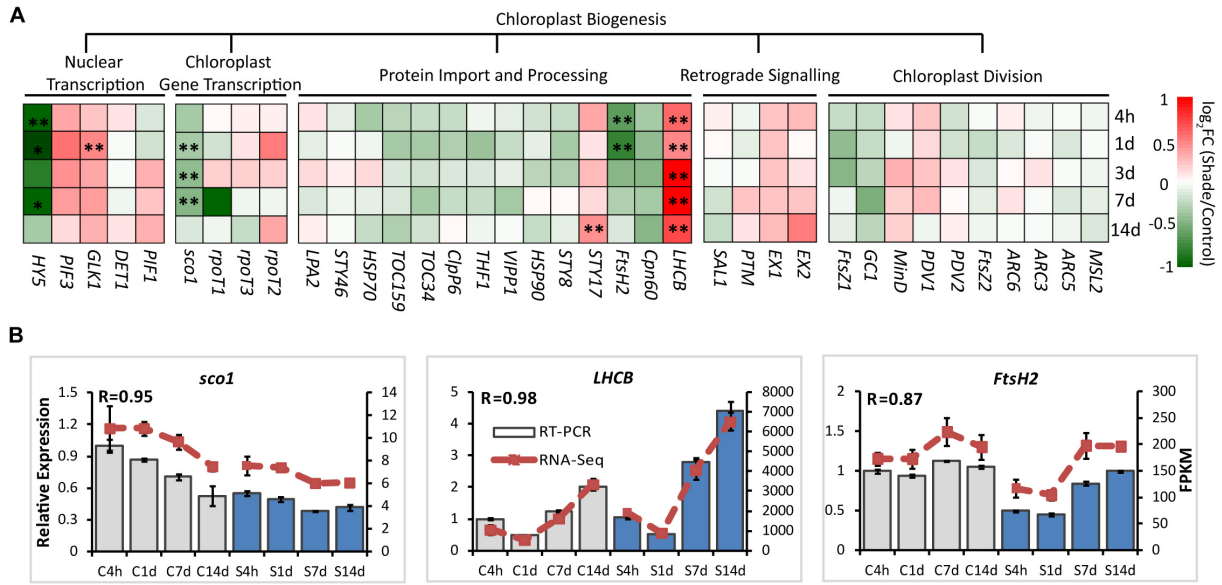

FIGURE 3 | Effect of shade on genes encoding key enzymes of the chlorophyll biosynthesis pathway. **(A)** Transcript abundance of 38 representative unigenes involved in the chlorophyll biosynthesis pathway generated from RNA-Seq analysis. Genes and TFs are classed into different subgroups according to their functions involved in the chloroplast biogenesis, including nuclear transcription, chloroplast gene transcription, protein import and processing, retrograde signaling and chloroplast division. **(B)** Transcript abundance of selected unigenes validated by the RT-PCR analysis. The column presents data generated from the RT-PCR analysis (gray column, data from the control treatment at four time points; blue column, data from the shade treatment at four time points). The line presents data generated from the RNA-Seq analysis. The transcript abundance in the control treatment at 4 h (C4h) was set at 1. R, the correlation factor between RT-PCR and RNA-Seq analysis for each gene/TF according to SPSS 13.0 software. 4h, 1d, 3d, 7d, and 14d indicate time points during the shade period. C, control treatment; S, shade treatment; FPKM, Fragment per kilobase of exon model per million mapped reads. Data shown are conducted of three independent biological replicates ($n = 3$). *Significant differences comparing the control treatment at each time point according to one-way ANOVA and a Fisher's LSD test at the 5% level (*$p \leq 0.05$, **$p \leq 0.01$; fold change ≥ 1.5). For abbreviations, see text.

interactions among these metabolites, genes/TFs and hormones were detected (**Figure 6A**).

To complement the interaction network, a matrix correlation analysis was conducted between genes/TFs and hormones and the major metabolites in the chlorophyll biosynthesis pathway (**Figure 6B**). Consistent with the information shown in the interaction network, strong positive and negative correlations were detected among integrators of light and hormone signal pathways (*CRY1*, *PHYA*, *HY5*, *DELLAs*, *EBF1*, *EIN3*, *sco1,* and *LHCB*), enzyme encoding genes in chlorophyll biosynthesis (*CAO*, *CHL1/2*, *HEMA1*, *NOL,* and *PORA*), major metabolites of the chlorophyll biosynthesis pathway (PBG, Mg-Proto IX, Chl *a,* and Chl *b*) and plant hormones (auxin, GA and CTK). Furthermore, most of these light- and hormone signal integrators (*CRY1*, *PHYA*, *DELLAs*, *EBF1,* and *EIN3*) showed a positive correlation with the accumulation of chlorophylls and metabolites (Proto IX, Mg-Proto IX, Chl *a,* and Chl *b*). In contrast, *HY5* and *sco1* were found to have a negative correlation with some metabolites (Proto IX, Mg-Proto IX, Chl *a,* and Chl *b*) and hormones (CTK and GA). This may indicate a negative regulation of HY5 and sco1 on these metabolites and hormones in the leaves under shade conditions. *GLK1*, which is reported to play a critical role in light regulation of chloroplast biogenesis in *Arabidopsis* (Waters et al., 2009). *GLK1* showed some positive correlation, at the transcriptional level, with many the metabolites (Urogen III, Proto IX, DPP, and Chl *a*/*b*) and hormones (CTK, GA, and auxin) quantified in this study. For instance, some positive correlation was detected between *GLK1* and Proto IX. Conversely, the changes of ACC, the ethylene precursor was found to be negatively correlated with *GLK1* expression.

DISCUSSION

Tea is a typical woody crop that prefers environments with a relatively low level of light intensity. Compared to other plants, *Camellia* tea plants can maintain good growth and condition for a long period even in an extremely low light environment. For example, when nearly 98% of the light was excluded by the shading nets, plants still grew well with new bud burst and chlorophylls concentrations increased after a 1 month treatment period (Yang et al., 2012). With such low-light tolerance, shading of plants has been widely used in tea plantations for decades, particularly for green tea with its high chlorophyll content requirement (Yamaguchi and Shibamoto, 1981; Shimoda et al., 1995). However, the molecular mechanism of chloroplast development, photosynthesis and chlorophyll biosynthesis underlying the shade effects remained largely unknown. From the current study, it is proposed that the

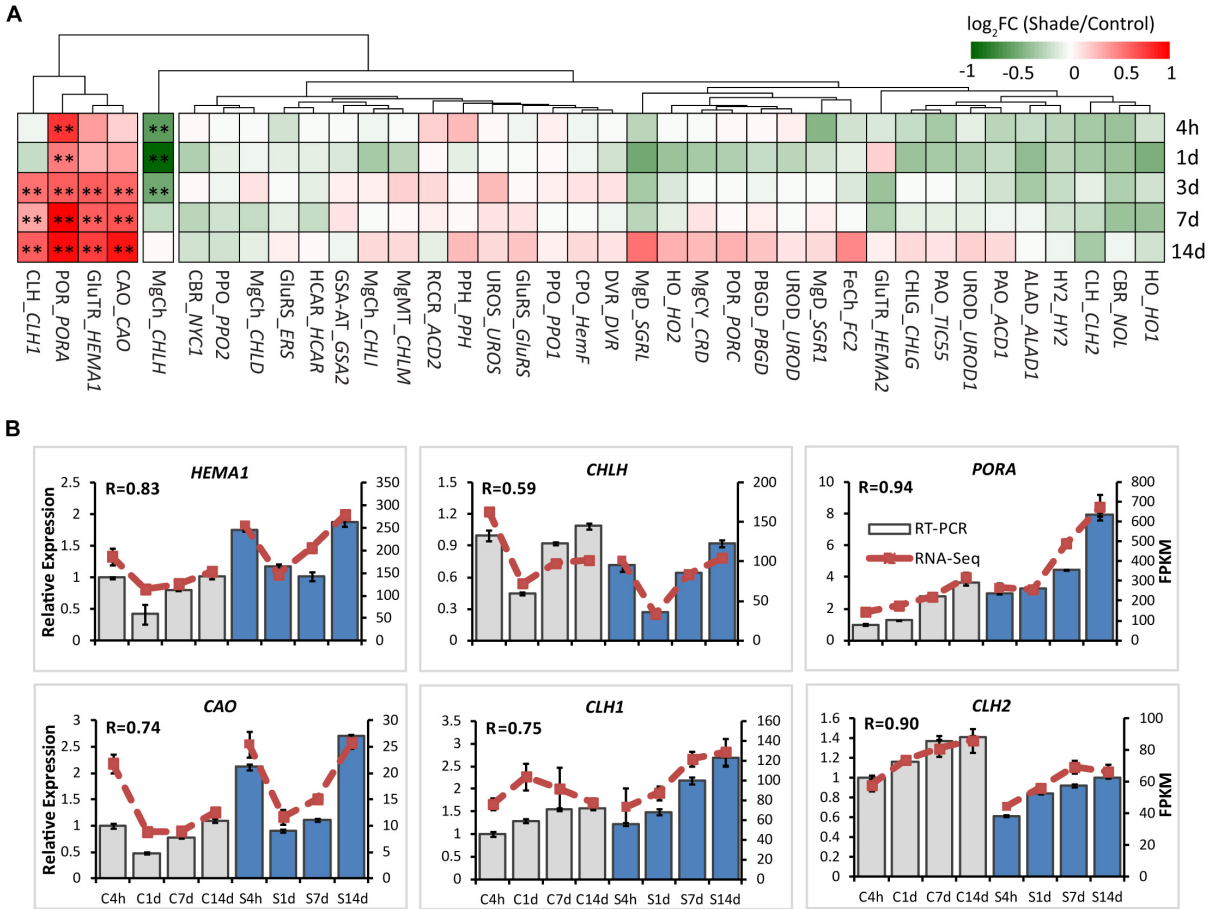

FIGURE 4 | Effect of shade on key genes and TFs involved in chloroplast biogenesis. **(A)** Transcript abundance of representative unigenes involved in chloroplast biogenesis generated from RNA-Seq analysis. **(B)** Transcript abundance of selected unigenes validated by the RT-PCR analysis. The column presents data generated from the RT-PCR analysis (gray column, data from the control treatment at four time points; blue column, data from the shade treatment at four time points). The line presents data generated from the RNA-Seq analysis. The transcript abundance in the control treatment at 4 h (C4h) was set at 1. R, the correlation factor between RT-PCR and RNA-Seq analysis for each gene/TF according to SPSS 13.0 software. Data shown are conducted of three independent biological replicates (n = 3). The annotations of 4h, 1d, 3d, 7d, 14d, C, S, FPKM and statistical label (*) are shown as in **Figure 3**. For abbreviations, see text.

shade-increased accumulation of chlorophylls in *C. sinensis* cv. Shuchazao leaves is the result of at least two factors: (a) positive changes in chloroplast development and (b) a light regulation of genes and potential TFs involved in the chlorophyll biosynthesis pathway. The changes of hormones, as a part of shade responses, presumably synergize with light to regulate chlorophyll biosynthesis in the tea crop.

Shade Expanded the Photosynthetic Apparatus

The biogenesis of a mature chloroplast is a complex and sophisticated process. Numerous genes and signal integrators participate in this process and together with light play crucial roles in the transition from a proplastid to a mature chloroplast (Waters and Langdale, 2009; Liu X. et al., 2017). Compared with findings in the model plant *Arabidopsis* (Lichtenthaler et al., 1981), similar responses to shade were observed in the leaves of Shuchazao. In the current study, the reduction of light stimulated the chloroplast development quantitatively (**Figure 1**). These changes included the abundance of chloroplasts, ultrastructural changes in the thylakoid membrane system and the formation of grana stacks. These were consistent with responses to shade reported in previous studies (Wu et al., 2016; Liu G. F. et al., 2017), with similar alterations in chloroplast ultrastructure observed in both the albino tea cultivars and yellow phenotype.

In *Arabidopsis*, a model for light regulation on chloroplast development has been summarized according to previous studies (**Figure 7A**, this model was modified from Waters and Langdale, 2009). Nuclear TF GLKs are required for transcription of genes encoding chloroplast proteins and provide the anterograde signal in response to light regulation (Oh and Montgomery, 2014). Simultaneously, LHCB acts as a bridge passing this anterograde signal from the nucleus to chloroplast to regulate the formation of the thylakoid membrane and grana development (Waters and

TABLE 1 | Leaf concentrations of endogenous phytohormones under control (C) and shade (S) conditions.

	CTK (ng/g)	GA (pmol/g)	Auxin (μmol/g)	ACC (μmol/g)	BR (ng/g)	SL (ng/g)
C1d	329 ± 3.4**	0.8 ± 0.1	0.10 ± 0.02	2.09 ± 0.31	42 ± 9.7	1090 ± 22.4
C3d	341 ± 53.5	0.8 ± 0.02*	0.11 ± 0.01	2.48 ± 0.02**	46 ± 2.2	878 ± 58.6
C7d	353 ± 24.4*	0.8 ± 0.01	0.12 ± 0.02*	1.79 ± 0.06	41 ± 9.6	1009 ± 51.7
C14d	377 ± 58.9	0.8 ± 0.1*	0.11 ± 0.02*	1.88 ± 0.04	39 ± 8.9	820 ± 34.5
S1d	469 ± 3.4**	0.8 ± 0.03	0.12 ± 0.01	1.85 ± 0.08	46 ± 11.9	909 ± 144.8
S3d	421 ± 13.8	1.0 ± 0.1*	0.13 ± 0.01	1.63 ± 0.15**	44 ± 4.7	880 ± 80.9
S7d	432 ± 6.6*	1.0 ± 0.1	0.17 ± 0.01*	1.96 ± 0.04	51 ± 5.4	1010 ± 29.3
S14d	447 ± 34.1	1.1 ± 0.1*	0.16 ± 0.02*	1.73 ± 0.14	45 ± 4.2	919 ± 72.4

*1d, 3d, 7d, and 14d indicate time points during the shade period. CTK, cytokinin; GA, gibberellins; ACC, 1-aminocyclopropane carboxylic acid; BR, brassinosteroid; SL, strigolactones. Data shown are from the value of three independent biological replicates (n = 3). *Significant differences comparing the control treatment at each time point according to one-way ANOVA test and a Fisher's LSD at the 5% significance level (*p ≤ 0.05, **p ≤ 0.01).*

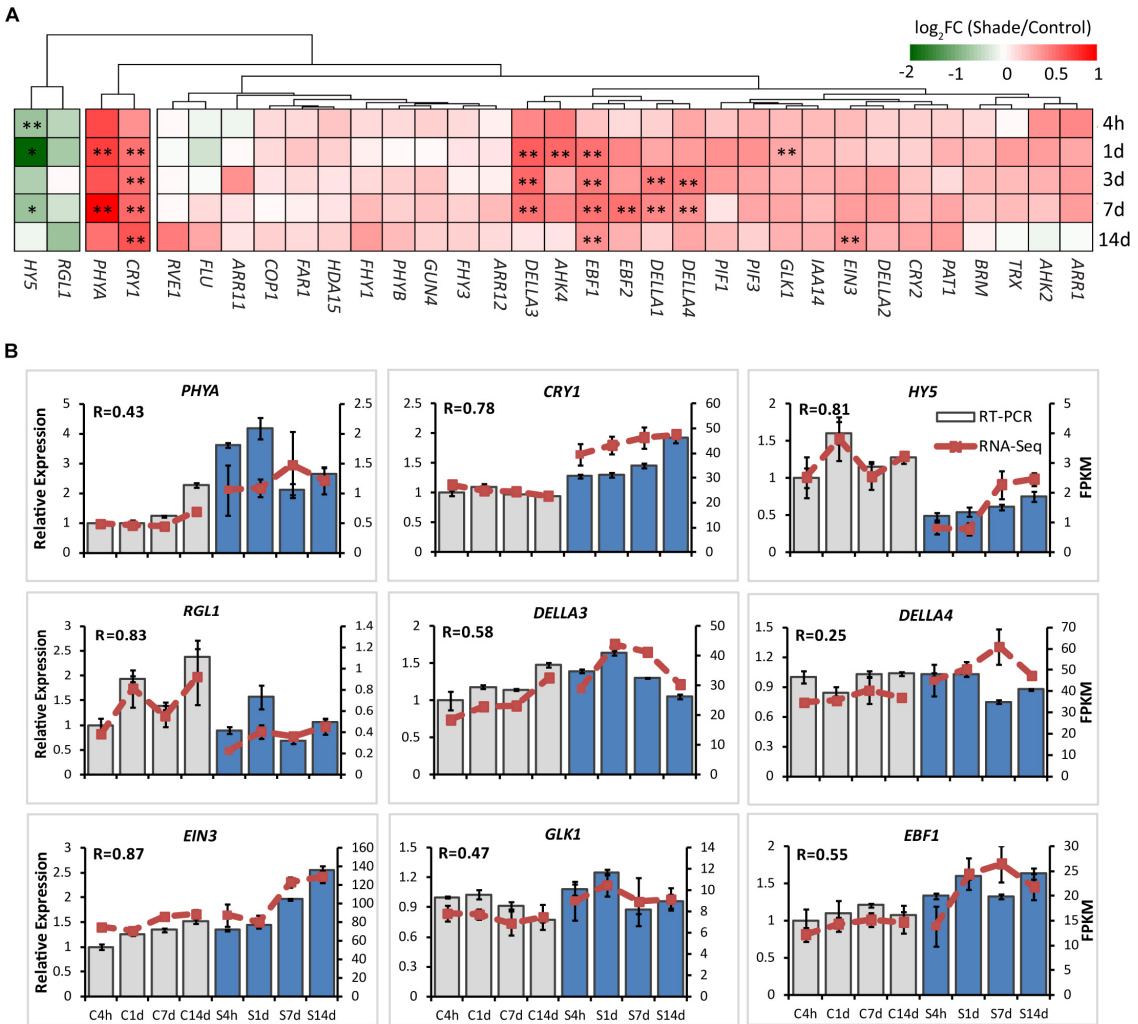

FIGURE 5 | Effect of shade on key genes and TFs involved in the light- and hormone regulation of chlorophyll biosynthesis. **(A)** Transcript abundance of representative unigenes involved in the light and hormone regulation of the chlorophyll biosynthesis generated from RNA-Seq analysis. **(B)** Transcript abundance of selected unigenes validated by the RT-PCR analysis. The column presents data generated from the RT-PCR analysis (gray column, data from the control treatment at four time points; blue column, data from the shade treatment at four time points). The line presents data generated from the RNA-Seq analysis. The transcript abundance in the control treatment at 4 h (C4h) was set at 1. R, the correlation factor between RT-PCR and RNA-Seq analysis for each gene/TF according to SPSS 13.0 software. Data shown are conducted of three independent biological replicates (*n* = 3). The annotations of 4h, 1d, 3d, 7d, 14d, C, S, FPKM and statistical label (*) are shown as in **Figure 3**. For abbreviations, see text.

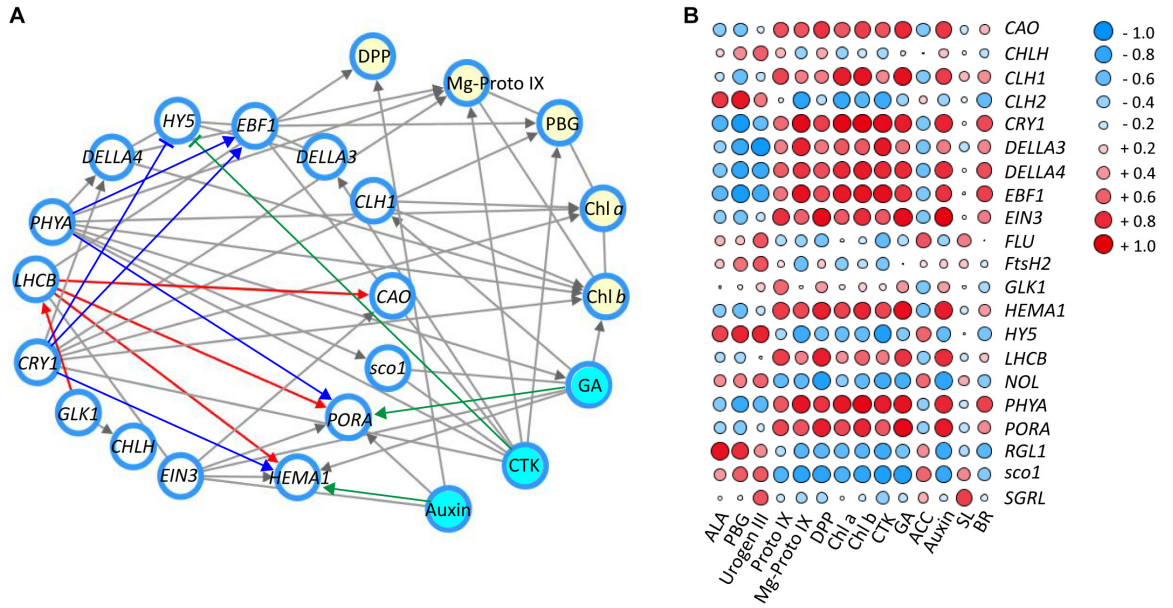

FIGURE 6 | The correlation of genes/TFs, chlorophylls and main precursors and hormones involved in chlorophyll biosynthesis in response to shade treatment. **(A)** Interaction network of main genes and TFs (white nodes), main compounds (yellow nodes) and hormones (blue nodes) involved in chlorophyll biosynthesis (relevant factor ≥ 0.85). **(B)** Correlation heatmap between genes and TFs, main compounds and hormones involved in chlorophyll biosynthesis (-1∼0, expression of genes are negatively correlated; 0, expression of genes are not correlated; 0∼1, expression of genes are positively correlated). Data shown are conducted of three independent biological replicates (n = 3), analyzed by the Speaman test in SPSS 13.0 software (IBM SPSS Software, https://www.ibm.com/analytics/datascience/predictive-analytics/spss-statistical-software). The network was conducted by Cytoscape (version 3.7.0; http://www.cytoscape.org/) (Shannon et al., 2003). The heatmap was visualized by the pheatmap package implemented in R (https://cran.r-project.org/web/packages/pheatmap/index.html). For abbreviations, see text.

Langdale, 2009). The data from the current study suggest that the regulation on chloroplast development by light in Shuchazao leaves is comparable to that of *Arabidopsis*. Compared with non-shaded leaves, an increase in the expression of *GLK1* was detected immediately in the shaded leaves. *LHCB*, the central signal bridge between the nucleus and chloroplast, showed a significant increase in the shaded leaves throughout the whole treatment period (**Figure 3**). Also, strong correlations were detected between the expression pattern of *LHCB* and the shade responses of downstream genes, *PORA*, *HEMA1,* and *CAO* (**Figure 6**). These findings are consistent with the model that *GLK* genes are up-regulated once the nucleus receives a light-limiting signal derived from the chloroplast, and, therefore, leading to higher expression of its target gene *LHCB* (Waters and Langdale, 2009; Oh and Montgomery, 2014). In addition, DELLAs stimulates photosynthesis and chlorophyll biosynthesis through regulating *LHCB*, *CHLH,* and *PORA*/*B*/*C* (Cheminant et al., 2011). Our data showed the expression of DELLAs (*DELLA3* and *DELLA4*) became activated in the shaded leaves (**Figure 5**). HY5 is supposed to be involved in the anterograde signal perception. Previous studies suggested that HY5 acts as a signal promoter in the light and hormone signaling pathways in regulating nuclear gene transcription and promoting seedling photomorphogenesis (Chen et al., 2004; Casal et al., 2014; Xu et al., 2016). Several nuclear encoding photosynthetic and chlorophyll biosynthesis genes, such as *CHLH*, *GUN4*, *PORC*, *CAO,* and *CHL27*, are the putative targets of HY5 (Lee et al., 2007). Recent findings suggest HY5 mediates the process of chlorophyll biosynthesis in plant roots and functions downstream of PHYB to modulate *PORA* expression through a Myb-like transcription factor REVEILLE1 (RVE1) (Jiang et al., 2016). Herein we found that the transcriptional level of *HY5* decreased significantly in leaves under the shade conditions (**Figure 5**). This decrease was probably due to the heavy limitation of light exposure (red, blue, and UV-B signals) under shading conditions, which overlays the intrinsic needs of chloroplast development in the opposite way.

Shade Stimulates the Gene Activities Involved in Chlorophyll Biosynthesis

In addition to the physical enhancement of the photosynthetic apparatus (thylakoid membrane systems) as shown in the ultrastructure alterations, metabolites and gene activities in the chlorophyll biosynthesis pathway showed a clear light regulatory role (**Figure 7B**). Significant increases were detected in the level of major metabolites, including My-Proto IX, DPP, Chl *a,* and Chl *b* (**Figure 2**). The accumulation of My-Proto IX increased immediately at 1d after commencing shade treatment, while the DPP was seen to increase at the later stages of shade treatment. The accumulation of Chl *a* and Chl *b* presented significant increases (around 2 x) in shaded leaves (**Figure 2C**), with a decrease in the ratio of Chl *a*/Chl *b* (**Figure 2B**).

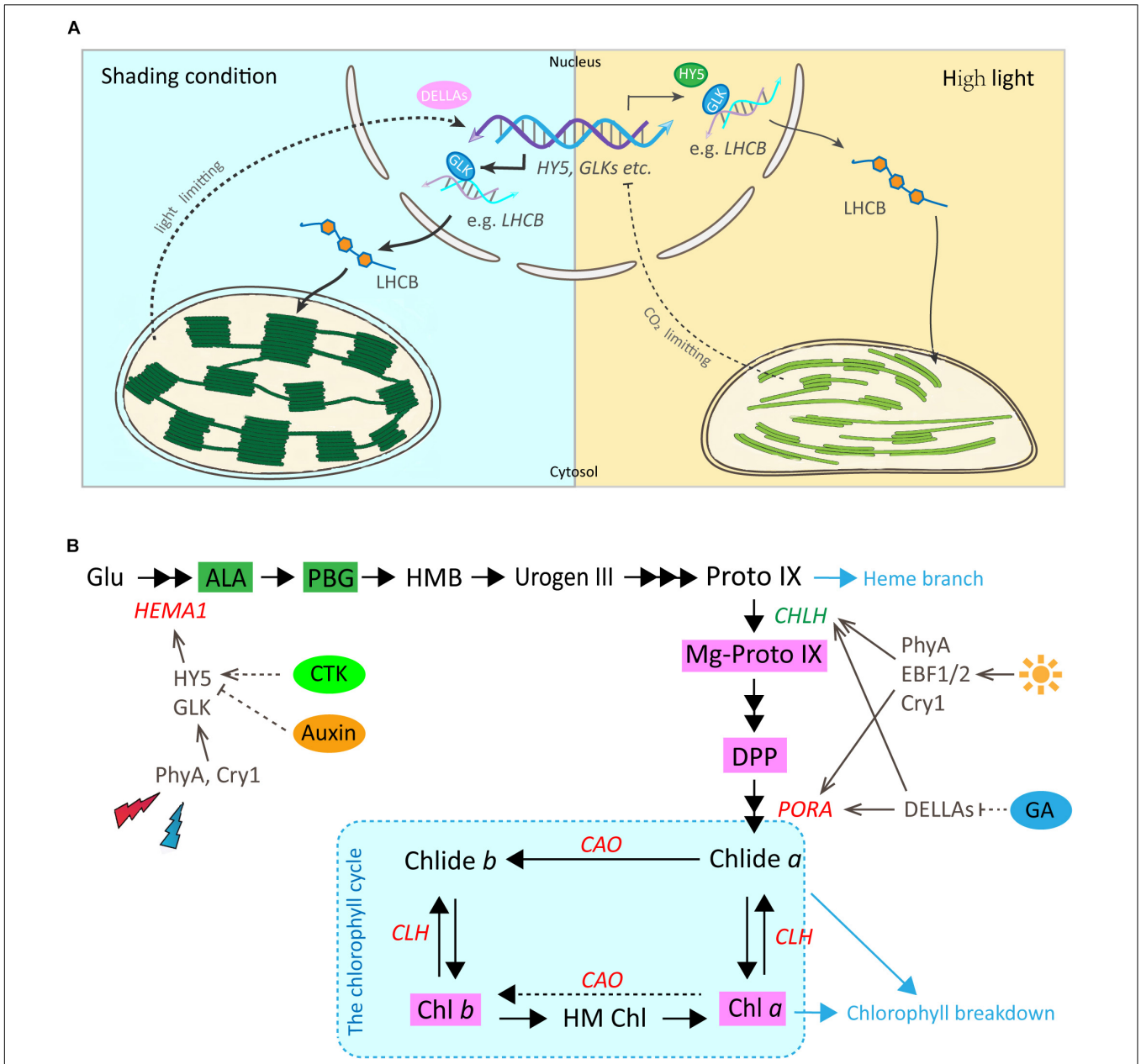

FIGURE 7 | Working models for the regulatory roles of light and hormones in chloroplast development and chlorophyll biosynthesis in Shuchazao leaves**. (A)** A predicted model for the regulatory role of light on chloroplast development by the GLK and other potential TFs in tea leaves (reproduced, in part, with permission from Waters and Langdale, 2009). Under the shade environment, the photosynthetic electron transfer chain is in an oxidized state and cannot provide sufficient ATP. Therefore, a light-limitation signal from the chloroplast is sent to the nucleus and GLK genes are up-regulated. Transcript levels of these GLK target genes increase leading to higher levels of proteins that function in light-harvesting (such as LHCB, etc.), and, therefore, assembly leads to higher specific chlorophyll levels, a lower Chl *a*/*b* ratio and more abundant grana. In contrast, when light is plentiful and the rate of CO_2 fixation is insufficient, this prompts a chloroplast-derived CO_2 fixation limiting signal to the nucleus. The transcriptions of GLK genes are repressed and the accompanying decrease in LHCB and chlorophyll-related gene transcripts, eventually results in a reduction in the thylakoid membrane and lower chlorophyll content. **(B)** A simplified regulatory network of chlorophyll biosynthesis by light and plant hormones in tea leaves (Red, increase in response to shade; Green, decrease in response to shade). The dashed portion indicates that further verifications are needed. For abbreviations, see text.

These can be explained by the changes in the activities of genes and TFs in chlorophyll biosynthesis (**Figure 4**). *PORA*, the well known light-regulated gene (Reinbothe et al., 1996), showed a clear up-regulation throughout the shade period. The transcript level of *CAO* increased significantly in the shaded leaves, especially at the later stage of the shade from 3d to 14d. A similar increase was detected in the expression of *CLH1*, which increased to nearly 2x

at 14d of shade treatment. In addition, light receptors and transcription factors that are believed to function in the chlorophyll biosynthesis pathway were also investigated. Previous studies reported HY5 plays a vital role in the convergence of blue, red and far-red light-signal pathways for regulating the transcription level of *HEMA1* (McCormac and Terry, 2002). In the present study, we found a significant reduction of *HY5* expression as discussed earlier, but a significant increase in the expression of *HEMA1*. Also, both the red/far-red light sensor *PHYA* and blue light receptor *CRY1* showed clear increases. These findings were consistent with the previous studies, implying a regulation of red/far-red and blue light signals on *HEMA1* expression through *HY5* (McCormac and Terry, 2002). Furthermore, light signal components, EBFs and DELLAs were detected to be light-regulated, suggesting a role of light regulation in tea leaves consistent with previous studies (Cheminant et al., 2011; Shi et al., 2016). Interestingly, *GLK1*, which was expected to have a strong correlation with the chlorophyll biosynthesis pathway, presented no significant connection with the metabolites measured in this study. It appears that *GLK1* functioned in the light regulation of chloroplast development rather than chlorophyll biosynthesis in tea leaves. Further analysis needs to be carried out at the protein level to investigate the role of *GLK1* in the tea leaves. In total, all of these data show the chlorophyll biosynthesis pathway, in particular, the "chlorophyll cycle" became much more active under the shade conditions to meet the requirements of photosynthetic pigments and energy needs.

The Involvement of Hormones in the Regulation of Chlorophyll Biosynthesis

Multiple hormone pathways may participate in mediating the shading responses in tea plants. HY5 is a key light signal component that integrates the light- and hormone signal pathways, including auxin, CTK, abscisic acid and GA (Lau and Deng, 2010). Previous studies have shown that auxin and CTK regulate *HY5*, at the protein level, to promote seedling photomorphogenesis (Lau and Deng, 2010). Conversely, HY5, together with its close homolog HY5 Homolog (HYH), repress auxin signaling by direct activation of its negative regulators Indole Acetic Acids 7 and 14 (IAA7 and IAA14) (Cluis et al., 2004; Sibout et al., 2006). In the present study, the transcription level of *HY5* was decreased, simultaneously significant increases were detected in the level of CTK, GA and auxin under shade conditions (**Figure 5** and **Table 1**). Also, strong negative correlations were detected between HY5 expressions and the level of hormones, CTK (relevant factor -0.86) and auxin (relevant factor -0.65) (**Figure 6**). Therefore, our data support the theory that HY5 negatively modulates auxin signaling to mediate responses to shade in tea plants. *HEMA1* is one of the target genes of HY5 (McCormac and Terry, 2002) and the expression of *HEMA1* is potentially regulated by both auxin and CTK signals in tea leaves as parts of the shade response (relevant factor between *HEMA1* and auxin is 0.7; relevant factor between *HEMA1* and CTK is 0.85). In addition DELLAs, a subfamily of the GRAS transcriptional regulators (Jiang and Fu, 2007), have been suggested as key nuclear-enriched repressors in the GA signaling pathway, which represses GA-regulated gene expression and seedling growth (Jiang and Fu, 2007; Cheminant et al., 2011). Also, DELLAs positively regulate the expression of genes involved in chlorophyll biosynthesis (*CHLH* and *PORC*) in a PIF dependent manner (Cheminant et al., 2011), and independently up-regulate photosynthesis (*LHCB*) and *PORA/B* gene expression. However, in our data, both the GA and *DELLA3* showed significant increases after shade treatment. Further studies can focus on the roles of CTK and GA signaling in mediating responses to shade in *Camellia sinensis*. Overall, based on the data generated in the current study, it is presumed that hormone changes, as a part of the shade response, may play some role in modulating chlorophyll biosynthesis in the tea leaves.

CONCLUSION

This study aimed to investigate the molecular mechanism underlying the shade-enhanced accumulation of chlorophylls in an important tea cultivar, *C. sinensis* cv. Shuchazao. Based on the data from the transcriptional expression of genes/TFs, alterations of metabolites and hormones, we proposed that the shade-enhanced accumulation of chlorophylls in Shuchazao leaves is modulated by a light-hormone network, functioning from ultrastructural construction of chloroplasts to gene activities of the chlorophyll biosynthesis pathway. Our findings showed the light-hormone mediated regulatory network on chlorophyll biosynthesis in tea leaves, which improves our understanding of the regulatory mechanism on chlorophyll biosynthesis and could provide new opportunities for the molecular breeding of tea cultivars with specific phenotypes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI accession PRJNA576575.

AUTHOR CONTRIBUTIONS

LL conducted this research, analyzed the data, and prepared the draft manuscript with assistance from NL, XL, SY, and WW for samplings and data analysis. XW provided supervision for the research. All of the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00256/full#supplementary-material

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Screening Tea Cultivars for Novel Climates: Plant Growth and Leaf Quality of *Camellia sinensis* Cultivars Grown in Mississippi, United States

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The United States (U.S.) consumed over 80 billion servings of tea, approximately 3.8 billion gallons, in the year of 2018. With the vast majority of tea demand being met by importation, the United States became the third largest tea importer worldwide after Russia and Pakistan. As demand for domestically produced tea increases and growers expressing increasing interest in growing and producing tea, tea production became an emerging industry in the United States. Compared to major tea producing countries with centuries of growing history, tea production in the United States is limited and requires research support in many aspect of tea production including selecting suitable cultivars adapted to local climatic conditions. This study evaluated nine tea cultivars, including 'BL1,' 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' 'Large Leaf,' 'Small Leaf,' 'Sochi,' and 'var. *assamica*,' for plant growth, leaf morphological characteristics, cold tolerance, and leaf biochemical compositions when grown in Mississippi United States with a subtropical climate. The nine tested cultivars had varying plant growth indices (PGI) and varying degrees of cold tolerance to freezing temperatures in winter, but resumed healthy growth the following spring. 'BL2' showed the highest PGI of 104.53 cm by February 2019, which might be helpful toward suppressing weed and early establishment of tea plantation. The nine cultivars also showed varying leaf characteristics in terms of leaf length, width, area, fresh and dry weights, and new shoot weight. There existed a diversity in leaf biochemical composition including soluble solids, carbohydrates, total polyphenols (TP), free amino acids (AA), L-theanine and caffeine among the nine cultivars and among different harvesting seasons of spring, summer, and fall within a certain cultivar. The nine cultivars in this study generally grow well in local environment. All tea samples collected from nine cultivars and three seasons were considered suitable for green tea processing with low TP/AA ratios ranging from 1.72 to 3.71 in this study.

Keywords: tea, *Camellia sinensis*, plant growth, cold tolerance, leaf quality, polyphenols, amino acids, caffeine

INTRODUCTION

Tea is the most popular beverage worldwide second only to water, with world consumption of 5.5 million tons in 2016 (Food and Agriculture Organization of the United Nations [FAO], 2018). The industry value for world tea consumption increased from $1.84 billion in 1990 to $12.66 billion in 2018, with a projected strong increasing trend over the next 10 years (Goggi, 2018; USDA, 2018). Tea is rich in a number of health beneficial compounds including catechin, caffeine, theanine, and other polyphenols. Polyphenols in tea are antioxidants believed to slow down aging, prevent certain types of cancer, and reduce risk of cardiovascular diseases (Sharangi, 2009; Lorenzo and Munekata, 2016). Americans consumed over 3.8 billion gallons, or more than 84 billion servings of tea in 2018, making the United States the third largest importer of tea after Russia and Pakistan, importing a total of 263 million pounds including black and green tea (Tea Association of the U.S.A., 2019). The vast majority of tea demand in the United States is met by importation. With increased demand for locally sourced food products, questions are raised regarding whether domestic production of tea is feasible.

Tea plant is a broad-leaved evergreen shrub, adapted to subtropical to tropical climates, with optimal growing temperatures of 18 to 30°C during the growing season and the ability to withstand temperatures from −16 to 40°C (Bhagat et al., 2010; Luo, 2015). They thrive in warm, humid climates with annual rainfall of 1,250 to 6,000 mm, favor humidity levels of 80 to 90% and elevations up to 2,000 m above sea level (Hajra, 2001). Successful tea plant production requires deep, light, well-drained, and acid soil with pH ranging from 4.5 to 5.5 (Willson and Clifford, 1992; Hajra, 2001; Ruan et al., 2007; Gascoyne et al., 2016). In the United States tea can be grown in USDA hardiness zones 6 to 9 (Dirr, 2009). Once established, a tea plantation can have commercial productivity for decades (Wang, 2016).

As United States growers seek to diversify their crops, tea plants can potentially serve as a high-value alternative crop. Tea production effort occurs in over 16 states in the United States, including Alabama, California, Florida, Georgia, Hawai'i, Idaho, Louisiana, Maryland, Michigan, Mississippi, New Jersey, New York, North Carolina, Oregon, South Carolina, Texas, Virginia, and Washington with the majority started over the past decade and having limited production (Zee et al., 2003; Walcott, 2012; Song et al., 2012; Bell, 2014; Hardin, 2017; LeCompte, 2018). The state of Mississippi is located in a subtropical climate, having annual average temperatures ranging from 17°C in the north to 20°C along the coast. Annual precipitation ranges from 1270 to 1650 mm (50–65 inches) and fairly evenly distribute throughout the year (Mississippi State University, 2019). There are currently three small scale commercial tea farms in Mississippi. Growing tea in Mississippi is subjected to challenges including periods of drought and flood, temperatures exceeding 35°C for over 100 days annually, and potential cold damage with lowest temperatures down to −14°C. Compared to centuries of growing history of tea in leading tea producing countries including China and India (Food and Agriculture Organization of the United Nations [FAO], 2015), there lacks research-based information to guide farmers on growing tea in the United States. The availability of suitable cultivars adapted to local climates is fundamental to an emerging tea industry in the United States.

Tea germplasms have been evaluated using morphology, biochemical compositions, molecular markers, and sensory evaluations (Feng et al., 2014; Li Y. et al., 2016; Wambulwa et al., 2016). Fresh leaf characteristics including leaf size, area, and fresh weight are commonly evaluated in breeding programs for yield potential. The final quality of tea product is highly associated with leaf biochemical composition including polyphenols, soluble solids, carbohydrates, amino acids (AA), theanine and caffeine (Willson and Clifford, 1992; Li X. et al., 2016). There exists large variations in physical characteristics and chemical composition in leaves of different germplasms (Willson and Clifford, 1992; Gai et al., 2019). Growing environment including soil, temperature, precipitation, light, seasonality, and altitude affect leaf characteristics and biosynthesis of important chemicals, thus the final tea quality (Lee et al., 2010; Wang et al., 2011; Wei et al., 2011; Han et al., 2017; Ahmed et al., 2019). This study is of the few recent research efforts to evaluate tea cultivars grown in Southeastern United States. The objectives of this study were to investigate plant growth and leaf morphology of nine tea cultivars; and to investigate chemical compositions in leaves of these cultivars in different seasons of the year (spring, summer, and fall) when grown in Mississippi, United States.

MATERIALS AND METHODS

Plant Cultivation

One-year-old tea plants propagated from stem cuttings grown in one-gallon containers were transplanted into the field located at the R. R. Foil Plant Research Center at Mississippi State University (USDA Hardiness Zone 8a; 33°29′N 88°47′W) in spring 2017. The nine tested cultivars included: 'BL1,' 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' 'Large leaf,' 'Small leaf,' 'Sochi,' and 'var. *assamica*.'. Tea plants were pruned to a uniform height of 30.48 cm (12 inch) at transplanting and grown in full sun in Stough fine sandy loam soil with a pH of 4.9 (Brent, 1973). Plants were planted in a double row hedge, with 0.76 m (2.5 ft) between plants within a row, 0.91 m (3 ft) between rows within the double row, and 1.83 m (6 ft) apart between double rows center-to-center. Each plant was fertilized with controlled release fertilizer 15N-3.9P-10K (Osmocote® Plus, 15-9-12, 8–9 months, ICL Specialty Fertilizers, Summerville, SC, United States) at a rate of 110 g per plant per year based on recommended medium rate. All plants were irrigated as needed through drip irrigation. Wheat straw was used between rows to aid weed control.

Plant Growth and Cold Tolerance

Each plant was measured for plant height, width 1 and width 2, where width 1 was the greatest width of an individual plant and width 2 was the perpendicular width to width 1, in February 2018 and 2019. Plant Growth Index (PGI) was calculated as the average of plant height, width 1, and width 2. Plants were evaluated for cold tolerance in February 2018 and 2019. The percentage

of foliage showing cold-damaged symptoms on each plant was recorded as described by Luo (2015). All plants were pruned to a height of 30.48 cm (12 inch) in 2018 and 50.80 cm (20 inch) in 2019 after plant growth and cold tolerance data were collected in February. Local monthly air temperature data, including average, maximum, and minimum temperatures, within the experiment duration were obtained from the USDA Natural Resources Conservation Service website (U. S. Department of Agriculture, National Resources Conservation Service [USDA-NRCS], 2019).

Leaf Characteristics

Leaf characteristics of each cultivar including individual leaf length, width, area, fresh, and dry weights were evaluated in Feb. 2018. For each cultivar, twenty most recent fully expanded leaves were collected from each replication (block) composed of 20 plants, with a total of four replications. Each leaf was measured for length and width (widest points apart). The twenty leaves from each replication were then passed through a leaf area meter (LI-3100C; LI-COR Biosciences, Lincoln, NE, United States) for the total leaf area, and an average was calculated for individual leaf area. Fresh weight of the 20 leaves from each replication was measured, and an average individual leaf fresh weight was calculated. The leaves were then oven dried at 60°C until no weight change for their average dry weight. Fresh weight of 100 new shoots in each cultivar, composed of one terminal bud and two leaves, were also measured with four replications.

Photosynthetic Activities

Plant photosynthetic activity was measured between 1,000 and 1,300 $_{HR}$ on September 20, 2018 using a portable photosynthesis system (LI-6400XT; LI-COR Biosciences, Lincoln, NE, United States). One plant from each replication was randomly selected to measure photosynthetic activities. For each plant, one most recent fully expanded leaf, not shaded by other leaves, was enclosed into a 2 cm^2 leaf chamber for the photosynthetic measurements. Photosynthetically active radiation (*PAR*) of 1500 μmol m^{-2} s^{-1} and reference CO_2 concentration of 400 μmol mol^{-1} were maintained inside the leaf chamber during measurements. Block temperature in the leaf chamber was maintained according to the air temperature on the measurement date. Net photosynthetic rate (P_n), stomatal conductance (g_s), and leaf transpiration rate (Trmmol) were measured on each selected plant.

Preparation of Tea Extract

New shoots containing one terminal bud and two leaves were harvested on 10 April, 12 July, and 18 October 2018 to represent tea harvests in spring (first flush of growth), summer, and fall. New shoots from each harvest were then oven-dried at 60°C and ground to pass a 40-mesh (0.42 mm) sieve using a Wiley mini mill (Thomas Scientific, Waltham, MA, United States). Dry shoot sample of 0.6 g were infused with 100 mL freshly boiled deionized water for 45 min. Then the supernatant was filtered through filter paper (Grade 1, GE Healthcare Bio-Sciences Corp., Marlborough, MA, United States) using a vacuum pump. After filtration, deionized water was added to the supernatant to reach a final volume of 100 mL. Three tea extracts (subsamples) were prepared for each replication and then used to test for biochemical compositions including soluble solids, carbohydrates, total polyphenols, free AAs, L-theanine and caffeine content. All biochemical compositions were presented as percentage on a dry weight basis.

Soluble Solids

Soluble solid content was measured following the protocol of Xu et al. (2018) with minor modifications. Tea extract of 50 mL was added to a weighed evaporation dish and evaporated to dryness, then oven-dried at 120°C (for approximately 2 h) to a constant weight and then cooled to room temperature in a desiccator. The residual solids were then measured to calculate soluble solid content.

Carbohydrates

Carbohydrates in tea extract were measured by modified anthrone-sulphuric acid method using dextrose as the standard as described by Fan et al. (2017). Anthrone-sulphuric acid solution (1 g L^{-1} anthrone dissolved in sulphuric acid) of 8 mL was added to 1 mL of tea extract. Then the mixture was placed in a water bath at 100°C for 7 min. After cooling to room temperature, absorbance of the solution at 620 nm was determined using a 1 cm photometer disposable cuvette and a spectrophotometer (Nicolet evolution 100, Thermo Scientific, Waltham, MA, United States). Dextrose, anthrone and sulfuric acid reagent were equal or above to ACS grade, purchased from Thermo Fisher Scientific (Waltham, MA, United States).

Caffeine

Caffeine content in tea extract was analyzed by high performance liquid chromatography (HPLC) (1260 Infinity II series; Agilent Technologies, Willington, DE, United States) according to Wang et al. (2019) with modifications. Tea extract was filtered through a 0.22 μm membrane. HPLC analyses were performed using a diode array detector (G1315C Diode-array Detector, Agilent Technologies) with an injection volume of 10 μL, flow rate of 1 mL min^{-1}, controlled oven temperature of 30°C, and a C18 column [Agilent TC-C18 (2), 4.6 mm × 250 mm, 5 μm; Agilent Technologies] plus a C18 guard column [Agilent TC-C18 (2) Grd, 4.6 mm × 12.5 mm; Agilent Technologies]. Mobile phase A consists of 0.5% acetic acid, 1% acetonitrile, and 2% methanol. Mobile phase B consists of 0.5% acetic acid, 10% acetonitrile, and 20% methanol. The elution program was 0–30 min: percentage of mobile phase A linearly decreased from 72.5 to 20.0%, percentage of mobile phase B linearly increased from 27.5 to 80.0%; 30–35 min: percentage of mobile phase A linearly increased from 20.0 to 72.5%, percentage of phase B linearly decreased from 80.0 to 27.5%; 35–40 min: 72.5% mobile phase A and 27.5% mobile phase B. Chromatograms were recorded at 280 nm. Caffeine used to develop calibration standard curves were purchased from Sigma-Aldrich (St. Louis, MO, United States). Mobile phase of HPLC grade were purchased from Thermo Fisher Scientific (Waltham, MA, United States).

Total Polyphenols

Total polyphenols in tea extract were estimated using the Folin-Ciocalteu method described by Singleton and Rossi (1965). The tea extract was diluted 25 times with deionized water. Then 2.5 mL of freshly prepared 10% (v/v) Folin & Ciocalteu's phenol reagent (Sigma-Aldrich Co., St. Louis, MO, United States) was added to 0.5 mL diluted tea extract. After 5 min of equilibration, 2 mL of 75 g L^{-1} Na_2CO_3 was added to the mixture, which was then placed at room temperature for 60 min. The absorbance of the solution at 765 nm was measured in a glass cuvette using a spectrophotometer. Folin-Ciocalteu reagent was purchased from Sigma-Aldrich Company. The calibration standard curve was obtained using gallic acid (Sigma-Aldrich Co.) as standard.

Free Amino Acids

The content of free AAs was determined by the ninhydrin dyeing method as described by Yin et al. (2009) with minor modifications using glutamic acid as standard. Ninhydrin solution (40 g L^{-1}) of 0.5 mL was added to 1.0 mL of tea extract in a test tube with cap. Then 0.5 mL of pH 8.0 buffer (95% v/v 0.067 mol L^{-1} $Na_2HPO_4{\cdot}12H_2O$ solution and 5% v/v 0.067 mol L^{-1} KH_2PO_4 solution) was added to the mixture. Test tubes with caps were then placed in a water bath at 100°C for 15 min. After cooling to room temperature, the solution was transferred to a volumetric flask and diluted to 25 mL with deionized water. Absorbance of the diluted solution at 540 nm was determined using a 1 cm photometer disposable cuvette and a spectrophotometer. Glutamic acid, ninhydrin, $Na_2HPO_4{\cdot}12H_2O$, KH_2PO_4 were equal or above to ACS grade, purchased from Thermo Fisher Scientific.

L-Theanine

L-theanine in tea extract was determined by HPLC as described by Li et al. (2019) with minor modification using L-theanine as standard. HPLC analyses were performed using the same detector and columns as mentioned in caffeine analyses. The injection volume was 20 μL of 0.22 μm membrane filtered tea extract, flow rate was 2 mL min^{-1}, controlled oven temperature was set at 27°C. Mobile phase A was 100% water and mobile phase B was 100% acetonitrile. The elution program was 0–7 min: 100% A; 7–9 min: percentage of mobile phase B linearly increased from 0 to 60%; 9–15 min: percentage of mobile phase B linearly decreased from 60 to 0%; 15–20 min: 100% A. Chromatograms were recorded at 210 nm. L-theanine used to develop calibration standard curves were purchased from Sigma-Aldrich (St. Louis, MO, United States).

Principal Components Analyses (PCA)

A PCA was conducted to investigate the relationship among nine tested cultivars using six biochemical descriptors including contents of soluble solids, carbohydrates, caffeine, total polyphenol, free AAs, L-theanine and the TP/AA ratios. Tea leaf samples used in the PCA were collected from each cultivar and from three harvesting seasons including spring, summer, and fall of 2018 as mentioned above. The PCA was conducted using software XLSTAT (version 2019; Addinsoft United States, New York, NY, United States).

Experimental Design and Data Analyses

The experiment was conducted in a completely randomized block design with four replications and cultivar as the experimental factor. There were 20 single-plant subsamples for each cultivar within each replication. The number of subsamples used for each variable varied and was specified in each methodology section. Significance of the cultivar effect was determined by the analysis of variance (ANOVA) using the PROC GLM procedure of SAS (version 9.4; SAS Institute, Cary, NC, United States). A logarithmic transformation was made on cold damage data (%) to meet the assumption of normality. Means were separated by Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. All statistical analyses were performed using SAS.

RESULTS

Local Air Temperatures

Within the experiment duration from January 2017 to February 2019, average monthly air temperature ranged from 3.9°C in January 2018 to 27.3°C in July 2017 (**Figure 1** and **Supplementary Figure S1**). Maximum monthly air temperature ranged from 20.6°C in January 2019 to 35.6°C in July 2017. Minimum monthly air temperature ranged from −13.9°C in January 2018 to 19.8°C in July 2018. The lowest air temperature in this study was −13.9°C, and occurred in January 2018 in which the winter was colder than normal in this region. The highest air temperature was 35.6°C and occurred in July 2017.

Plant Growth Indices

Tea cultivars varied in size with plant growth indices ranging from 38.22 to 59.34 cm in February 2018 (**Table 1**, **Supplementary Figure S1**). 'BL2' had the highest PGI of 59.34 cm. The second highest PGIs were in 'Black Sea,' 'Christine's Choice,' and 'Large Leaf' ranging from 50.44 to 53.36 cm. 'BL1,' 'Small Leaf,' and 'var. *assamica*' had the lowest PGIs ranging from 38.22 to 41.25 cm. In February 2019, the trend of PGI among cultivars was similar to 2018, ranging from

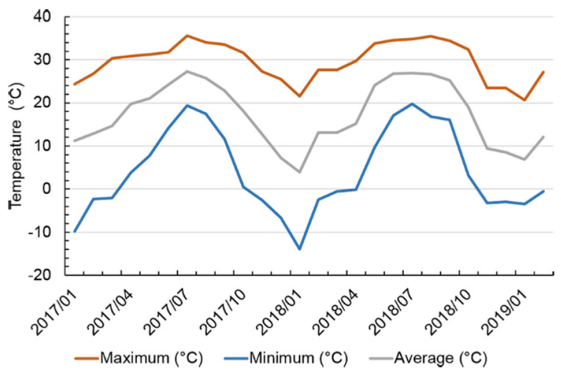

FIGURE 1 | Maximum, minimum, and average air temperatures on a monthly basis in Starkville, MS, United States from January 2017 to February 2019. Air temperature data were obtained from the website of USDA Natural Resources Conservation Service (https://wcc.sc.egov.usda.gov/reportGenerator/).

63.75 to 104.53 cm. 'BL2' had the highest PGI of 104.5 cm, and 'BL1,' 'Small Leaf,' and 'var. *assamica*' had the lowest PGIs of 69.33, 63.75, and 74.62 cm, respectively. 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' and 'Large Leaf' had comparable PGIs, but lower than 'BL2,' ranging from 83.97 to 92.92 cm. From February 2018 to February 2019, PGIs of all cultivars increased by 62.0–83.6%.

Cold Tolerance

Measured in February 2018, 'Christine's Choice' showed the highest percentage of cold damage, with an average of 34.67% foliage on an individual plant showing symptoms (**Table 2**). 'Dave's Fave' showed the second highest percentage of cold damage of 18.2% foliage per plant. 'BL2,' 'Small Leaf,' and 'Sochi' had the least cold damage with average cold-damaged foliage of 2.08–3.85%. In February 2019, cold damage on tea plants was not as severe compared to February 2018 due to a generally mild winter. 'Dave's Fave' and 'Small Leaf' had the highest percentage of cold-damaged foliage ranging from 9.83 to 9.90%. In comparison, 'BL2' and 'Large Leaf' showed the least cold-damaged foliage at 1.90 and 1.94%, respectively.

Leaf and Shoot Characteristics

The nine tested cultivars had varying leaf shapes and sizes (**Table 3**). 'Large Leaf' had the largest leaf length of 9.61 cm per leaf. Lower than 'Large Leaf,' 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' 'Sochi,' and 'var. *assamica*' had similar leaf lengths ranging from 8.04 to 8.73 cm per leaf. 'BL1' and 'Small Leaf' had the lowest leaf lengths averaging less than 8 cm per leaf. In terms of leaf width, 'Dave's Fave' and 'Large Leaf' had the widest leaves and were wider than the other seven cultivars. The smallest leaf width was in 'BL1' averaging 2.99 cm per leaf. The other six cultivars had leaf widths ranging from 3.40 cm in 'Small Leaf' to 4.12 cm in 'Christine's Choice.'

TABLE 1 | Plant growth index (PGI) of nine tea cultivars grown in Mississippi, United States.

	Plant growth index (cm)	
Cultivar	**February 2018**	**February 2019**
BL1	38.22 ± 1.19 e	69.33 ± 3.19 ef
BL2	59.34 ± 1.08 a	104.53 ± 1.22 a
Black Sea	53.36 ± 0.9 b	88.97 ± 1.46 b
Christine's Choice	50.44 ± 0.71 b	83.97 ± 4.18 bc
Dave's Fave	46.19 ± 0.65 c	84.79 ± 1.09 bc
Large Leaf	51.92 ± 0.76 b	92.92 ± 0.99 b
Small Leaf	39.35 ± 0.84 e	63.75 ± 1.46 f
Sochi	45.00 ± 0.86 cd	79.81 ± 1.77 cd
var. *assamica*	41.25 ± 1.29 de	74.62 ± 3.42 de
P-value	<0.0001	<0.0001

One-year-old tea plants were transplanted into the field in spring 2017. Plant growth index was calculated as the average of plant height, width 1 (widest points apart), and width 2 (perpendicular to width 1) and measured in February 2018 and February 2019. Different lower-case letters within a column suggest significant difference among cultivars indicated by Tukey's HSD test at $P \leq 0.05$.

TABLE 2 | Cold damage of nine tea cultivars in February 2018 and February 2019 grown in Mississippi, United States.

	Cold damage (% foliage per plant)	
Cultivar	**February 2018**	**February 2019**
BL1	6.11 ± 1.34 def	5.64 ± 0.66 bc
BL2	3.25 ± 0.36 fg	1.94 ± 0.25 d
Black Sea	7.51 ± 1.24 cde	6.73 ± 0.54 ab
Christine's Choice	34.67 ± 2.08 a	4.79 ± 0.57 c
Dave's Fave	18.15 ± 2.18 b	9.83 ± 1.63 a
Large Leaf	7.32 ± 1.13 c	1.90 ± 0.18 d
Small Leaf	3.85 ± 0.55 efg	9.90 ± 1.10 a
Sochi	2.08 ± 0.23 g	5.03 ± 0.67 c
var. *assamica*	13.71 ± 3.21 cd	7.40 ± 1.28 bc
P-value	< 0.0001	<0.0001

Cold damage on each plant was evaluated as the percentage of foliage showing cold-damaged symptoms and became brown in color. Different lower case letters within a column suggest significant difference among cultivars indicated by Tukey's HSD test at $P < 0.05$.

Single leaf areas of the nine cultivars ranged from 13.11 cm^2 in 'BL1' to 29.43 cm^2 per leaf in 'Large Leaf' (**Table 3**). 'BL2,' 'Christine's Choice,' 'Dave's Fave,' 'Sochi,' and 'var. *assamica*' had comparable single leaf areas ranging from 19.66 to 26.07 cm^2 per leaf. 'BL1' had the smallest single leaf area, lower than any of the other eight cultivars.

Individual leaf fresh or dry weight shared a similar trend with leaf area (**Table 3**). 'Large Leaf' and 'Dave's Fave' had the greatest leaf weight, fresh (1.40 g and 1.17 g per leaf, respectively) or dry (0.58 g and 0.52 g per leaf, respectively). The five cultivars 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Sochi,' and 'var. *assamica*' had comparable leaf fresh weights ranging from 0.80 to 0.99 g per leaf, and dry weights ranging from 0.37 to 0.44 g per leaf. 'BL1' and 'Small Leaf' had the lowest leaf fresh weights (0.50 g and 0.61 g per leaf, respectively) and dry weights (0.22 g and 0.28 g per leaf, respectively).

Fresh weight of 100 new shoots of the nine cultivars ranged from 28.85 g in 'Small Leaf' to 74.60 g in 'Large Leaf' (**Table 3**). 'Dave's Fave,' 'Christine's Choice' had intermediate new shoot weight of 63.4 and 48.26 g per 100 shoots, respectively. 'BL1,' 'Black Sea,' and 'Small Leaf' had the comparable lowest fresh weight of 100 new shoots.

Photosynthetic Activity Measurements

Tea cultivars did not vary significantly in net photosynthetic rate (Pn), stomatal conductance (g_s), or leaf transpiration rate (Trmmol) when measured in September 2018 (**Table 4**). Pn ranged from 5.53 $\mu mol\ m^{-2}\ s^{-1}$ in 'var. *assamica*' to 11.51 $\mu mol\ m^{-2}\ s^{-1}$ in 'BL2.' Stomatal conductance and leaf transpiration rate ranged from 0.10 to 0.19 $mol\ m^{-2}\ s^{-1}$ and 3.19 to 5.69 $mmol\ m^{-2}\ s^{-1}$, respectively, suggesting cultivars were at similar physiological and water status.

Soluble Solids

Among nine cultivars, soluble solids in tea extract ranged from 32.6 to 36.02%, 32.89 to 38.68%, and 31.87 to 37.15% on a dry

TABLE 3 | Leaf and shoot characteristics of nine tea cultivar grown in Mississippi, United States.

Cultivar	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Fresh leaf weight (g)	Dry leaf weight (g)	New shoot weight (g per 100 shoot)
BL1	7.52 ± 0.22 c	2.99 ± 0.06 e	13.11 ± 0.85 e	0.50 ± 0.06 e	0.22 ± 0.03 e	35.50 ± 1.91 ef
BL2	8.60 ± 0.13 b	3.60 ± 0.07 cd	20.74 ± 0.97 bcd	0.80 ± 0.06 cd	0.37 ± 0.03 cd	38.48 ± 1.78 de
Black Sea	8.04 ± 0.17 bc	3.57 ± 0.06 cd	19.66 ± 1.62 cd	0.80 ± 0.08 cd	0.37 ± 0.04 cd	29.23 ± 1.36 f
Christine's Choice	8.65 ± 0.15 b	4.12 ± 0.07 b	23.88 ± 1.43 bc	0.99 ± 0.08 bc	0.44 ± 0.03 bc	48.26 ± 2.21 c
Dave's Fave	8.18 ± 0.08 bc	4.76 ± 0.06 a	26.07 ± 0.83 ab	1.17 ± 0.06 ab	0.52 ± 0.02 ab	63.40 ± 2.98 b
Large Leaf	9.61 ± 0.10 a	4.80 ± 0.06 a	29.43 ± 0.69 a	1.40 ± 0.04 a	0.58 ± 0.02 a	74.60 ± 2.86 a
Small Leaf	7.56 ± 0.18 c	3.40 ± 0.08 d	16.62 ± 0.60 de	0.61 ± 0.04 de	0.28 ± 0.02 de	28.85 ± 1.67 f
Sochi	8.73 ± 0.16 b	3.72 ± 0.07 c	22.92 ± 1.35 bc	0.92 ± 0.02 bc	0.42 ± 0.01 bc	39.95 ± 1.17 de
var. *assamica*	8.06 ± 0.17 bc	3.61 ± 0.10 cd	20.85 ± 1.34 bcd	0.84 ± 0.08 cd	0.38 ± 0.04 cd	43.73 ± 1.18 cd
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Different lower case letters within a column suggest significant difference among cultivars indicated by Tukey's HSD test at $P < 0.05$.

TABLE 4 | Net photosynthetic rate, stomatal conductance, and leaf transpiration rate of nine tea cultivar grown in Mississippi, United States.

Cultivar	P_n (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	Trmmol (mmol m^{-2} s^{-1})
BL1	9.81 ± 0.55	0.19 ± 0.03	5.69 ± 0.05
BL2	11.51 ± 1.08	0.14 ± 0.04	4.35 ± 0.66
Black Sea	6.68 ± 1.14	0.14 ± 0.04	4.07 ± 0.74
Christine's Choice	6.93 ± 0.90	0.14 ± 0.02	4.19 ± 0.21
Dave's Fave	8.03 ± 2.69	0.15 ± 0.03	4.66 ± 0.54
Large Leaf	10.86 ± 1.64	0.17 ± 0.03	5.03 ± 0.15
Small Leaf	7.98 ± 0.99	0.15 ± 0.05	4.29 ± 0.85
Sochi	9.11 ± 2.89	0.17 ± 0.07	4.62 ± 1.18
var. *assamica*	5.53 ± 1.59	0.1 ± 0.04	3.19 ± 0.98
P-value	0.26	0.90	0.53

Pn, net photosynthetic rate; g_s, stomatal conductance; Trmmol, leaf transpiration rate. $P > 0.05$ indicates no significant difference among cultivars within a column by Tukey's HSD test.

weight basis in spring, summer, and fall of 2018, respectively (**Figure 2A**). In spring, there was no difference in soluble solid content among the nine cultivars. In summer, six cultivars including 'BL1,' 'BL2,' 'Christine's Choice,' 'Small Leaf,' 'Sochi,' and 'var. *assamica*' had comparable soluble solids content ranging from 36.97 to 38.84%. 'Dave's Fave' had the lowest soluble solids content of 32.89%. In fall, nine cultivars generally had similar soluble solids with 'Dave's Fave' having the lowest soluble solids of 31.87%, lower than 'BL1,' 'Christine's Choice,' 'Small Leaf,' and 'Sochi.' There was generally no difference in soluble solids content among seasons within a certain cultivar, except for 'Sochi' having higher soluble solids content in summer and fall than in spring.

Carbohydrates

Carbohydrates in nine cultivars ranged from 3.33 to 4.31%, 3.00 to 4.16%, and 4.24 to 5.99% in spring, summer, and fall, respectively (**Figure 2B**). In spring, 'BL1,' 'Black Sea,' 'Dave's Fave,' and 'Large Leaf' had comparable highest carbohydrates ranging from 3.94 to 4.31%, with' Christine's Choice' having the lowest carbohydrate content of 3.33%. In summer, 'BL1' and 'Christine's Choice' had the highest carbohydrates at 4.16 and 4.02% respectively, higher than 'BL2,' 'Black Sea,' 'Dave's Fave,' 'Large Leaf,' 'Small Leaf,' or 'Sochi.' In fall, 'Dave's Fave' and 'Small Leaf' had the highest carbohydrates at 5.99 and 5.53%, respectively. 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Large Leaf,' and 'Sochi' had comparable low carbohydrate content ranging from 4.24 to 4.76%. Within each cultivar, carbohydrates in fall were the highest among the three tested seasons. In seven cultivars including 'BL1,' 'BL2,' 'Black Sea,' 'Dave's Fave,' 'Large Leaf,' 'Small Leaf,' and 'var. *assamica*,' spring carbohydrates were higher than summer, whereas in 'Christine's Choice' higher carbohydrates was found in summer than in spring.

Caffeine

Caffeine content in nine cultivars ranged from 2.06 to 2.92%, 2.67 to 3.49%, and 1.53 to 2.46% in spring, summer, and fall, respectively (**Figure 2C**). The trend of caffeine levels among nine cultivars was generally similar in all three seasons. 'Sochi' had the highest levels of caffeine among cultivars in spring and summer. 'Dave's Fave,' 'Large Leaf,' and 'Small Leaf' generally had the lowest caffeine levels in all three seasons. All nine cultivars shared the same trend of caffeine levels across seasons, where cultivars had the highest caffeine content in summer, spring the second, with fall having the lowest caffeine content.

Total Polyphenols (TP)

Total polyphenols in nine cultivars ranged from 7.99 to 14.53%, 10.75 to 14.36%, and 5.83 to 10.55% in spring, summer, and fall, respectively (**Figure 2D**). In spring, 'Black Sea' and 'Sochi' had the highest total polyphenol content of 14.53 and 13.82%, respectively. Lower than 'Black Sea' or 'Sochi,' 'BL1,' 'BL2,' 'Christine's Choice,' and 'var. *assamica*' had comparable total polyphenol ranging from 11.72 to 11.98%. 'Dave's Fave' had the lowest total polyphenol of 7.98%. In summer, 'BL1,' 'Chiristina's Choice,' 'Small Leaf,' and 'Sochi' had comparable highest total polyphenol content ranging from 13.15 to 14.36%, whereas 'Dave's Fave' had the lowest total polyphenol of 10.75%. In fall, the highest polyphenol content was found in 'Christine's Choice' (10.11%), 'Small Leaf' (10.55%), and 'Sochi' (9.48%), and

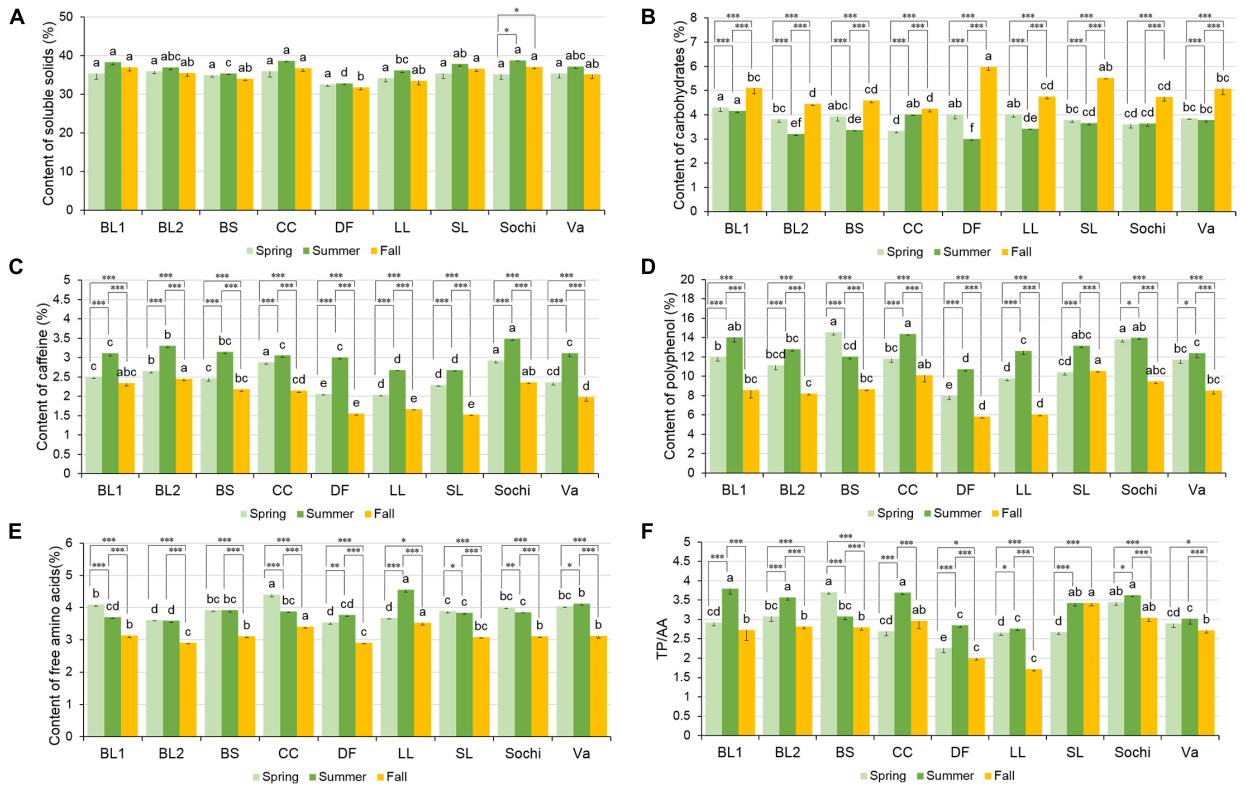

FIGURE 2 | Content of soluble solids (dry weight%) **(A)**, carbohydrates (dry weight%) **(B)**, caffeine (dry weight%) **(C)**, total polyphenols (dry weight%) **(D)**, free amino acids (AA) (dry weight%) **(E)**, and ratio between total polyphenols and free amino acids (TP/AA) **(F)** in tea extract of nine cultivars grown in Mississippi, United States. 'BS' stands for cultivar 'Black Sea,' 'CC' stands for cultivar 'Christine's Choice,' 'DF' stands for cultivar 'Dave's Fave,' 'LL' stands for cultivar 'Large Leaf,' 'SL' stands for cultivar 'Small Leaf,' 'Va' stands for cultivar 'var. *assamica*.' Different lower case letters on top of the same colored bars suggest significant difference in soluble solid content among nine cultivars within one harvesting season indicated by Tukey's HSD test at $P \leq 0.05$; * suggests significant difference within one cultivar among different harvest seasons at $P \leq 0.05$; ** suggests significant difference within one cultivar among different harvest seasons at $P \leq 0.01$; *** suggests significance different within one cultivar among different harvest seasons at $P \leq 0.0001$.

the lowest polyphenol was found in 'Dave's Fave' (5.83%) and 'Large Leaf' (6.05%). All cultivars except 'Black Sea' shared a similar trend in total polyphenols across seasons: highest total polyphenol was found in summer and lowest in fall. 'Black Sea' had the highest polyphenol of 14.53% in spring, higher than summer of 12.03%, with fall being the lowest of 8.69%.

Free Amino Acids (AA)

The content of free AAs in nine cultivars ranged from 3.54 to 4.41%, 3.59 to 4.56%, and 2.91 to 3.54% in spring, summer, and fall, respectively (**Figure 2E**). In spring, 'Christine's Choice' had the highest total free AAs at 4.41%, with 'BL2,' 'Dave's Fave,' and 'Large Leaf' having the lowest total AAs ranging from 3.54 to 3.67%. In summer, 'Large Leaf' had the highest free AAs at 4.56%. In fall, 'Christine's Choice' and 'Large Leaf' had the highest total free AAs of 3.41 and 3.54%, respectively. Five cultivars, 'BL1,' 'Black Sea,' 'Small Leaf,' 'Sochi,' and 'var. *assamica*,' had comparable free AAs ranging from 3.09 to 3.14%. Cultivars varied in trends of free AAs among the three seasons. In 'BL1,' 'Christine's Choice,' 'Small Leaf,' and 'Sochi,' greatest free AAs were found in spring, summer second, with fall having the lowest AAs. In 'BL2' and 'Black Sea,' spring and summer had comparable free AAs, both higher than fall. In 'Dave's Fave,' 'Large Leaf,' and 'var. *assamica*' greatest free AAs were found in summer, spring the second, with fall being the lowest.

L-Theanine

The content of L-theanine in nine cultivars ranged from 0.28 to 0.87%, 0.33 to 0.99%, and 0.47 to 0.85% in spring, summer and fall, respectively (**Table 5**). In spring, 'Christine's Choice' had the highest L-theanine content of 0.87%, and 'Small Leaf' had the second highest L-theanine content of 0.68%, with 'Dave's Fave' having the lowest content of 0.28%. Six cultivars including 'BL1,' 'BL2,' 'Black Sea,' 'Large Leaf,' and 'Small Leaf' had comparable intermediate L-theanine content ranging from 0.36 to 0.47%. In summer, 'Large Leaf' had the highest L-theanine content of 0.99%, with 'BL1,' 'BL2,' 'Dave's Fave,' and 'Sochi' having comparable lowest content of 0.33 to 0.41%. In fall, six cultivars including 'Black Sea,' 'Christine's Choice,' 'Large Leaf,' 'Small Leaf,' 'Sochi,' and 'var. *assamica*' had comparable L-theanine content of 0.67 to 0.85%, with 'BL1' having the lowest content of 0.47%. Among seasons, L-theanine content was generally the highest in the fall among all cultivars. 'BL2,' 'Black Sea,' 'Sochi,' and 'var. *assamica*' had comparable L-theanine contents in spring and

TABLE 5 | Content of L-theanine in tea extract in three harvest seasons and nine cultivars grown in Mississippi, United States.

	L-theanine (%)		
Cultivar	**Spring**	**Summer**	**Fall**
BL1	0.47 ± 0.04 Acd	0.33 ± 0.02 Bd	0.47 ± 0.05 Ad
BL2	0.36 ± 0.03 Bde	0.33 ± 0.02 Bd	0.59 ± 0.03 Abcd
Black Sea	0.43 ± 0.04 Bd	0.51 ± 0.03 Bc	0.74 ± 0.07 Aab
Christine's Choice	0.87 ± 0.10 Aa	0.53 ± 0.04 Bbc	0.85 ± 0.05 Aa
Dave's Fave	0.28 ± 0.02 Ce	0.36 ± 0.03 Bd	0.53 ± 0.06 Acd
Large Leaf	0.42 ± 0.03 Bd	0.99 ± 0.09 Aa	0.84 ± 0.06 Aa
Small Leaf	0.68 ± 0.04 Ab	0.53 ± 0.03 Bbc	0.67 ± 0.05 Aabc
Sochi	0.44 ± 0.03 Bd	0.41 ± 0.02 Bcd	0.76 ± 0.05 Aab
var. *assamica*	0.56 ± 0.04 Bc	0.64 ± 0.04 Bb	0.77 ± 0.04 Aab
P-value	<0.0001	<0.0001	<0.0001

Different lower case letters within a column suggest significant difference in L-theanine content among nine cultivars within one harvesting season indicated by Tukey's HSD test at $P < 0.05$. Different capitalized letters within a row suggest significant different in L-theanine content among three seasons within a certain cultivar indicated by Tukey's HSD test at $P < 0.05$.

summer. 'BL1,' 'Christine's Choice,' and 'Small Leaf' had higher L-theanine content in spring than in summer, with 'Dave's Fave' and 'Large Leaf' having higher content in summer than spring.

TP to AA Ratio

The ratio between total polyphenol and free amino acids (TP/AA) among nine cultivars ranged from 2.26 to 3.71, 2.77 to 3.80, and 1.72 to 3.42 in spring, summer, and fall, respectively (**Figure 2F**). In spring, the highest ratios of TP/AA were found in 'Black Sea' and 'Sochi,' with 'Dave's Fave' having the lowest TP/AA ratio of 2.26. In summer, the five cultivars 'BL1,' 'BL2,' 'Christine's Choice,' 'Small Leaf,' and 'Sochi' had comparable high ratios of TP/AA ranging from 3.43 to 3.80. In fall, the highest ratios of TP/AA were found in 'Christine's Choice,' 'Small Leaf,' and 'Sochi,' with 'Dave's Fave' and 'Large Leaf' having the lowest ratios. Six cultivars 'BL1,' 'BL2,' 'Christina's Choice,' 'Dave's Fave,' 'Large Leaf,' and 'Sochi' had higher ratios of TP/AA ratios in summer than in spring or fall. 'Black Sea' and 'var. *assamica*' had higher TP/AA ratios in spring and summer than in fall.

Principle Component Analyses (PCA)

The first and second principle components (PC1 and PC2) in PCA accounted for 57.62 and 18.14% of the variation among the nine cultivars tested in three seasons, respectively (**Figure 3**). The positive PC1 dimension was largely correlated with contents of soluble solids, caffeine, total polyphenols, AAs, and TP/AA that had higher values in spring or summer. PC1 was negatively correlated with content of carbohydrates and L-theanine that had higher values in fall. The positive PC2 dimension was largely correlated with contents of L-theanine and AAs. PC2 was negatively correlated with content of carbohydrates, soluble solids and TP/AA. Based on the PCA results, tea leaves of nine cultivars harvested from fall were generally characterized with high content of carbohydrates. Except for 'Dave's Fave' and 'Large Leaf,' tea leaves collected from spring which were characterized with a high content of AAs. Except for 'Black Sea,' leaves of the other cultivars collected in summer were characterized with high contents of total polyphenols and caffeine.

DISCUSSION

The nine tested cultivars in this study, including 'BL1,' 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' 'Large leaf,' 'Small leaf,' 'Sochi,' and 'var. *assamica*' showed diverse results in plant growth, leaf morphology and chemical composition. Within each cultivar, leaf chemical composition also varied among harvesting seasons in spring, summer, and fall. Despite some cold damage during winter, the nine tested cultivars generally demonstrated healthy growth and satisfactory adaptation to the growing environment in Mississippi since spring 2017.

At this current stage of tea industry in the United States, availability of superior tea cultivars are of the most limiting factors. To our knowledge, 'BL1,' 'BL2,' 'Black Sea,' 'Christine's Choice,' 'Dave's Fave,' 'Large leaf,' 'Small leaf,' and 'Sochi' are *Camellia sinensis* var. *sinensis*. Var. *assamica* is *Camellia sinensis* var. *assamica*. As for sources of tested cultivars, two cultivars 'BL1' and 'BL2' were donated by Robert E. "Buddy" Lee from Transcend Nursery in Louisiana, they were grown in the nursery since mid-1970s and survived down to −13°C during the 1980s. The remaining seven cultivars were obtained from Camellia Forest Nursery in North Carolina. Through personal communication with the nursery, 'Black Sea' was collected from Batumi region of Georgia and grown in the nursery since 2015; 'Christine's Choice' and 'Dave's Fave' were collected from China and grown in the nursery since 2008; 'Sochi' was collected from Sochi region of Russia and grown in the nursery since 2008; 'Large Leaf,' 'Small Leaf,' and 'var. *assamica*' were grown in the nursery since 1970s, 1970s, and 2000 with unknown original sources, respectively. The exact genotype of tea cultivars remain unclear. Research is in progress to investigate genetic background of tested cultivars using single nucleotide polymorphisms (Yamashita et al., 2019), and will be discussed in future research.

Tea is a long-lived woody perennial plant with decades of commercial productivity (Wang, 2016). Previous studies in traditional tea producing countries focused on profitable tea plants that were over 4 years and up to 100 years in age (Han et al., 2007; Kamau et al., 2008; Chan et al., 2009; Kumar et al., 2015). Few studies have been conducted in newly transplanted tea fields. The tea plants in our field were pruned back to 30 cm in February 2018 (2 years old) and to 50 cm in February 2019 (3 years old), respectively. The increase in PGI of tea plants in our study ranged from 62.0 to 83.6% within a year, which is consistent with previous studies (Green, 1971; Kulasegaram and Kathiravetpillai, 1972; Ahmed et al., 2014). When tea plants have a high growth rate and tea canopy covers 90% of tea fields, weed control becomes less of a concern (Wang, 2016). Fast plant growth and increases in PGI are thus beneficial for newly established tea fields to suppress weed growth (Anderson, 2010). Larger plant sizes, measured by PGI, may serve as an indicator for yield, which is one of the most important consideration in

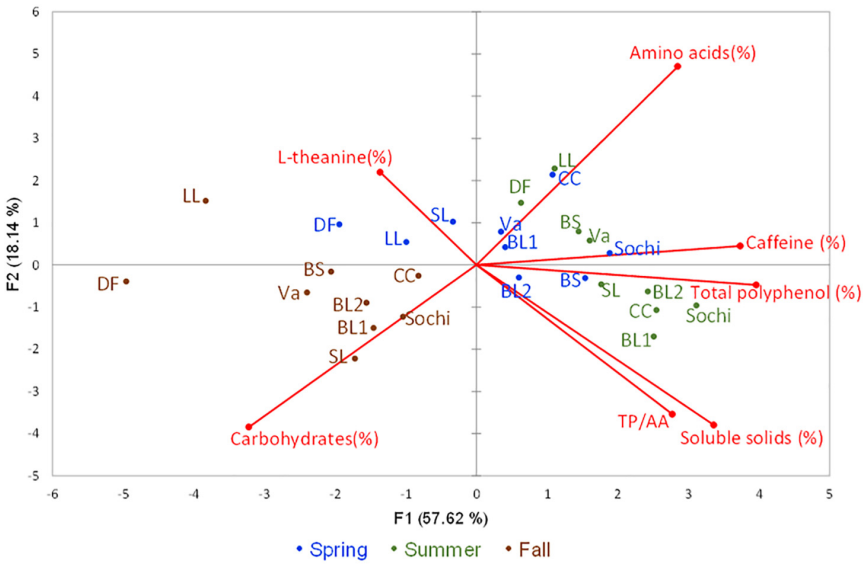

FIGURE 3 | Principle component analyses (PCA) in nine tea cultivars grown in Mississippi, United States with six descriptors. The principal components scatterplot was generated based seven descriptors including contents of soluble solids, carbohydrates, total polyphenols, caffeine, L-theanine, and the ratios between total polyphenols and free amino acids (TP/AA) using leaf samples collected from nine test tea and from three harvesting seasons from each cultivar in spring (blue color), summer (green color), and fall (brown color) of 2018. The first and second principal components accounted for 75.75% of the total variation. 'BS' stands for cultivar 'Black Sea,' 'CC' stands for cultivar 'Christine's Choice,' 'DF' stands for cultivar 'Dave's Fave,' 'LL' stands for cultivar 'Large Leaf,' 'SL' stands for cultivar 'Small Leaf,' 'Va' stands for cultivar 'var. *assamica*.'

commercial tea production. Therefore, tea plants with higher PGI may establish faster, decrease weed pressure, and potentially become profitable at a younger age. In our study, 'BL2' had the highest PGI, followed by 'Black Sea,' 'Christine's Choice,' and 'Large Leaf.'

Leaf morphology, including size and weight, are correlated with tea yield (Karthigeyan et al., 2008). Of the nine tested cultivars, 'Large Leaf' had the largest leaf length (9.61 cm), width (4.80 cm), area (29.43 cm^2), and fresh (1.40 g) and dry weights (0.58 g) on a single leaf basis and the highest 100 new shoot weight of 74.60 g. Rajkumar et al. (2010) reported *C. sinensis* var. *assamica* to be a large leaf variety with leaf length of 13.05 cm and width of 5.35 cm when grown in India. In our study, leaf size of var. *assamica* was among the smallest of the nine tested cultivars, which may have been affected by genetic background, plant age, and growing environment (Parkhurst and Loucks, 1972; Forrester et al., 2017). 'BL1' had the smallest leaf length of 7.52 cm and width of 2.99 cm, appearing to be larger than some small leaf cultivars reported from China (Karthigeyan et al., 2008). Leaf morphology also affects suitability of tea cultivars to make certain types of tea. For instance, most black tea is made from large leaf varieties (Astill et al., 2001). As plants age, leaf morphology of the tested cultivars may change.

Within this study duration, from spring 2017 to spring 2019, the lowest air temperature was reported to be −13.9°C in January 2018 (U. S. Department of Agriculture, National Resources Conservation Service [USDA-NRCS], 2019), which was approximately the historical extreme in this region. Tea plants can tolerate lowest air temperatures between −6 and −16°C, varying among varieties and different growth environments (Bi et al., 2014). With approximate 1 week of minimum air temperature below −6°C in January 2018, 'Christine's Choice' had the most severe cold damage in February 2018, with over 34% of foliage showing cold damage symptoms. However, PGIs of 'Christine's Choice' increased by 66% in spring 2019, suggesting the cultivar resumed vigorous growth after relatively severe cold damage. It was reported that cold damage on shoots may serve as a pinching agent, reducing apical dominance, as seen in *Cordyline*, and may promote production of more lateral shoots (Harris et al., 2001). In addition to the pinching effect of cold damage, all plants were pruned to a fixed height (30.48 and 50.80 cm in 2018 and 2019, respectively). Removal of apical dominance also promote lateral growth during the growing season. Compared to 2018, minimum air temperature in Jan 2019 was −3.5°C, resulting in cold damage on all cultivars below 10%. Lu et al. (2017) reported that tea cultivars varied in their critical cold temperature below which cell damage increased rapidly in tea leaves. This might explain the more severe cold damages in January 2018 compared to January 2019.

A wide range of variation in leaf chemical composition existed among the nine tested cultivars and among the three harvest seasons. This is in agreement with previous reports, where a high variability in soluble solids, total polyphenols, carbohydrates, AAs and caffeine content was found due to differences in gene expression and environmental conditions among cultivars and harvest seasons (Xu W. et al., 2012; Jayasekera et al., 2014; Fang et al., 2017). The content of soluble solids affects sensory characteristics of tea extract, including cream formation, viscosity, mouth feel and taste (Xu Y. Q. et al., 2012; Wang et al., 2019). Eight tested cultivars had similar soluble solids contents

among the three harvest seasons, except that 'Sochi' had the highest soluble solids content in summer and lowest in spring. In comparison, the nine tested cultivars showed more variations among three seasons in their contents of carbohydrates, total polyphenols, free AAs, and caffeine.

With a number of reports around the world confirming seasonal effects on content of total polyphenol and AAs (Hilton et al., 1973; Gulati and Ravindranath, 1996; Yao et al., 2005; Chen et al., 2010; Lee et al., 2010; Jayasekera et al., 2014), few studies investigated the seasonal effects on carbohydrate content in tea leaves. In this study, the PCA results clearly separated fall samples from spring or summer due to the higher carbohydrate content in each cultivar (**Figure 3**). Such results indicate seasonality significantly affects carbohydrate content in tea leaves. A previous study reported the accumulation of carbohydrates contributes to increased cold tolerance of tea plants (Yue et al., 2015), suggesting that increasing carbohydrate levels in the fall might be related to plants adaptation to decreasing temperatures.

There are three fundamental tastes in tea extract including umami, sweetness, and bitterness. AAs, especially are an important contributor to the umami taste, which is especially important for green tea quality (Narukawa et al., 2008). The TP/AA ratio is a widely-used parameter to evaluate suitability of tea leaves to make certain types of tea. A previous study reported increased TP/AA with increasing temperatures (Han et al., 2017). Most of our results agreed with this finding except for 'Black Sea'. The TP/AA ratios found in the nine tested cultivars in this study, ranging from 1.72 to 3.80 with total polyphenol content ranging from 5.83 to 14.53%, are considered suitable for green tea processing as black tea requires higher TP/AA ratios (Karori et al., 2007; Ai et al., 2011; Han et al., 2017). The low TP/AA ratios may have resulted from the young age of tea plants used in this study, having limited ability for biosynthesis of phenolics (Wang, 2013).

L-theanine is an important AAs in tea for its health benefits in reducing blood pressure, stress and anxiety, and improving memory and learning ability. L-theanine also contributes to flavor of tea infusion including caramel and umami tastes and alleviate bitterness (Yokogoshi et al., 1995; Juneja et al., 1999; Narukawa et al., 2008; Yang et al., 2013). Compared to the lowest AAs content in fall being among all seasons, L-theanine content was generally the highest in fall. This was consistent with Wang's (2013) in investigating seasonal effects on tea leaves grown in China. While green tea harvested from spring were considered most popular with optimum quality in terms of flavor and taste (Liu et al., 2015), tea harvested from fall may contain higher health beneficial compounds considering the highest contents of total polyphenols and L-theanine among three seasons in tested cultivars. Future research will be conducted to investigate changes of individual AA agent among season and over time as tea plants mature.

CONCLUSION

The nine cultivars tested in this study varied in plant size, leaf characteristics (including leaf length, width, area, fresh and dry weights, and new shoot weight), and leaf biochemical compounds including carbohydrates, TP, AA, caffeine, and L-theanine. 'BL2' showed the highest PGI of 104.53 cm by February 2019, a beneficial characteristic toward weed suppression and early establishment of tea plantation. 'Large Leaf' and 'Dave's Fave' had the largest leaves in terms of individual leaf area, fresh, dry weights, and new shoot weight. Biochemical compounds in tea cultivars differed among harvesting seasons of spring, summer, and fall. With TP/AA ratios ranging from 1.72 to 3.71, the tested nine cultivars are generally considered suitable for green tea processing in three seasons at current growth stage, which may change over time as plants mature and requires further investigation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GB, QZ, and JL designed and set up experiments. QZ and QW carried out experiments. QZ and TL analyzed experimental results. GB, RH, and TL provided essential instruments and technical guidance. TL and QZ wrote the manuscript that was revised and approved by GB, RH, QW, and JL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00280/full#supplementary-material

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Integrated Analysis of Long Non-coding RNAs (lncRNAs) and mRNAs Reveals the Regulatory Role of lncRNAs Associated With Salt Resistance in *Camellia sinensis*

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Tea plant (*Camellia sinensis*), an important economic crop, is seriously affected by various abiotic stresses, including salt stress, which severely diminishes its widespread planting. However, little is known about the roles of long non-coding RNAs (lncRNAs) in transcriptional regulation under salt stress. In this study, high-throughput sequencing of tea shoots under salt-stress and control conditions was performed. Through sequencing analysis, 16,452 unique lncRNAs were identified, including 172 differentially expressed lncRNAs (DE-lncRNAs). The results of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of their cis- and trans-target genes showed that these DE-lncRNAs play important roles in many pathways such as the galactinol synthase (GOLS), calcium signaling pathway, and interact with transcription factors (TFs) under salt stress. The data from the gene-specific antisense oligodeoxynucleotide-mediated reduction in the lncRNA *MSTRG.139242.1* and its predicted interacting gene, *TEA027212.1* (Ca^{2+}*-ATPase 13*), in tea leaves revealed that *MSTRG.139242.1* may function in the response of tea plants to high salinity. In addition, 12 lncRNAs were predicted to be target mimics of 17 known mature miRNAs, such as *miR156*, that are related to the salt-stress response in *C. sinensis*. Our results provide new insights into lncRNAs as ubiquitous regulators in response to salt stress in tea plants.

Keywords: *Camellia sinensis*, long non-coding RNA, salt stress, antisense oligodeoxynucleotide suppression, endogenous target mimic

INTRODUCTION

Tea plant [*Camellia sinensis* (L.) O. Kuntze] is an important economic crop in many countries, such as China, Japan, and India. Tea made from tea plant leaves is one of the most consumed drinks worldwide. However, tea plants are seriously affected by many abiotic stresses, such as cold, salt, and drought stresses, due to their specific environmental requirements, which severely diminish

the widespread planting of tea plants (Upadhyaya and Panda, 2013). Therefore, it is important for the tea industry to breed tea plant species that exhibit strong resistance and adaptability. Salt stress is a form of abiotic stress that severely affects the growth of tea plants and the quality of tea products (Upadhyaya and Panda, 2013). In our previous report, we performed an Illumina RNA sequencing (RNA-Seq) to compare the transcriptomes of tea plants treated with and without NaCl, and many differentially expressed mRNAs involved in signal transduction pathways, transcription factors (TFs), and other functional genes under salt stress were identified (Wan et al., 2018). Previous reports have also shown that many mRNAs and proteins in tea plants, such as *CsSnRK2* (Zhang et al., 2018), *CsAQP* (Yue et al., 2014), and *CsVQ* (Guo et al., 2018), respond to salt stress. Long non-coding RNAs (lncRNAs) are important regulatory factors that respond to various abiotic stresses in plants; however, their functions in responding to salt stress have not been studied in tea plants. Therefore, the identification of important lncRNAs that respond to salt stress in tea plants is necessary.

LncRNAs are non-coding RNAs that are longer than 200 nt and usually have low protein-coding potential (Mercer et al., 2009). According to their genomic origins, lncRNAs are broadly divided into four types: intronic lncRNAs, intergenic lncRNAs (lincRNAs), sense lncRNAs, and antisense lncRNAs (Liu et al., 2012). LncRNAs are considered "transcriptional noise" because of their poor conservation and the limited evidence for their functions (Ponjavic et al., 2007). However, increasing research has revealed that lncRNAs function in transcriptional regulation and epigenetic gene regulation, and some lncRNAs function as cis- or trans-regulators in various biological processes (Ponting et al., 2009). In mammals, many lncRNAs have been proven to be related to a wide range of diseases, especially cancers and neurodegenerative diseases (Ma et al., 2012). In recent years, a large number of lncRNAs that are important in plant growth and development (Heo and Sung, 2011; Ding et al., 2012), biotic stress responses (Cui et al., 2017), and abiotic stress responses (Swiezewski et al., 2009; Qin et al., 2017) have been identified. For example, the reduced expression of the rice lncRNA *LDMAR* can lead to male sterility under long-day conditions in rice (Ding et al., 2012). In *Arabidopsis*, the antisense lncRNA *FLORE* can regulate the circadian clock to photoperiodic flowering (Henriques et al., 2017). A nucleus-localized lncRNA, *DRIR*, can enhance drought- and salt-stress tolerance in *Arabidopsis* (Qin et al., 2017). Currently, with the development of deep-sequencing technology, genome-wide lncRNA analyses have been performed on many species, such as *Arabidopsis* (Liu et al., 2012), rice (Zhang Y.C. et al., 2014), *Zea mays* (Boerner and McGinnis, 2012), wheat (Shumayla et al., 2017), and other species (Zhang and Chen, 2013). However, in many non-model plants, studies on lncRNAs are rather limited. Recently, Varshney et al. (2019) discovered 33,400 putative lncRNAs in different tissues of tea plants, which was the first report of lncRNAs in tea plants.

In this study, to explore the early response of lncRNA under salt stress in tea plants, we reanalyzed the transcriptomes of tea plants treated with and without NaCl based on the latest tea tree genome (Wei et al., 2018). LncRNAs were identified and classified systematically. The functions of these lncRNAs were predicted, and differentially expressed lncRNAs (DE-lncRNAs) that responded to salt stress were identified. In addition, the cis-targeting and trans-targeting relationship between lncRNAs and mRNAs and the possible interactions between lncRNAs and abiotic stress-related miRNAs were found to be involved in salt stress in tea plants. Notably, in our previous report, the Ca^{2+}-transporting ATPase was shown to be involved in the salt-stress response in tea plants (Wan et al., 2018), and in this study, the differentially expressed Ca^{2+}-transporting ATPase 13 (*TEA027212.1*) was proven to be co-expressed with the lncRNA *MSTRG.139242.1*, which indicates that this lncRNA may participate in Ca^{2+} signal transduction in response to salt stress. In addition, six DE-lncRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) validation to confirm the reliability of the expression levels obtained from the RNA-Seq transcriptome. Overall, the results obtained in our study provide a valuable resource for studying lncRNAs involved in salt stress in tea plants and will enhance our understanding of the putative regulatory functions of lncRNAs in plants.

MATERIALS AND METHODS

Plant Materials and NaCl Treatments

One-year-old "Pingyangtezao" tea plant cutting seedlings with consistent growth were pre-incubated in nutrient solution under standard growth conditions (temperature: 25 ± 3°C, air relative humidity: 60–70%, photoperiod: 12 h light/12 h dark) for 2 months. These tea plants were used for stress assays under the same growth conditions. In order to analyze the early response of tea plant to high salinity and evaluate the early salt responsive genes in the tea plant leaves, salt treatment was conducted by soaking the roots in nutrient solution that contained 250 mM NaCl (salt treatment) or standard nutrient solution (control), and fresh leaf samples (first and second leaves) were randomly collected at 4 h when tea plants began to show the symptoms of salt stress and wilt slightly after treatment. Control and NaCl treatment were both repeated three times (Control1, Control2, and Control3 and Salt1, Salt2, and Salt3).The samples were immediately immersed in liquid nitrogen and stored at −80°C until RNA-Seq, qRT-PCR validation, and further analysis.

Analysis of Transcriptomic Data Based on the Tea Tree Reference Genome

In our previous report, we sequenced six tea plant samples using RNA-Seq technology. A total of 79.40 Gb of clean data was obtained, and the Q30 base percentage of each sample was greater than 92.93% (Wan et al., 2018). The identification of lncRNAs was executed according to the pipeline shown in **Figure 1A**. All RNA-Seq datasets were aligned to the reference genome of *C. sinensis* var. *sinensis* (Wei et al., 2018) using the HISAT2 system[1] (Kim et al., 2015) to reconstruct the transcriptome. After the alignment, the StringTie software[2] (Pertea et al., 2016) was

[1]http://ccb.jhu.edu/software/hisat2/index.shtml

[2]https://ccb.jhu.edu/software/stringtie/index.shtml

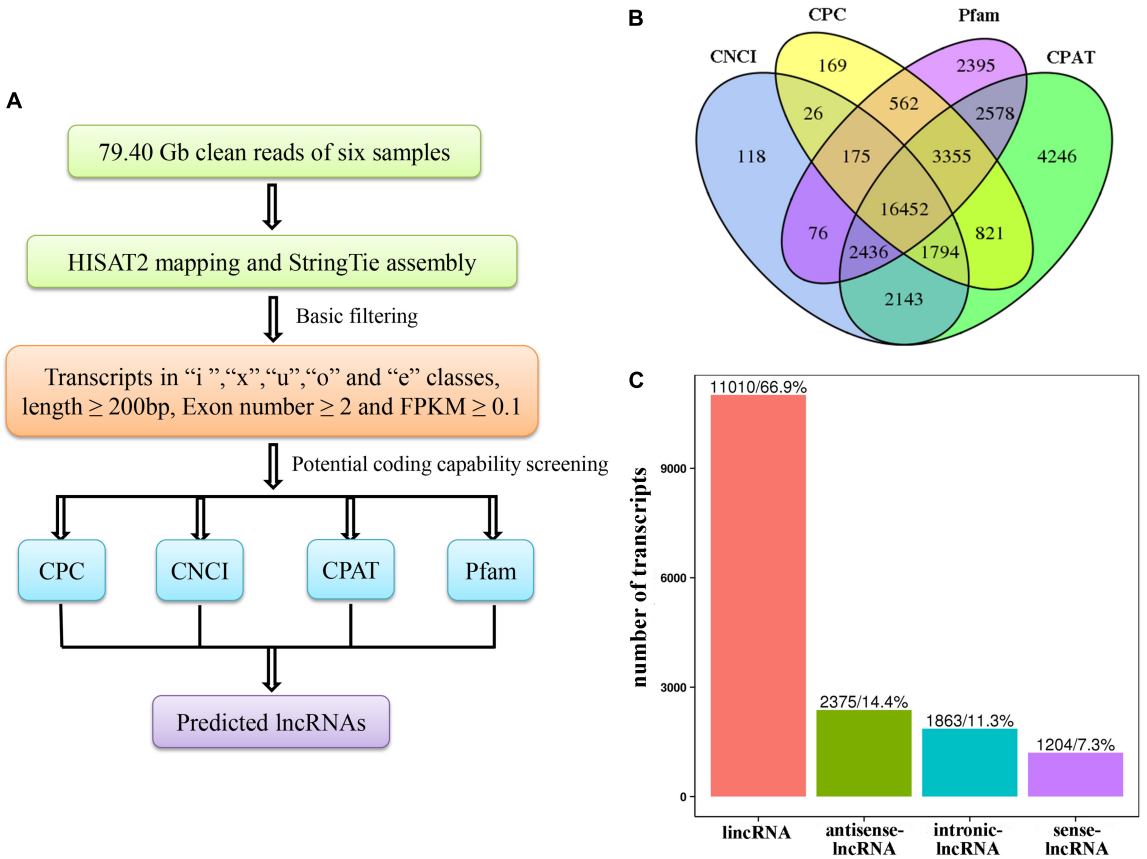

FIGURE 1 | (A) Pipeline for the identification of lncRNAs in *Camellia sinensis*. **(B)** Filtering of the candidate long non-coding RNAs (lncRNAs). Venn diagrams of coding potential analysis using four methods [Coding Potential Calculator (CPC), Coding-Non-Coding Index (CNCI), Coding Potential Assessment Tool (CPAT), and Protein family (Pfam)]. Those simultaneously shared by the four methods were predicted as candidate lncRNAs and used in subsequent analyses. **(C)** Classification of lncRNAs.

used to assemble reads into transcripts and for quantification. The assembled transcripts were annotated using the gffcompare program,[3] and the unknown transcripts were used to screen for putative lncRNAs.

Identification of LncRNAs in Tea Plants

The prediction of lncRNAs consisted of two parts: basic filtering and potential coding capability screening. For basic filtering, transcripts with a class code of "i," "x," "u," "o," and "e" (Lv et al., 2013), a length ≥ 200 bp, an exon number ≥ 2, and a fragments per kilobase of transcript per million mapped reads (FPKM) value ≥ 0.1 (Kelley and Rinn, 2012) were selected. Subsequently, four methods, including Coding Potential Calculator (CPC)[4] (Kong et al., 2007), Coding-Non-Coding Index (CNCI)[5] (Sun L. et al., 2013), Coding Potential Assessment Tool (CPAT)[6] (Wang et al., 2013), and Protein family (Pfam)[7] (Finn et al., 2014) protein domain analysis were used to evaluate the coding capabilities of the transcripts, and potential lncRNAs were filtered out by combining the four results. Next, the identified lncRNAs were classified into four categories: lincRNA, antisense-lncRNA, intronic-lncRNA, and sense-lncRNA using cuffcompare.[8] In addition, lncRNAs and mRNAs were comparatively analyzed according to their transcript length and exon number.

Prediction of Potential Target Genes of LncRNAs

The prediction of potential target genes of lncRNAs was based on the two interaction modes between lncRNAs and mRNAs. First, because lncRNA can regulate the expression of nearby genes, we searched coding genes 100 kb upstream and downstream of lncRNAs for cis-target genes. The other interaction mode of lncRNA and mRNA is due to the complementary pairing of bases. The LncTar (Li et al., 2015) tool was used for target gene prediction of lncRNAs. The free energy and standard free energy of paired sites were calculated, and the target genes with

[3]http://ccb.jhu.edu/software/stringtie/gffcompare.shtml
[4]http://cpc.cbi.pku.edu.cn/
[5]http://www.ncbi.nlm.nih.gov/pubmed/23892401
[6]http://lilab.research.bcm.edu/cpat/
[7]http://pfam.xfam.org/
[8]http://cole-trapnell-lab.github.io/cufflinks/cuffcompare/

standard free energy threshold < -0.1 were considered trans-target genes of lncRNAs.

Identification and Functional Analysis of DE-LncRNAs

The expression levels of lncRNAs were quantified using FPKM values with the StringTie software. Differential expression analysis of the NaCl and Control groups was performed using the DESeq R package (1.10.1).[9] The resulting *P*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. LncRNAs with an adjusted value of $P < 0.01$ and an absolute value of log2 (fold change) > 1 found by DESeq were considered as differentially expressed.

Functional analyses of DE-lncRNAs were performed by annotation and classification of their cis- and trans-target genes. BLASTX alignment (value of $E < 10^{-5}$) (Altschul et al., 1997) between target genes and public databases was performed, which included the Clusters of Orthologous Groups (COG) of proteins (Tatusov et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al., 2004). Gene Ontology (GO) classifications were conducted using Blast2GO and WEGO software (Ashburner et al., 2000).

Prediction of LncRNAs as Endogenous Target Mimics for miRNAs Under Salt Stress

The 172 DE-lncRNAs and 539 known conserved mature miRNAs of *C. sinensis* (Zhu and Luo, 2013; Zhang Y. et al., 2014; Jeyaraj et al., 2017a,b; Sun et al., 2018) were submitted to psRNATarget[10] using a maximum expectation of 2.0 (Wu et al., 2013; Dai et al., 2018) for miRNA–lncRNA interaction prediction. Less than three mismatches and G/U pairs were allowed within the lncRNA and miRNA pairing regions. The co-expression network was established using Cytoscape,[11] based on the DE-lncRNAs, target mRNAs of DE-lncRNAs, and miRNAs interacting with DE-lncRNAs.

LncRNA and Target mRNA Suppression in Tea Plants Using Antisense Oligonucleotides (AsODNs)

A gene-suppression assay in tea plants using antisense oligonucleotides (AsODNs) was conducted according to Liu et al. (2018). ODN sequences were selected using the Soligo software.[12] The sequences of input and selected ODNs are shown in **Supplementary Data Sheet S1: Table S1**. To increase the efficiency of gene suppression, two or three independent ODNs were synthesized of each gene and mixed in equal proportions during treatment. The ODNs were purified by HPLC, and the concentration was diluted to 10 μM. Fresh tea shoots with a bud and two leaves were excised and inserted into centrifuge tubes containing 1 ml of mixed ODNs for treatment. All leaves from the shoots were harvested after 24 h of treatment, and each treatment had three biological repeats. At the same time, a random non-sense control was used (**Supplementary Data Sheet S1**: **Table S1**). A nucleotide BLAST search of the tea tree genome CDS and lncRNA sequences with this sequence was used to verify that the sequences did not overlap with other sequences.

qRT-PCR

qRT-PCR was performed to validate the expression patterns of salt-responsive lncRNAs. Total RNA was isolated from the six tea samples (Salt1, Salt2, Salt3, Control1, Control2, and Control3) using TRNzol reagent (TIANGEN, Beijing, China). First-strand cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China), after which qRT-PCR detection was completed using the EvaGreen qPCR MasterMix-No Dye kit (ABM, Richmond, BC, Canada) on a StepOne Plus PCR instrument (ABI, United States) following the manufacturers' protocols. The tea plant *Csβ-actin* gene was chosen as the reference gene in accordance with previous methods (Wan et al., 2018), and the primers used in this assay are shown in **Supplementary Data sheet S1: Table S2**. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and the data are presented as the mean ± SD from three independent biological replicates. Group differences were tested using one-way ANOVA and Duncan's test, and significant differences among various treatment groups are represented by different letters ($P < 0.05$). The SPSS 20.0 software was used to determine significant differences between the Control and 250 mM NaCl treatment data.

RESULTS

Identification of LncRNAs in *C. sinensis*

To identify salt-responsive RNAs in *C. sinensis*, six cDNA libraries from three controls (Control1, Control2, Control3) and three salt-treated (Salt1, Salt2, Salt3) leaf samples that were treated with 250 mM NaCl for 4 h were constructed and sequenced using an Illumina HiSeq2500 platform in our previous report (Wan et al., 2018). In this report, the RNA-Seq results were aligned to the latest tea tree genome, and the alignment results are shown in **Table 1**. The mapped ratio of each sample ranged from 88.99 to 91.10%. After the data were filtered with CPC, CNCI, Pfam, and CPAT, 16,452 candidate lncRNAs were obtained from the six samples (**Figure 1B**). Then, the identified lncRNAs were classified into four categories: 11,010 lincRNAs (66.9%), 2,375 antisense-lncRNAs (14.4%), 1,863 intronic-lncRNAs (11.3%), and 1,204 sense-lncRNAs (7.3%) (**Figure 1C**).

To understand the differences between lncRNAs and mRNAs in sequence and structure, lncRNAs were analyzed and compared with mRNAs, according to the transcript length and number of exons. The average length of lncRNAs was 822 nucleotides, and the average length of mRNAs was 1,348 nucleotides. As shown in **Figure 2A**, 75.19% of lncRNAs were < 1,000 nucleotides, while only 54.28% of mRNAs were < 1,000 nucleotides. In addition, 94.44% of lncRNAs had two (83.15%) or three (11.29%) exons,

[9] http://www.bioconductor.org/packages/release/bioc/html/DESeq.html
[10] https://plantgrn.noble.org/psRNATarget/
[11] http://cytoscapeweb.cytoscape.org/
[12] http://sfold.wadsworth.org/cgi-bin/soligo.pl

TABLE 1 | Overview of the genome alignment result.

Sample	Total reads	Mapped reads	Uniq mapped reads	Multiple mapped reads	Reads map to " + "	Reads map to "–"
Control1	112,129,142	101,328,235 (90.37%)	70,079,537 (62.50%)	31,248,698 (27.87%)	3,8880,996 (34.68%)	3,8760,991 (34.57%)
Control2	130,302,020	118,699,443 (91.10%)	76,309,916 (58.56%)	42,389,527 (32.53%)	43,511,636 (33.39%)	43,518,412 (33.40%)
Control3	78,517,558	71,255,097 (90.75%)	47,395,231 (60.36%)	23,859,866 (30.39%)	26,782,952 (34.11%)	26,888,454 (34.25%)
Salt1	119,801,158	106,611,134 (88.99%)	80,043,176 (66.81%)	26,567,958 (22.18%)	43,845,279 (36.60%)	43,746,111 (36.52%)
Salt2	84,340,792	75,281,525 (89.26%)	54,731,132 (64.89%)	20,550,393 (24.37%)	30,080,568 (35.67%)	29,993,645 (35.56%)
Salt3	110,082,884	98,490,021 (89.47%)	71,042,037 (64.54%)	27,447,984 (24.93%)	39,127,799 (35.54%)	39,106,854 (35.52%)

while 10.05% of mRNAs had two exons and 46.99% of mRNAs had only one exon (**Figure 2B**).

DE-lncRNAs Involved in NaCl Stress

In this study, 172 DE-lncRNAs in tea plants were identified by DESeq using the FPKM value, including 101 upregulated and 71 downregulated lncRNAs (**Figure 3** and **Supplementary Table S1**), suggesting that these lncRNAs may function in response to salt stress.

To analyze the potential functions of these lncRNAs, the predicted cis-target genes encoding genes 100 kb upstream or downstream of these DE-lncRNAs and trans-target genes predicted by complementary pairing of bases were searched and annotated. In total, 250 cis- and 421 trans-target genes of these DE-lncRNAs were predicted (**Supplementary Tables S2, S3**). Furthermore, GO and KEGG enrichment analyses were used to investigate the potential functions of the cis- and trans-target genes, respectively. As shown in **Figures 4A,B**, 116 cis-target and 181 trans-target genes were annotated in the GO

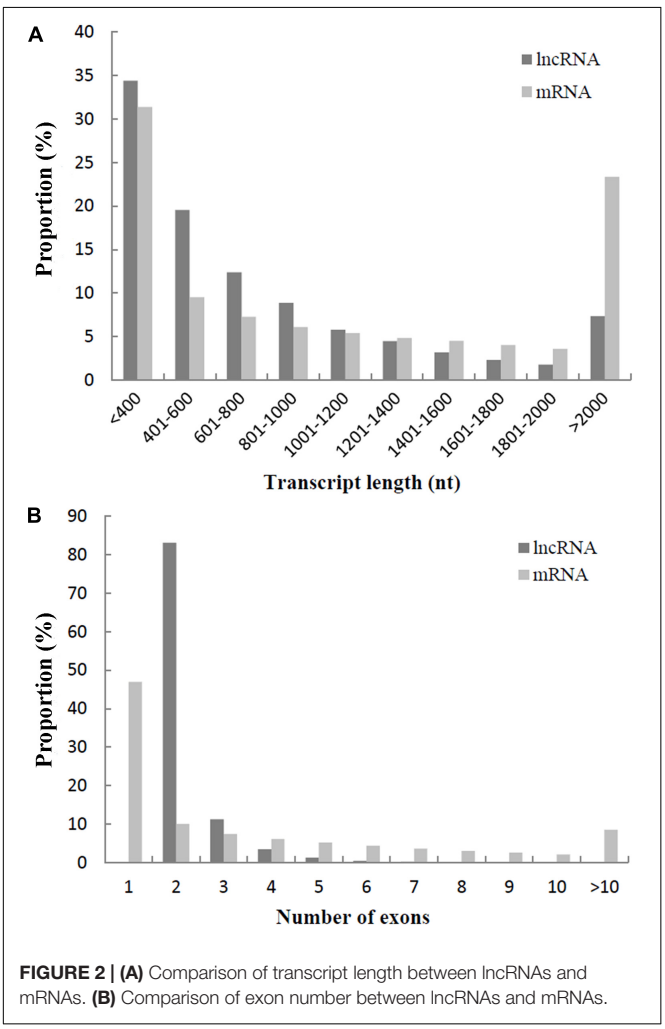

FIGURE 2 | (A) Comparison of transcript length between lncRNAs and mRNAs. **(B)** Comparison of exon number between lncRNAs and mRNAs.

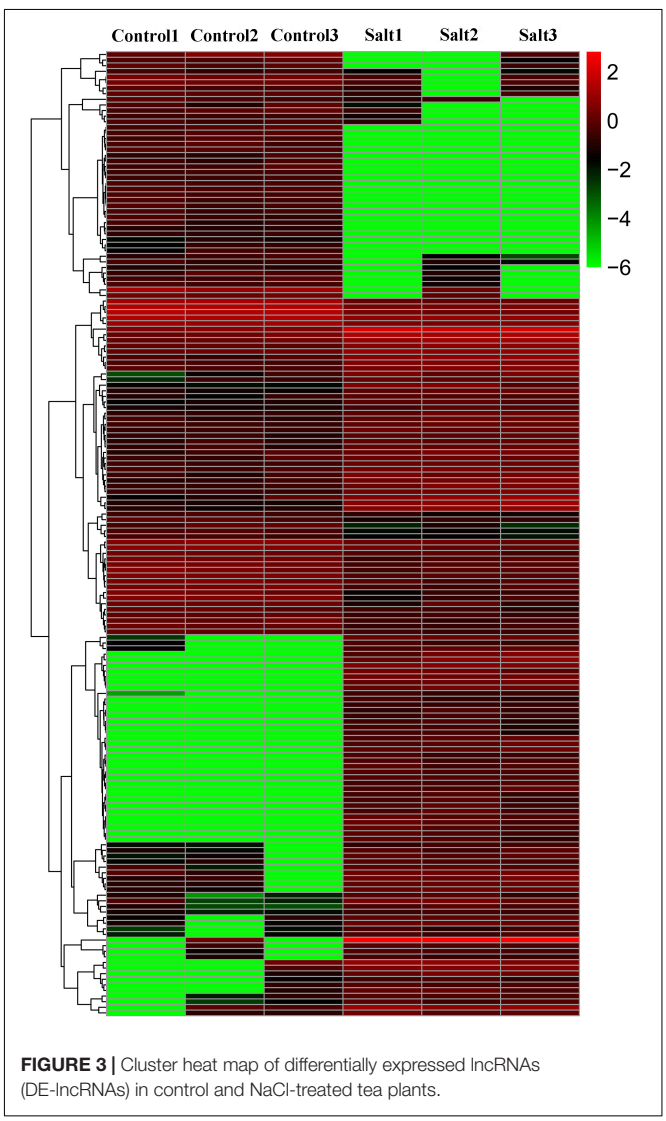

FIGURE 3 | Cluster heat map of differentially expressed lncRNAs (DE-lncRNAs) in control and NaCl-treated tea plants.

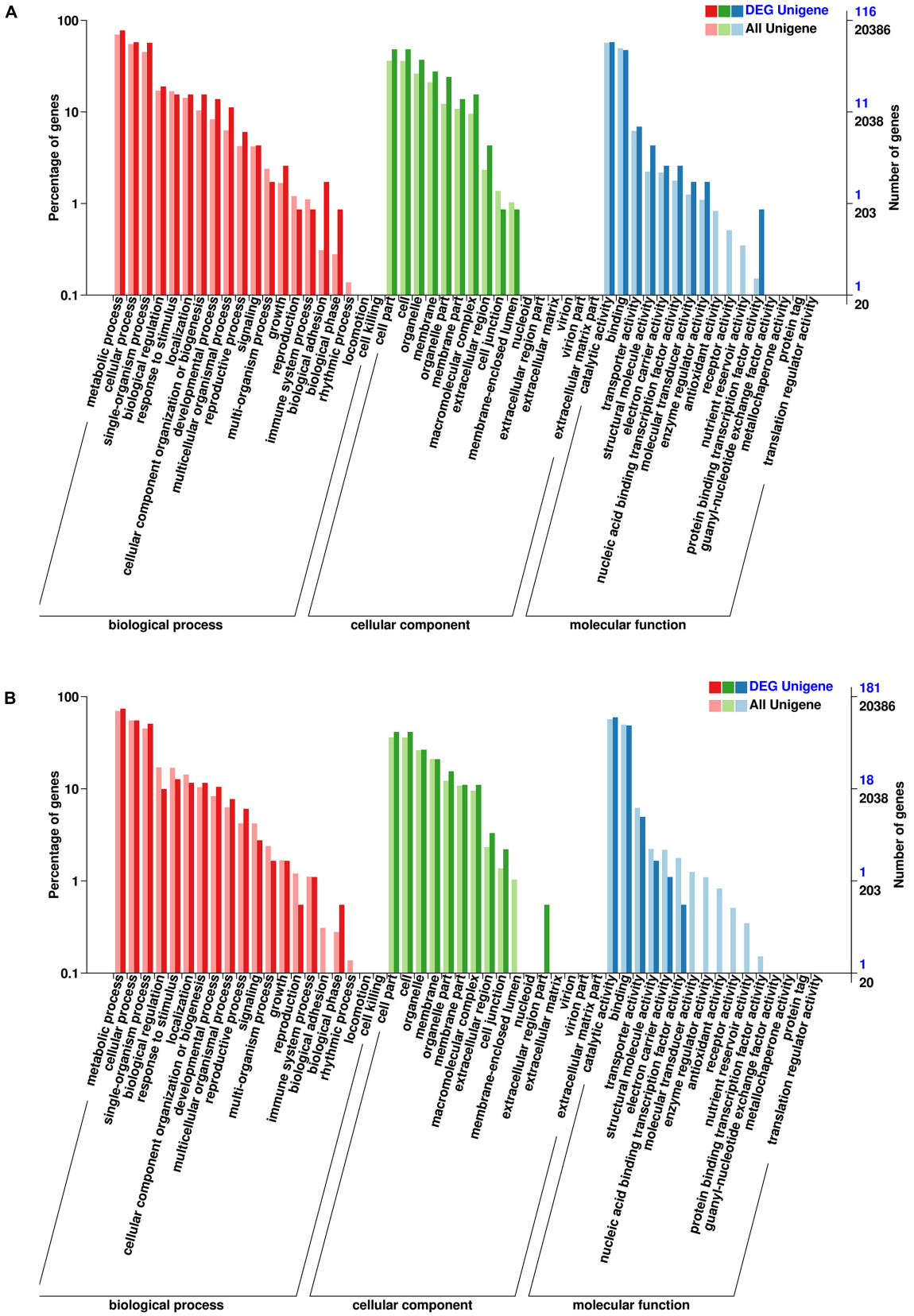

FIGURE 4 | (A) Gene Ontology (GO) enrichment of cis-target genes of the DE-lncRNAs. **(B)** GO enrichment of trans-target genes of the DE-lncRNAs.

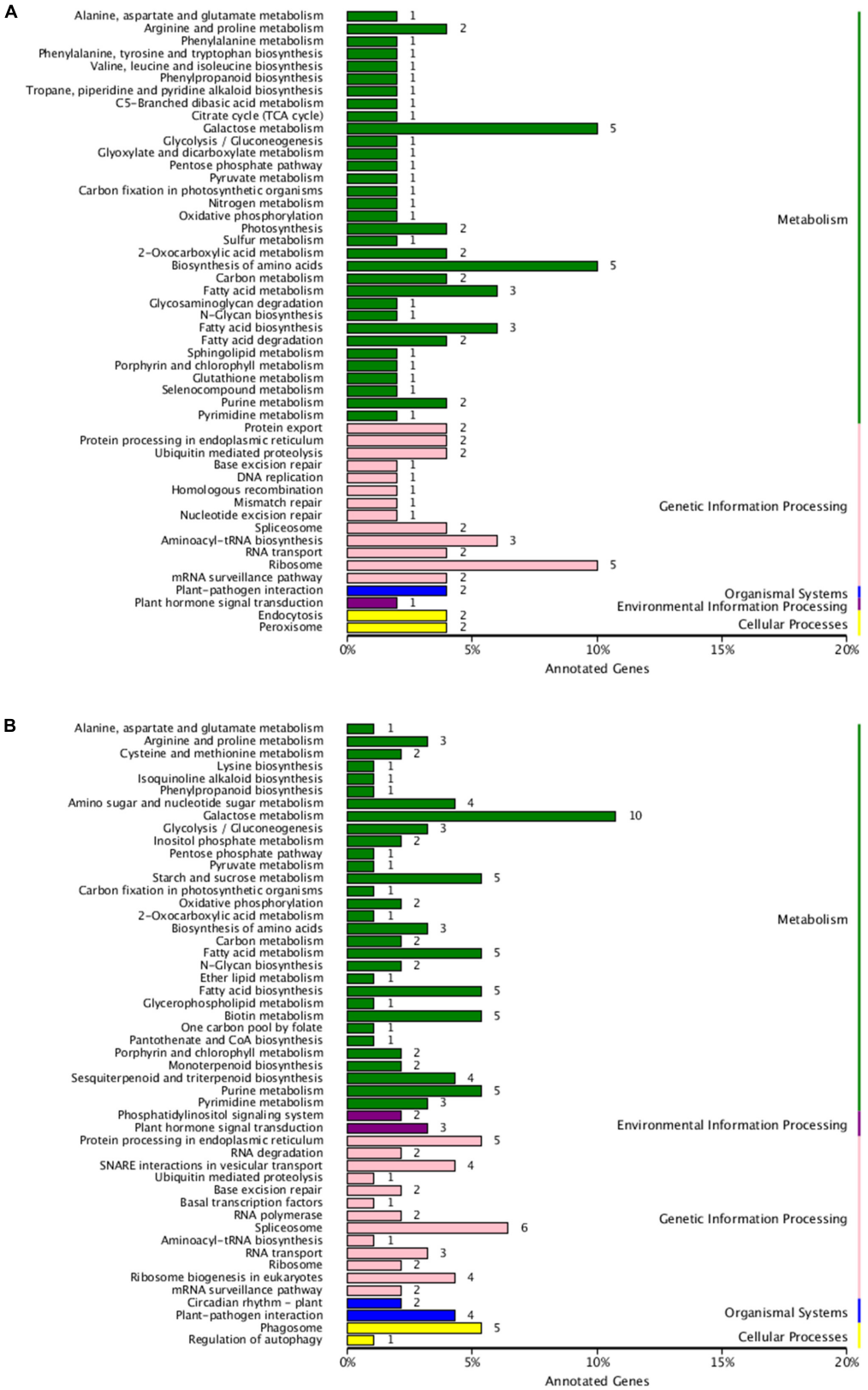

FIGURE 5 | (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of cis-target genes of the DE-lncRNAs. **(B)** KEGG enrichment of trans-target genes of the DE-lncRNAs.

database. For both cis- and trans-target genes, "Cell part" (GO: 0005623) was the largest subcategory in the cellular component category; "Catalytic activity" (GO: 0003824) in the molecular function category, and "Metabolic process" (GO: 0008152) in the biological process category were the most abundant terms. In addition, 50 cis- and 93 trans-target genes were assigned to 50 and 63 KEGG pathways, respectively (**Figures 5A,B**), which mainly included "Galactose metabolism" (ko00052), "Biosynthesis of amino acids" (ko01230), and "Ribosome" (ko03010). The results showed that these pathways may play important roles in the salt-stress response of tea plants.

Interactions of DE-lncRNAs With mRNAs

Recent studies in plants have found that lncRNAs may co-express with nearby coding genes, thereby regulating the downstream targets of the coding genes and exerting biological functions (Cui et al., 2017, 2018). Interestingly, in this study, 42 differentially expressed coding genes spaced less than 100 kb away from 35 DE-lncRNAs were identified (**Figure 6** and **Supplementary Data Sheet S1: Table S3**). These coding genes were annotated as auxin-responsive protein (*TEA005327.1*), Ca^{2+}-transporting ATPase 13 (*TEA027212.1*), vacuole membrane protein KMS1-like (*TEA033827.1*), etc. In addition, 67 differentially expressed coding genes were predicted to be trans-target genes of 23 DE-lncRNAs (**Figure 6** and **Supplementary Data Sheet S1: Table S4**). These trans-target genes were annotated as galactinol synthase (GOLS) 2 (*TEA006804.1*), WRKY TF 31 isoform X2 (*TEA005334.1*), ethylene-responsive TF ABR1 (*TEA015017.1*), MYC2 TF (*TEA000833.1*), etc. These differentially expressed mRNAs may interact with corresponding lncRNAs and participate in the salt-stress response through various pathways.

LncRNAs Participate in Galactinol Synthase, Calcium Signaling Pathway, and Interact With Transcription Factors Under Salt Stress

Synthesis of sugars and signal transduction pathways are crucial participants in regulatory networks of plant responses

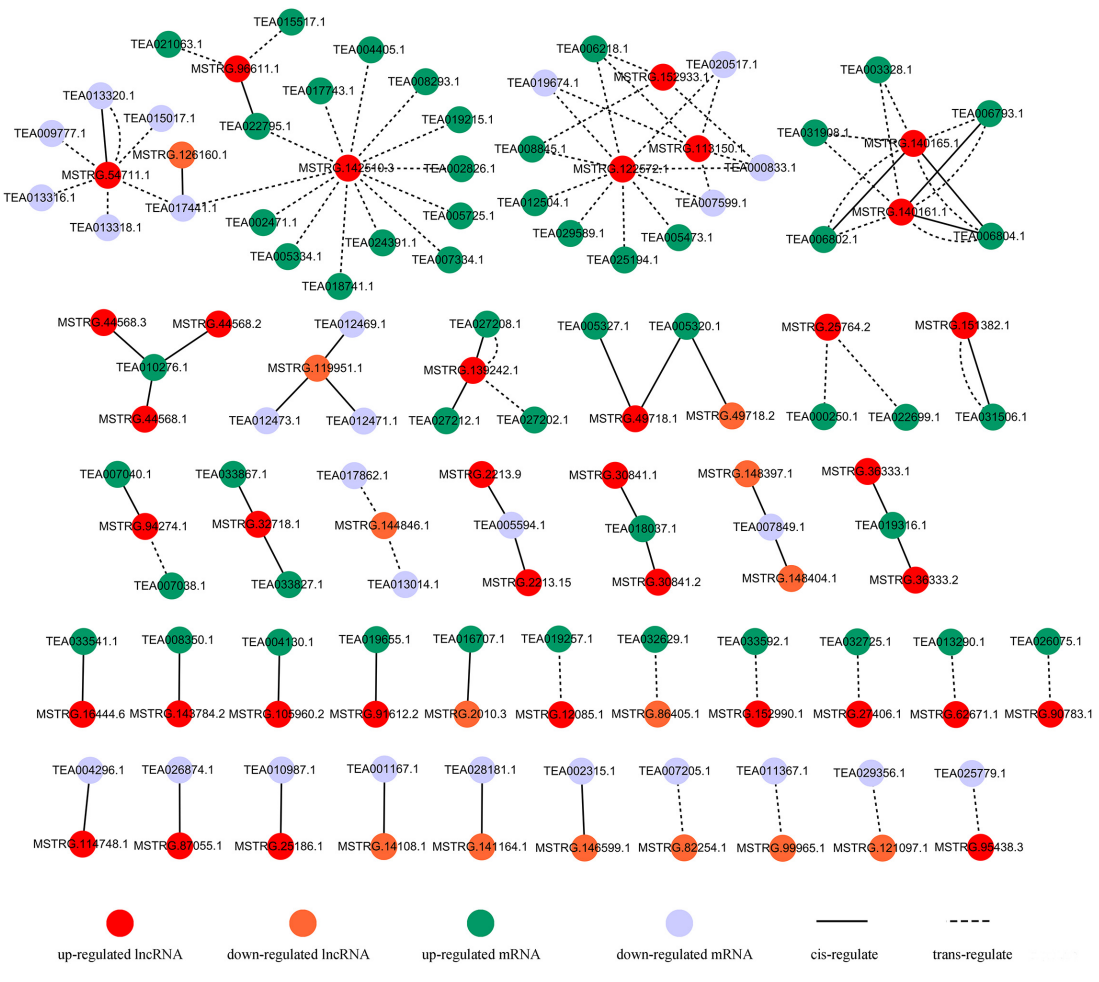

FIGURE 6 | Predicted interaction networks among lncRNAs and mRNAs; details are shown in **Supplementary Data Sheet S1: Tables S3, S4**.

to salt stress. As presented in **Table 2**, eight cis/trans target genes GOLS 2 were identified to be upregulated under salt stress. Similarly, six cis/trans target genes related to the Ca^{2+} signaling pathway were identified, including genes encoding calcium-transporting ATPase, calmodulin-interacting protein, Ca^{2+}-dependent protein kinases (CDPKs), and CBL-interacting protein kinases (CIPKs). Most of these genes were upregulated except CIPK 23 (TEA032544.1).

In addition, many TF families play vital roles in regulating plant resistance mechanisms under abiotic stress. In the present study, 23 DE-lncRNA target genes were identified, including HSF, GATA, MADS-box, bHLH, ERF, WRKY, ICE, LHY, and MYC TFs. Except all of the bHLH, LHY and MYC TFs and some of the TFs in GATA and MADS families were downregulated; the rest of the TFs were upregulated (**Table 3**).

Interactions of DE-lncRNAs With miRNAs

In total, 12 lncRNAs were predicted to be target mimics of 17 known mature miRNAs in *C. sinensis* (**Table 4**). Interestingly, four miRNAs, *lja-miR7539*, *csn-miR156h*, *ath-miR156i*, and *csn-miR156h*, were predicted to be targeted by more than one lncRNA. Similarly, five lncRNAs, *MSTRG.56302.5*, *MSTRG.116911.1*, *MSTRG.36615.10*, *MSTRG.55773.2*, and *MSTRG.92784.12*, were predicted to be targets of more than one miRNA. Thus, it can be inferred that these lncRNAs and miRNAs may form a complex regulatory network in response to salt stress.

LncRNA *MSTRG.139242.1* May Interact With Its Nearby mRNA*TEA027212.1*

Among all of the target genes of the DE-lncRNAs, we found an upregulated coding gene, *TEA027212.1* (Scaffold765 1270655–1272793), which was annotated as Ca^{2+}-transporting ATPase 13, that was located only 5,911 bp adjacent to the upregulated lncRNA *MSTRG.139242.1* (Scaffold765 1278704–1412939) according to the tea tree genome. To verify their expression relationship, a gene suppression assay in tea plants using AsODNs was conducted with *MSTRG.139242.1* and *TEA027212.1*. qRT-PCR results showed that after gene suppression of lncRNA *MSTRG.139242.1*, its expression level was not significantly reduced until 12–24 h, but *TEA027212.1*expression level was significantly reduced from 0 to 12 h (**Figure 7A**). However, gene suppression of *TEA027212.1* from 0 to 6 h significantly reduced *MSTRG.139242.1* transcript levels from 6 to 12 h (**Figure 7B**). These results indicate that the lncRNA *MSTRG.139242.1* may be regulated by its nearby coding gene, *TEA027212.1*, and may be involved in Ca^{2+} transport in response to salt stress in tea plants.

TABLE 2 | LncRNA target genes involved in galactinol synthase and calcium signaling pathway.

Target gene	Regulation	Annotation	Interaction mode
TEA006791.1	Up	Galactinol synthase 2	Cis/trans
TEA006802.1	Up	Galactinol synthase 2	Cis/trans
TEA006793.1	Up	Galactinol synthase 2	Cis/trans
TEA006811.1	Up	Galactinol synthase 2	Cis/trans
TEA006804.1	Up	Galactinol synthase 2	Cis/trans
TEA011903.1	Up	Galactinol synthase 2	Trans
TEA031908.1	Up	Galactinol synthase 2	Trans
TEA003328.1	Up	Galactinol synthase 2	Trans
TEA027212.1	Up	Calcium-transporting ATPase 13	Cis
TEA020781.1	Up	Calcium-dependent protein kinase 16	Cis
TEA027208.1	Up	Calcium-transporting ATPase 13	Cis/trans
TEA023759.1	Up	Calmodulin-interacting protein 111	Trans
TEA019257.1	Up	Calcium-dependent protein kinase 28	Trans
TEA032544.1	Down	CBL-interacting protein kinase 23	Trans

qRT-PCR Validation

To confirm the reliability of the expression levels of lncRNAs obtained from the RNA-Seq transcriptome, six DE-lncRNAs, *MSTRG.143784.2*, *MSTRG.16444.6*, *MSTRG.32718.1*, *MSTRG.139242.1*, *MSTRG.49718.1*, and *MSTRG.151316.8*, were randomly selected for qRT-PCR validation. As shown in **Figure 8**, the expression levels of these lncRNAs closely corresponded to the transcript level estimated from the sequence data, which indicates the reproducibility and accuracy of the RNA-Seq results.

Data Deposition

The sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database[13] under accession number

[13]http://www.ncbi.nlm.nih.gov/Traces/sra

TABLE 3 | LncRNA target transcription factors.

Target gene	Regulation	Interaction mode	Annotation
TEA022795.1	Up	Cis/trans	HSF
TEA009409.1	Down	Cis	GATA transcription factor 15
TEA013465.1	Down	Cis	GATA transcription factor 28
TEA006583.1	Up	Cis	MADS-box transcription factor
TEA011370.1	Down	Cis	bHLH70
TEA008966.1	Up	Cis	MADS-box transcription factor 23
TEA002032.1	Up	Cis	GATA transcription factor 24
TEA017704.1	Up	Cis	ERF118
TEA002471.1	Up	Trans	WRKY7
TEA013512.1	Up	Trans	ICE1
TEA011367.1	Down	Trans	LHY
TEA007038.1	Up	Trans	ERF4
TEA024930.1	Down	Trans	bHLH155
TEA008962.1	Down	Trans	MADS-box transcription factor 23
TEA015017.1	Up	Trans	ERF110 (ABR1)
TEA022018.1	Down	Trans	bHLH74
TEA007969.1	Up	Trans	WRKY transcription factor 31
TEA010590.1	Down	Trans	bHLH transcription factor 1
TEA010741.1	Down	Trans	MYC1
TEA005334.1	Up	Trans	WRKY transcription factor 31
TEA021401.1	Up	Trans	ERF4
TEA000833.1	Up	Trans	MYC2
TEA005142.1	Up	Trans	WRKY6

TABLE 4 | Putative targets of lncRNAs for miRNAs.

miRNA Acc.	Target Acc.	Expect	UPE	miRNA-aligned fragment	Target-aligned fragment	Inhibition
ath-miR156i	MSTRG.56302.5	1	11.063	GAGAGAGAGAGAGAGAGCAG	UUUCUCUCUCUCUCUCUCUC	Cleavage
csn-miR156h	MSTRG.116911.1	1	4.055	UGAGAGAGAGAGAGAGAGCAU	CCCCUCUCUCUCUCUCUCUCA	Cleavage
gma-miR1533c	MSTRG.118391.1	1	10.118	AAAAUAAAAAUAAUAAUAA	UUAUUUUUAUUUUUAUUUU	Cleavage
lja-miR7539	MSTRG.36615.10	1	19.202	GAGAGAGAGAGAGCGAGAGG	CCUCUCUCUCUCUCUCUCUC	Cleavage
	MSTRG.56302.5	1	10.734	GAGAGAGAGAGAGCGAGAGG	UCUCUCUCUCUCUCUCUCUC	Cleavage
	MSTRG.55773.2	1	1.11	GAGAGAGAGAGAGCGAGAGG	UCUCUCUCUCUCUCUCUCUC	Cleavage
	MSTRG.92784.12	1	6.259	GAGAGAGAGAGAGCGAGAG	CUCUCUCUCUCUCUCUCUC	Cleavage
ath-miR426	MSTRG.106114.4	1.5	5.662	AUUUGGAAAAGGAAAGAGAAAAG	UUUUUCUUUUUUCUUUUCUAAAU	Cleavage
ath-miR5021	MSTRG.26810.3	1.5	6.423	AGAGAAGAAGAAGAAGAAAA	UCUUCUUCUUCUUCUUCUUU	Cleavage
ath-miR5998b	MSTRG.143628.1	1.5	7.673	UUAGUUUUUGUUUUGUUUUGU	AAAAAACAAAACAAAAACAAA	Cleavage
csn-miR156h	MSTRG.92784.12	1.5	6.259	UGAGAGAGAGAGAGAGAGCA	CUCUCUCUCUCUCUCUCUCG	Cleavage
	MSTRG.56302.5	1.5	6.06	UGAGAGAGAGAGAGAGAGCAU	UCUCUCUCUCUCUCUCUCUCG	Cleavage
lja-miR7539	MSTRG.91236.3	1.5	7.404	GAGAGAGAGAGAGCGAGAGG	ACUCUCUCUUUCUCUCUCUC	Cleavage
ppe-miR6281	MSTRG.92784.12	1.5	6.259	AUGAGAGAGAGAGAGAGUGAG	CUCUCUCUCUCUCUCUCUCGU	Cleavage
ath-miR156i	MSTRG.36615.10	2	17.653	GAGAGAGAGAGAGAGAGCAG	GCCCUCUCUCUCUCUCUCUC	Cleavage
	MSTRG.55773.2	2	1.11	GAGAGAGAGAGAGAGAGCAG	CAUCUCUCUCUCUCUCUCUC	Cleavage
	MSTRG.116911.1	2	4.044	GAGAGAGAGAGAGAGAGCAG	CCCCUCUCUCUCUCUCUCUC	Cleavage
ath-miR5021	MSTRG.88836.3	2	5.697	AGAGAAGAAGAAGAAGAAAA	CUAUGUUCUUCUUCUUCUCU	Cleavage
csn-miR156h	MSTRG.36615.10	2	18.775	UGAGAGAGAGAGAGAGAGCAU	GCCCUCUCUCUCUCUCUCUCU	Cleavage
	MSTRG.55773.2	2	1.11	UGAGAGAGAGAGAGAGAGCAU	CAUCUCUCUCUCUCUCUCUCU	Cleavage
lja-miR7539	MSTRG.116911.1	2	4.044	GAGAGAGAGAGAGCGAGAGG	CCCCUCUCUCUCUCUCUCUC	Cleavage
mtr-miR2586a	MSTRG.123314.2	2	18.404	AGAGGUGUCCGUGCUUCAU	AUGAAGCAUGGACACAUCU	Cleavage
ppe-miR6281	MSTRG.116911.1	2	4.06	AUGAGAGAGAGAGAGAGUGAG	CCCUCUCUCUCUCUCUCUCAC	Cleavage
ptc-miR6462a	MSTRG.56302.5	2	5.5	UCUCUUUUGCAUUUUUGCUGCC	GAGAGCAAAGAUGAAGAAGAGA	Translation

SRP107589. LncRNA sequences identified are attached in **Supplementary Table S4**.

DISCUSSION

Salt stress is an important factor that affects the growth of plants, including tea plants, under natural conditions, and the mechanism of the response to salt stress and salt stress-resistance breeding have been studied for a long period. Over the past decade, a number of genes involved in salt stress have been identified and verified, in addition to their regulatory pathways that mediate the transduction of stress signals and the process of the salt-stress response, such as the Ca^{2+} signaling pathway, ABA pathway, and MAPK cascade (Boudsocq and Sheen, 2009; Cheong and Kim, 2010; Yoshida et al., 2014; Abbasi et al., 2016; Zhu, 2016). In tea plants, many coding genes, such as *AQP*, *VQ*, and *SnRK2*, participate in the salt-stress response (Yue et al., 2014; Guo et al., 2018; Zhang et al., 2018). However, a few studies have been performed on the identification and mechanism of salt stress-related non-coding RNAs, especially lncRNAs, although lncRNAs have been proven to be involved in many biological processes in plants (Ponjavic et al., 2007; Ponting et al., 2009). In this study, we systematically identified tea plant lncRNAs based on the latest tea tree genome to find lncRNAs associated with salt stress and preliminarily analyzed their possible interactions with mRNAs or miRNAs. A number of lncRNAs were identified for the first time to be specifically expressed under high concentrations of NaCl and involved in the stress response.

A total of 16,452 candidate lncRNAs from six tea leaf samples were identified in this study. Similar to the lncRNAs identified in other species (Liu et al., 2012; Shuai et al., 2014; Zhu et al., 2015; Tian et al., 2016; Ma et al., 2018), the average length of lncRNAs in tea plants was much shorter than that of mRNAs, and most lncRNAs (66.9%) were lincRNAs. In some plants, functional lncRNAs that were differentially expressed under specific conditions were identified using transcriptome sequencing, and the functions were validated. For example, Zhu et al. (2015) identified 490 lncRNAs that were significantly upregulated in tomato ripening mutants using RNA-Seq, and two novel lncRNAs, *lncRNA1459* and *lncRNA1840*, were proven to function in the delay of fruit ripening by gene silencing and mutagenesis (Li et al., 2018). Ding et al. (2014) conducted RNA-seq of salt-treated *Arabidopsis* seedlings, and Qin et al. (2017) obtained a putative lncRNA named *DRIR* that was later proven to increase tolerance to drought and salt stress. Similarly, 172 lncRNAs with various functions were found to be differentially expressed under NaCl stress in our results. Thus, these lncRNAs are involved in the salt-stress response of tea plants, and the results can provide candidate genes for salt tolerance studies in tea plants.

Previous studies have reported that lncRNAs play important roles in a variety of biological processes in response to stress by acting directly or indirectly on functional genes in plants (Wu et al., 2013; Fatica and Bozzoni, 2014; Wang et al., 2017).

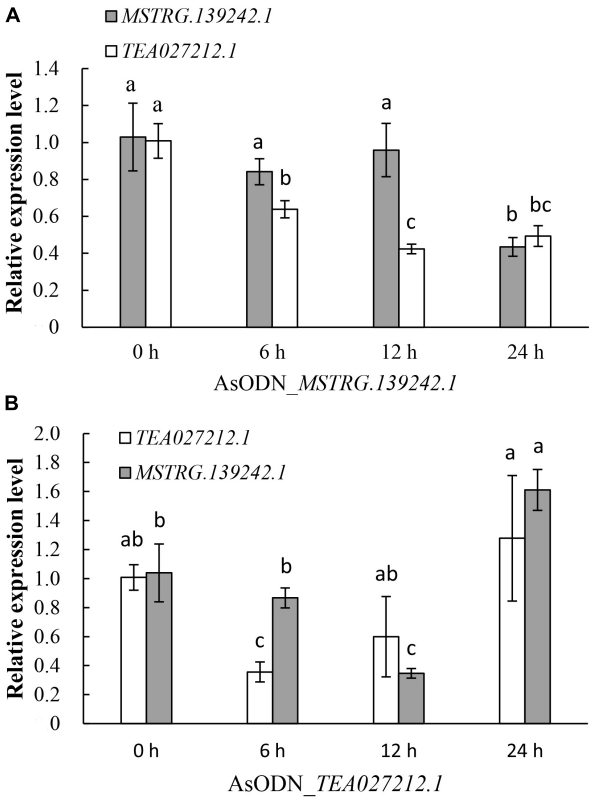

FIGURE 7 | Quantitative real-time PCR (qRT-PCR) analysis of the lncRNA *MSTRG.139242.1* and its target mRNA*TEA027212.1*, using tea leaves treated with AsODN_*MSTRG.139242.1* and AsODN_*TEA027212.1* for 0, 6, 12, and 24 h, respectively. Different letters in the columns represent a significant difference in the qRT-PCR results between the control and antisense oligodeoxynucleotide (AsODN)-treated individuals ($P < 0.05$).

Currently, the interactions between lncRNAs, mRNAs, and miRNAs are the main research hotspots. Thus, in this study, the interactions between lncRNAs and mRNAs, and between lncRNAs and miRNAs were predicted.

To the best of our knowledge, lncRNAs can negatively or positively regulate the expression of protein-coding genes by acting in cis or in trans; lncRNAs work in cis when their target genes are on the same chromosome and within a close distance, and lncRNAs work in trans when they affect genes on other chromosomes (Kornienko et al., 2013). In addition, recent studies found that some cis-acting lncRNAs also have the ability to act in trans (Martianov et al., 2007; Schmitz et al., 2010). In this study, possible cis- and trans-target genes were predicted and annotated to explore the possible functions of the DE-lncRNAs. In total, 250 cis- and 421 trans-target genes of these DE-lncRNAs were predicted. GO and KEGG analyses indicated that these lncRNAs participated in various pathways, such as catalytic activity, galactose metabolism, and biosynthesis of amino acids in response to salt stress. Notably, 42 cis- and 67 trans-target genes were differentially expressed under salt stress. Among these target genes, Ca^{2+}-transporting ATPase (*TEA027212.1*) was reported to be an important gene in Ca^{2+} signaling under abiotic stress (Wilkins et al., 2016), which suggests that its corresponding lncRNA, *MSTRG.139242.1*, may interact with Ca^{2+}-transporting ATPase and may be involved in this process. Thus, a gene suppression assay of *MSTRG.139242.1* and *TEA027212.1* (Ca^{2+}-transporting ATPase 13) using AsODNs was conducted and suggested that lncRNA *MSTRG.139242.1* may be regulated by its nearby coding gene, *TEA027212.1*. In a previous study, the tomato lncRNA *lncRNA33732* activated by *WRKY1* induces *RBOH* expression and conferred resistance to *Phytophthora infestans* infection (Cui et al., 2018). In this study, we can speculate that lncRNA *MSTRG.139242.1* may interact with its nearby mRNA TEA027212.1 in response to salt stress. In addition, an auxin-responsive protein (*TEA005327.1*) and ethylene-responsive TF ABR1 (*TEA015017.1*), the target genes of the lncRNAs *MSTRG.49718.1* and *MSTRG.54711.1*, respectively, have been reported to be involved in the auxin, ethylene, and ABA signal transduction pathways in response to abiotic stresses in other plants (Pandey et al., 2005; Jain and Khurana, 2009; Feng et al., 2015), indicating that these two lncRNAs may participate in hormone signal transduction in response to salt stress in tea plants. We speculate that the expression of these target mRNAs may be regulated by their upstream lncRNAs in response to salt stress.

In addition, it is worth noting that many lncRNA target genes were classified as GOLS, calcium signaling, and some crucial TFs. GOLS was reported to be a key enzyme in the synthesis of raffinose family oligosaccharides (RFOs) and catalyzes the condensation of UDP-galactose with myo-inositol to produce galactinol as the sole donor for the synthesis of RFOs. In addition, RFOs are used for the transport and storage of carbohydrates and as compatible solutes for protection against abiotic and biotic stresses (Peters and Keller, 2009; Philippe et al., 2010). Sun Z.B. et al. (2013) have reported that *TsGOLS2* enhances tolerance to high salinity and osmotic stresses in *Arabidopsis thaliana*. Thus, in the present study, lncRNAs targeting the eight upregulated *GOLS* genes may participate in RFO synthesis in response to salt stress. The Ca^{2+} signaling pathway was also reported to mediate plant response to salt stress, which starts with Ca^{2+} transporters such as Ca^{2+}-ATPases, Ca^{2+} sensors, and relay proteins such as CaM, CMLs, CDPKs, CBLs, and CIPKs (Wilkins et al., 2016). LncRNAs targeting calcium-transporting ATPase, calmodulin-interacting protein, CDPKs and CIPKs in this study were predicted to be involved in salt-stress response through the Ca^{2+} signaling pathway. TFs have long been an important topic in plant resistance research, and some TF families such as WRKY, bHLH, ERF, GATA, and MYC play significant roles in salt stress adaptation (Lindemose et al., 2013). Here, the results provide many candidate lncRNAs, which may interact with vital TFs and response to salt stress.

On the other hand, lncRNAs can also interact with miRNAs. Some miRNAs have been found to respond to different environmental stresses (Zhang, 2015). Some lncRNAs can bind to miRNAs, thus inhibiting the effect of miRNAs on downstream genes. This type of lncRNA is termed an endogenous target mimic (eTM) (Wu et al., 2013). Jiang et al. (2019) reported that the tomato *lncRNA23468* functions as an eTM to modulate NBS-LRR genes by mimicking miR482b in the tomato–*P. infestans* interaction. In this study, 12 lncRNAs were predicted to be target mimics of 17 known mature miRNAs.

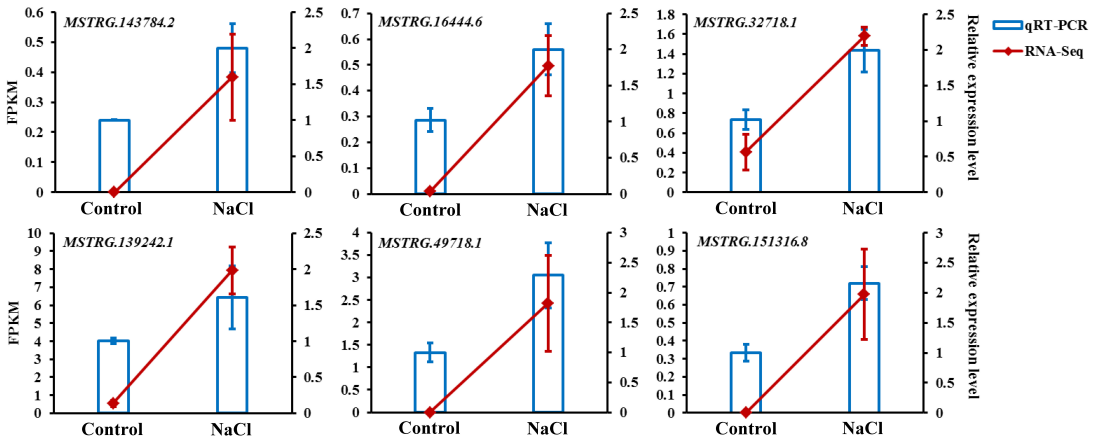

FIGURE 8 | qRT-PCR validation of six DE-lncRNAs selected from the RNA-Seq transcriptome. The expression levels of the RNA-Seq results were calculated using the FPKM value presented on the left axis; qRT-PCR relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method presented on the right axis; the values shown are the means ± standard deviation of $n = 3$ replicates.

Interestingly, five lncRNAs, *MSTRG.56302.5*, *MSTRG.116911.1*, *MSTRG.92784.12*, *MSTRG.36615.10*, and *MSTRG.55773.2*, were predicted to be eTMs of *miR156*. According to previous reports, *miR156* is induced by drought, and salinity stresses in several different plant species specifically regulates downstream *SPL* TFs and affects plant morphology, phase change, and seed germination (Stief et al., 2014). Thus, the five lncRNAs may influence the effect of *miRNA156* on *SPL* in response to salt stress. These results provide candidate lncRNAs for exploring the interactions between lncRNAs and miRNAs in response to salt stress.

CONCLUSION

In the present study, genome-wide identification of lncRNAs was performed using high-throughput RNA-Seq and bioinformatics analysis. lncRNAs (16,452) were identified in tea plants, among which, 172 lncRNAs were differentially expressed and responsive to salt stress by cis- or trans-interaction with important coding genes. These lncRNAs may participate in GOLS, calcium signaling pathway, and interact with TFs in response to salt stress. Notably, 35 DE-lncRNAs were predicted to interact with 42 differentially expressed coding genes, which may participate in pathways such as the auxin response, ABA, and Ca^{2+} signal transduction pathways under salt stress. AsODN suppression of the lncRNA *MSTRG.139242.1* and its predicted interacting gene, *TEA027212.1* (Ca^{2+}-ATPase 13), indicated that *MSTRG.139242.1* interacts with Ca^{2+}-ATPase 13 in the Ca^{2+}-transport pathway in response to high salinity in tea plants. In addition, 12 lncRNAs were predicted to be target mimics of 17 known mature miRNAs in *C. sinensis*, thereby affecting the expression of downstream functional genes. This study can provide a source of lncRNAs and benefit an in-depth understanding of the function and regulatory mechanisms in tea plant response to salt stress.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YYu and YYa designed the experiments. SW and YZ performed the experiments and data analysis. SW and YZ wrote the manuscript. WW and QX provided valuable advice on the manuscript. LH and MD revised the manuscript. All authors discussed the results and contributed to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00218/full#supplementary-material

TABLE S1 | Statistics of DE-lncRNAs.

TABLE S2 | Statistics of cis-target genes of DE-lncRNAs.

TABLE S3 | Statistics of trans-target genes of DE-lncRNAs.

TABLE S4 | LncRNA sequences identificated in this study.

DATASHEET S1 | Sequences for AsODN assay, primers used in qRT-PCR and differentially expressed target genes.

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Physiological and Defense Responses of Tea Plants to Elevated CO_2: A Review

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Rising atmospheric carbon dioxide, an important driver of climate change, has multifarious effects on crop yields and quality. Despite tremendous progress in understanding the mechanisms of plant responses to elevated CO_2, only a few studies have examined the CO_2-enrichment effects on tea plants. Tea [*Camellia sinensis* (L.)], a non-deciduous woody perennial plant, operates massive physiologic, metabolic and transcriptional reprogramming to adapt to increasing CO_2. Tea leaves elevate photosynthesis when grown at CO_2–enriched environment which is attributed to increased maximum carboxylation rate of RuBisCO and maximum rates of RuBP regeneration. Elevated CO_2-induced photosynthesis enhances the energy demand which triggers respiration. Stimulation of photosynthesis and respiration by elevated CO_2 promotes biomass production. Moreover, elevated CO_2 increases total carbon content, but it decreases total nitrogen content, leading to an increased ratio of carbon to nitrogen in tea leaves. Elevated CO_2 alters the tea quality by differentially influencing the concentrations and biosynthetic gene expression of tea polyphenols, free amino acids, catechins, theanine, and caffeine. Signaling molecules salicylic acid and nitric oxide function in a hierarchy to mediate the elevated CO_2-induced flavonoid biosynthesis in tea leaves. Despite enhanced synthesis of defense compounds, tea plant defense to some insects and pathogens is compromised under elevated CO_2. Here we review the physiological and metabolic responses of tea plants to elevated CO_2. In addition, the potential impacts of elevated CO_2 on tea yield and defense responses are discussed. We also show research gaps and critical research areas relating to elevated CO_2 and tea quality for future study.

Keywords: climate change, tea quality, elevated CO_2, secondary metabolism, catechin, theanine, caffeine, plant defense

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INTRODUCTION

Increasing atmospheric CO_2 is the most prominent driver of global warming. At present, global atmospheric CO_2 concentration is 407.65 ppm (recorded in September 2019[1]), which was only 270 ppm during the preindustrial era. Over the last 200 years, such an unparalleled increase in atmospheric CO_2 occurred due to massive human anthropogenic activities such as deforestation,

[1] http://www.esrl.noaa.gov/gmd/ccgg/trends/

fossil-based fuel combustion, rapid urbanization and industrialization (Ahuja et al., 2010). The concentration of atmospheric CO_2 is still increasing, and it will possibly reach 800 ppm by the end of the 21st century (IPCC, 2014). It is inferred that with increasing CO_2, the adversities of extreme climate events such as heatwave, drought, and frost will be increasing, which will variously affect tea yield, quality and ecosystem (Li et al., 2018, 2019a; Ahmed et al., 2019). Nonetheless, tea plantations play a significant role in CO_2 sequestration and thus tea gardens can be useful in mitigating global warming (Pramanik and Phukan, 2020).

Tea is the most popular beverage consumed across the seven continents (Macfarlane and Macfarlane, 2004), even though its cultivation is limited to Asia and Africa. Green tea is basically manufactured from the species *Camellia sinensis* (L.) Kuntze through rapid roasting of fresh leaves to avoid oxidation (Han et al., 2016). The popularity of green tea is increasing day by day, not only for its pleasant flavor but also for numerous health benefits such as anti-inflammatory, anti-cancer, anti-obesity, and anti-allergic effects on humans (Kim et al., 2009; Siamwala et al., 2013; Mancini et al., 2017). Due to the increasing demand for tea, areas belonging to tea cultivation are increasing in tea growing regions including China (Han et al., 2016, 2018). Tea is a long-living commercial beverage crop that can remain productive for a century if the gardens are well managed. The long life span of tea plants compels them to face environmental challenges years after years through physiological adaptations to changing climate (Larson, 2015; Li et al., 2017). Drivers of climate change differentially affect tea yield and quality on a spatiotemporal basis (Wijeratne et al., 2007; Han et al., 2016). Although the effects of climate change on the yield of food crops have extensively been studied, its impact on tea has received less attention. In particular, research on the effect of elevated CO_2 on tea is still in its infancy (Ahmed et al., 2019). However, recently more attention has been paid to the issue and inter-governmental initiatives have been taken to address climate change effects on tea under the umbrella of the Food and Agriculture Organization of the United Nations (Han et al., 2018). Several research papers on the effect of CO_2 on tea yield and quality were published in last 3 years (Hui et al., 2016; Li et al., 2016, 2017, 2018, 2019a; Roy et al., 2019; Pramanik and Phukan, 2020). However, a comprehensive review of the effect of elevated CO_2 on tea plants is still missing. In this review, we intend to summarize key physiological and metabolic processes associated with the tea quality in response to elevated CO_2. Besides, the potential impact of elevated CO_2 on tea yield and defense has been discussed. We also try to find out research gaps and critical research areas on the effect of elevated CO_2 on tea quality for future study.

GROWTH AND BASIC PHYSIOLOGICAL RESPONSES TO ELEVATED CO_2 IN TEA PLANTS

Evidence from a number of studies shows that elevated CO_2 improves leaf number, leaf area index, branches, shoot length, root length, and overall biomass accumulation in C_3 plants (Huang et al., 2007; Kimball, 2016). Similarly, exposure of tea plants to elevated CO_2 (800 μmol mol^{-1}) even for 24 days increases plant height, shoot dry weight and root dry weight (Li et al., 2017). Recently, Li et al. (2019a) also showed that exposure of the 1-year-old tea seedlings to 770.5 μmol mol^{-1} CO_2 concentration in open-top chambers for 60 days significantly increases biomass accumulation in terms of fresh weights of leaves (+15.04%), roots (+22%), and whole plants (+16.26%). Since the yield of tea is the sum of buds and young leaves, elevated CO_2-induced promotion in shoot biomass greatly contributes to tea yield.

In C_3 plants, such as tea, elevated CO_2 stimulates the CO_2 assimilation rate by providing sufficient substrates (i.e., CO_2) required for photosynthetic reactions (**Figure 1**). This eventually leads to the enhanced supply of energy-rich compounds, such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) under elevated CO_2. Notably, stomata as a basic channel for gas exchange plays an important role in CO_2 and O_2 exchange between plant and atmosphere. A leaf gas exchange analysis showed that exposure of tea seedlings to elevated CO_2 for 60 days increases the net photosynthetic rate (+20%) and intercellular CO_2 concentrations (+15.74%); however, it decreases the stomatal conductance (−5.52%) and transpiration rate (−9.40%) in tea leaves (Li et al., 2019a). Intriguingly, the increase in net photosynthetic rate is much higher (+87.9%) in case of short duration (24 days) CO_2-enrichment treatment, suggesting a potential photosynthetic acclimation following prolonged exposure to elevated CO_2 in tea plants (Li et al., 2017). This raises the question of how tea plants increase the photosynthetic rate under reduced stomatal conductance. In C_3 plants, the activity of ribulose-1,5-bis-phosphate (RuBP) carboxylase/oxygenase (RuBisCO) is critical for CO_2 assimilation (Eisenhut et al., 2019; Thomey et al., 2019). Elevated CO_2 increases maximum carboxylation rate of RuBisCO and maximum rates of RuBP regeneration in tea plants (Li et al., 2017), thus facilitating carboxylation over oxygenation of RuBP (Amthor, 1997; Li et al., 2013), which potentially contributes to increased CO_2 assimilation in tea leaves (Li et al., 2017). Although exposure of tea plants to elevated CO_2 (648–65 μmol mol^{-1}) for 45 days does not significantly affect photosynthetic pigment content (Hui et al., 2016), a 3-month long CO_2 enrichment (750 μmol mol^{-1}) results in 18.4, 22.0, and 20.1% increased chlorophyll *a*, chlorophyll *b*, and carotenoid concentrations in tea shoots (Jiang et al., 2005). Through respiration, a part of the carbon is consumed by leaves, buds, shoots, and roots, and the rest is released as CO_2 via stomata to the atmosphere (Huang et al., 2007; Eisenhut et al., 2019). Ultimately, respiration supplies energy in the form of ATP to plant cells. In tea plants, elevated CO_2 (800 μmol mol^{-1}) promotes total respiration rate (+28.9–53.6%), which is attributed to concurrent increases in the salicylhydroxamic acid (SHAM)-resistant respiration as well as the cyanide (CN)-resistant respiration (Li et al., 2017), suggesting that elevated CO_2 triggers both electron transport pathways, i.e., the ATP-coupling SHAM-resistant cytochrome pathway and the CN-resistant alternative pathway utilized by plant mitochondria. Notably, the alternative respiration greatly contributes to redox homeostasis by minimizing the excess

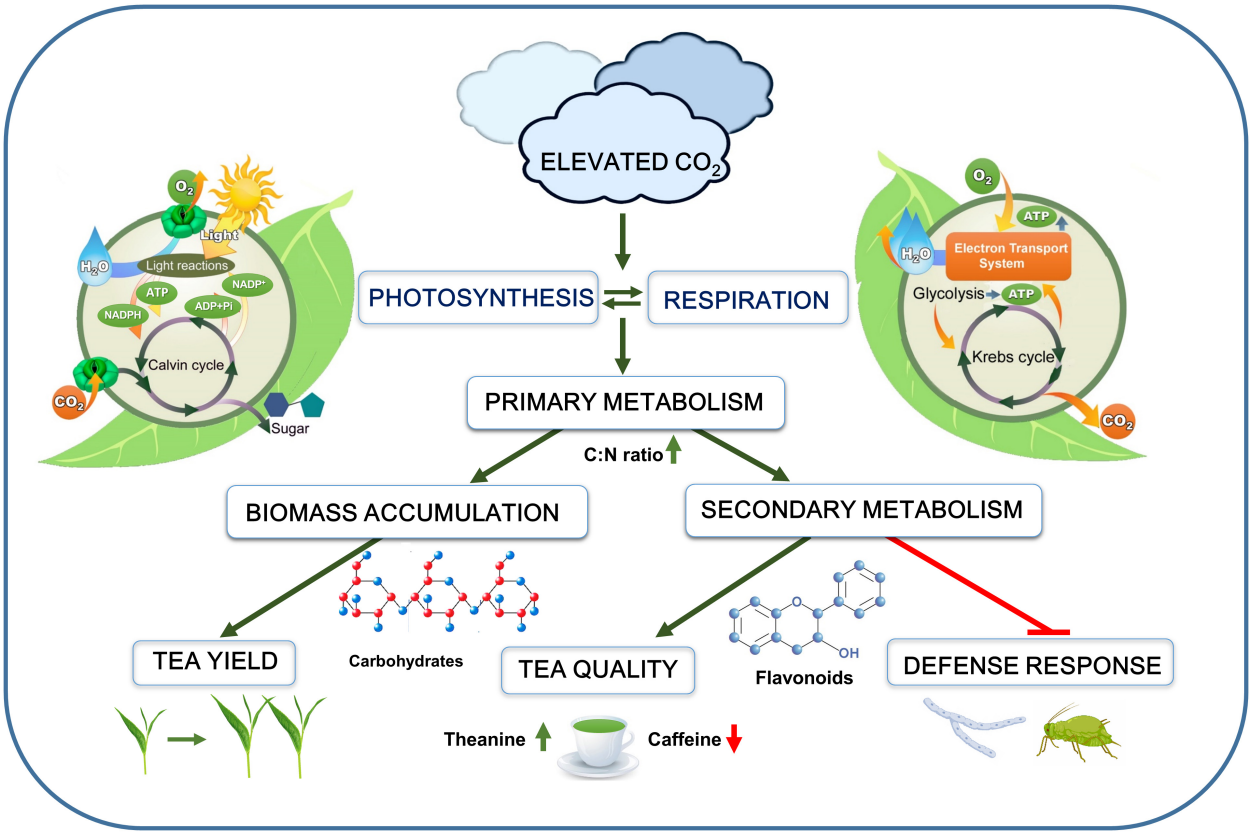

FIGURE 1 | Schematic representation of the overall effect of elevated CO_2 on tea plants. Elevated CO_2 increases photosynthesis and respiration, leading to enhanced primary metabolism. Stimulation of primary metabolism increases biomass accumulation as well as carbon-flow toward secondary metabolic pathways. Elevated CO_2 increases concentrations of carbohydrates (starch and sugars), flavonoids, theanine, and salicylic acid, but it decreases caffeine content in tea leaves. Despite enhanced production of secondary metabolites and defense compounds, tea plant defense against aphids and necrotrophic fungi is greatly compromised under elevated CO_2. The green arrows indicate promotion, while the red arrows or blunt arrow-heads indicate inhibition.

production of reactive oxygen species via the mitochondrial electron transport chain (Gong et al., 2020). It is believed that elevated CO_2-induced photosynthesis enhances energy demand which triggers respiration as well.

MECHANISMS OF ELEVATED CO_2-INDUCED CHANGES IN PRIMARY AND SECONDARY METABOLISM

Among numerous plant metabolites, only a minor portion of metabolites are directly used for plant growth and development, commonly termed as "primary metabolites"; however, numerous other metabolites, commonly termed as "secondary metabolites," are used for various functions, including plant defense against biotic and abiotic stresses (Zhao et al., 2013, 2019). When tea plants are grown under elevated CO_2, tea leaves accumulate more soluble sugar, sucrose and starch (Li et al., 2017, 2019a). However, elevated CO_2 (770.5 μmol mol^{-1}) decreases free fatty acid content with no effect on soluble protein content in tea leaves (Li et al., 2019a). In addition to the influence on nutrient compositions, elevated CO_2 affects functional components of tea leaves. For instance, a 24 days or 60 days exposure of tea plants to elevated CO_2 significantly increases the concentrations of free amino acids, theanine, and tea polyphenols, but it decreases the content of caffeine in tea leaves (Li et al., 2017, 2019a). Tea polyphenols are the major antioxidant compounds in tea leaves that provide astringency to tea infusion (Chen et al., 2018; Li et al., 2019b). Nonetheless, polyphenols have numerous health benefits, such as relieving inflammation and oxidative stress (Zhao et al., 2016; Du et al., 2018; Fei et al., 2018). Under elevated CO_2, tea polyphenols such as total catechins (major flavonoids) as well as (−)-epigallocatechin (EGC) and (−)-epigallocatechin-3-gallate (EGCG) concentrations become high (Li et al., 2017). A couple of comprehensive studies on the effect of elevated CO_2 on tea quality show that elevated CO_2 upregulates the expression of genes encoding key enzymes required for the biosynthesis of phenylpropanoids that act as precursors of different catechins. More importantly, the transcript levels of *ANTHOCYANIDIN REDUCTASE (CsANR)* remarkably upregulates (>500%) under elevated CO_2, which encodes specific enzymes that catalyze the conversion of anthocyanidins into epicatechins (Li et al., 2017, 2019a). Interestingly, elevated CO_2 also increases the concentrations of salicylic acid (SA) in tea leaves compared with

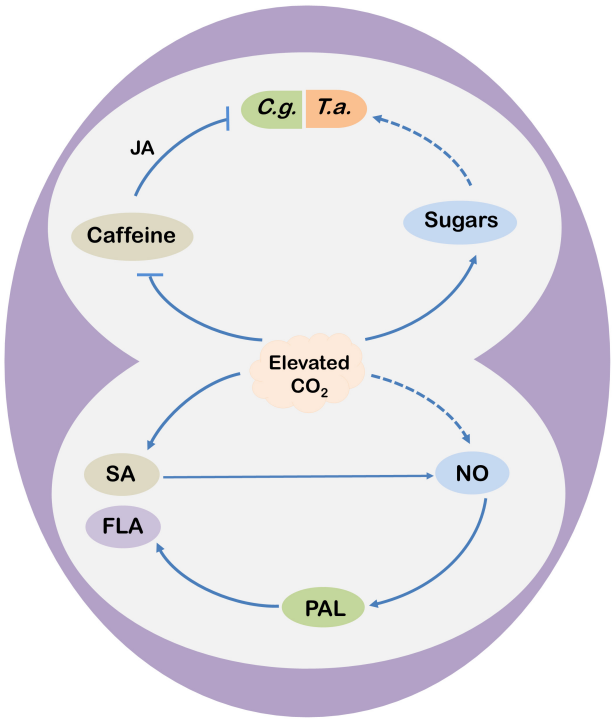

FIGURE 2 | A schematic model showing potential mechanisms of elevated CO_2-induced changes in tea functional components and defense responses. Elevated CO_2 enhances salicylic acid (SA) concentrations in tea leaves that trigger nitric oxide (NO) accumulation and subsequent flavonoid (FLA) biosynthesis by stimulating the activity of phenylalanine ammonia-lyase (PAL) under elevated CO_2 conditions. Nonetheless, elevated CO_2 may also promote FLA biosynthesis via NO in a SA-independent manner. Meanwhile, elevated CO_2 inhibits caffeine biosynthesis, leading to reduced accumulation of jasmonic acid (JA) via the lipoxygenase (LOX)-dependent pathway. Decreased JA biosynthesis compromises caffeine-induced resistance to *C. gloeosporioides* (*C. g.*, the anthracnose fungus) in tea plants. On the other hand, elevated CO_2 increases the concentrations of soluble sugars, soluble proteins, and free fatty acids in tea leaves, which might contribute to the increased population abundance of *T. aurantii* (*T. a.*; the tea aphid) under elevated CO_2. Arrows indicate promotion, while blunt arrow-heads indicate inhibition. Dotted lines are assumptions.

the ambient CO_2 (Li et al., 2017, 2019a). It has been further revealed that SA mediates elevated CO_2-induced flavonoid biosynthesis in tea leaves and SA acts downstream of CO_2 and enhances nitric oxide (NO) production to increase flavonoid biosynthesis (Li et al., 2019c). However, a SA-independent NO production pathway may also function under elevated CO_2 (**Figure 2**).

Theanine is a kind of non-protein amino acid that comprises about 50% of total amino acid content in tea leaves and it imparts the "umami" taste of tea (Vuong et al., 2011; Cheng et al., 2017). Theanine has numerous health benefits including improvement of memory, reduction of blood pressure, stress relief and stimulation of relaxation (Unno et al., 2017). Studies have revealed that elevated CO_2 increases theanine concentration in tea leaves (Li et al., 2017, 2019a). However, concentrations of some essential amino acids are differentially modulated under elevated CO_2 in tea leaves (Li et al., 2017, 2019a). There are also some discrepancies in existing literature relating to the elevated CO_2 effect on theanine content, which is largely attributed to the exposure duration in different studies. For instance, Li et al. (2017) and Li et al. (2019a) exposed tea seedlings to elevated levels of CO_2 (800 and 770 μmol mol^{-1}) for 24 and 60 days, respectively. They found a significant increase in leaf theanine concentration under elevated CO_2 conditions. However, Jiang et al. (2006) exposed tea seedlings to elevated CO_2 (750 μmol mol^{-1}) for 6 months and found a significant decrease in leaf theanine content. It is highly likely that long-term exposure to elevated CO_2 would affect total carbon and nitrogen pools, causing an increase in the C:N ratio, which might also affect the biosynthesis of nitrogenous compounds, including theanine. Likewise, Hui et al. (2016) also found a negative effect of elevated CO_2 on amino acid (including theanine) content, which eventually increased polyphenols to amino acid ratio in tea leaves.

In addition to flavonoids and theanine, caffeine accumulation also depends on environmental factors (Li et al., 2016, 2017). Studies have revealed that caffeine content declines when tea plants are grown under elevated CO_2 conditions (Li et al., 2016, 2017, 2019a). Accordingly, transcript analysis of caffeine synthetic pathway genes reveals that elevated CO_2 remarkably suppresses the expression of *INOSINE 5′-MONOPHOSPHATE DEHYDROGENASE (CsTIDH)*, *s-ADENOSYL-L-METHIONINE SYNTHASE (CssAMS)* and *TEA CAFFEINE SYNTHASE 1 (CsTCS1)* in tea leaves (Li et al., 2017).

DEFENSE RESPONSE OF TEA PLANTS AS INFLUENCED BY ELEVATED CO_2

A general perception is that elevated CO_2-induced stimulation of primary metabolism promotes secondary metabolism in plants, leading to enhanced synthesis of defense-related compounds (Noctor and Mhamdi, 2017). Although there is no discrepancy regarding the enhancement of SA levels under elevated CO_2, changes in jasmonic acid (JA) levels were found different in two studies in tea plants. A study that used 2-year-old Longjing 43 tea seedlings showed that exposure of tea plants to elevated CO_2 (800 μmol mol^{-1}) for 14 days significantly decreases JA concentrations (Li et al., 2016); however, recently Li et al. (2019a) reported that exposure of 1-year-old Longjing Changye tea seedlings to elevated CO_2 (770 μmol mol^{-1}) for 60 days significantly increases JA concentrations (+98.6%) in tea leaves (Li et al., 2019a). Despite the increased accumulation of these metabolites under elevated CO_2, plant resistance to some insects and pathogens is compromised in tea plants when grown under elevated CO_2 (Li et al., 2019a). This raises another outstanding question, how tea plants balance growth and defense while improving tea quality under high levels of atmospheric CO_2 concentrations.

Pathogens and insects invade plants in search of nutrients. While pathogens may reside inside plant cells to complete their life-cycle, insects mostly feed on plants. Therefore, palatably of host leaves matters to insect pests, which is largely dependent on the nutrient constituents of the plant organs

(Alba et al., 2014). Studies have revealed that elevated CO_2 alters nutrient constituents as well as defense compounds in leaves, leading to significant changes in insect infestation (Robinson et al., 2012; Li et al., 2019a). Thus the growth and development of herbivorous and sap-sucking insects is indirectly altered by elevated CO_2 through its direct effects on plant biomass and nutrient compositions (Kazan, 2018; Li et al., 2019a). In general, elevated CO_2 triggers population growth of sap-sucking insects such as aphids (e.g., *Myzus persicae*), whiteflies (e.g., *Bemisia tabaci*), and planthoppers (e.g., *Nilaparvata lugens*) (Jiang et al., 2016; Li et al., 2019a). However, the growth, survival rates and population density of most leaf-chewing insects are suppressed by elevated CO_2 possibly because of the potential deterioration of nutrient quality of their feeds (Coll and Hughes, 2008; Kazan, 2018). In a recent study, Li et al. (2019a) showed that the population abundance of tea aphid (*Toxoptera aurantii*) significantly increased (+4.24–41.17%) when these aphids were fed on tea seedlings grown under elevated CO_2. Although the study concludes that a reduction in caffeine content under elevated CO_2 is potentially involved in reduced defense against aphids, such a low decrease (−3.38%) in caffeine content questions this claim. Therefore, it is possible that improved leaf nutrient status under elevated CO_2, i.e., increased soluble sugars, soluble proteins and free amino acids might play a major role in increased tea aphid abundance due to the characteristics features of aphids, such as short life span, high body weight, increased fecundity and population growth under elevated CO_2. Notably, stomatal closure and subsequent minimization of transpirational water losses can result in improved leaf water status, which promotes the infestation of pea aphid (*Acyrthosiphon pisum*) in *Medicago truncatula* (Sun et al., 2015; Kazan, 2018). As elevated CO_2 also reduces the transpiration rate in tea plants by regulating stomatal conductance, the leaf water status may play a role in increased tea aphid abundance.

The role of caffeine in tea plant defense against pathogenic fungus has been reported under elevated CO_2. A reduction in caffeine content in tea leaves under elevated CO_2 conditions increases the susceptibility of the tea plants to *Colletotrichum gloeosporioides*, which causes anthracnose, brown blight and dieback disease of tea in different geographical locations depending on the weather conditions (Li et al., 2016). Interestingly, exogenous application of caffeine suppresses the necrotic lesions caused by *C. gloeosporioides*, which is attributed to the caffeine-induced elevation of endogenous JA content under elevated CO_2 conditions in tea leaves. The study also revealed that caffeine-induced increase in JA levels is attributed to JA biosynthesis through the lipoxygenase (LOX) pathway under elevated CO_2 in tea leaves (**Figure 2**). Based on this study, it is quite clear that caffeine plays a vital role in tea plant defense against necrotrophic fungal pathogens in tea plants; however, this response is compromised under elevated CO_2 due to reduced biosynthesis of caffeine and JA (Li et al., 2016). Conversely, elevated CO_2 improves the resistance of *Arabidopsis thaliana* to the fungal plant pathogen *Plectosphaerella cucumerina* and the oomycete pathogen *Hyaloperonospora arabidopsidis* by stimulating the JA-dependent and SA-dependent defense priming, respectively (Williams et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

Surveys of literature show that the effects of elevated CO_2 on tea were mostly studied by exposing the plants to a range of artificially enriched CO_2 levels (550, 650, 750, and 800) for various durations (24 days, 45 days, 60 days, and 6 months) in open-top chambers or controlled closed chambers, suggesting that studies using free air CO_2 enrichment (FACE) for long-duration are needed to better understand the realistic responses of tea plants to climate change. Notably, about a 100-year life span of tea plants allows them to witness and experience gradual changes in atmospheric CO_2 concentrations in a single generation, which possibly compels the tea plants to operate massive physiologic, metabolic and transcriptional reprogramming to adapt to changing climate. The two levels of CO_2, 550 and 800 that are frequently used for CO_2-enrichment studies, are predicted atmospheric CO_2 concentrations of the year 2050 and 2100, respectively. Thus, it is quite unusual that tea plants will experience such high levels of CO_2 overnight, which is possibly the main limitation of the existing research. Therefore, it will be more meaningful to explore how small changes in atmospheric CO_2 levels would affect tea quality.

Climate change is a cumulative effect of multiple factors, such as changes in temperatures, radiation, precipitation, and CO_2 levels. Thus it is important to study the combined effects of multiple factors along with elevated CO_2 to better mimic the real-world situations. Availability or deficiency of essential macro and micro elements and their effect on tea quality under elevated CO_2 conditions can also be considered from the point of nutraceutical value. In this regard, the role of elevated CO_2 in the regulation of γ-aminobutyric acid (GABA), a non-proteinogenic amino acid in tea with numerous health benefits, should be considered. Moreover, safety factors such as the occurrence of toxicants, mycotoxins, and contaminants in tea under elevated CO_2 remain largely unknown. In this aspect, biotic factors such as insect herbivory and pathogen infection can also be included. Notably, how the quality of so-called "bug-bitten tea," a kind of Oolong tea that is produced under special circumstances upon infestation with tea green leafhoppers (*Empoasca vitis*), is influenced by elevated CO_2, could be an interesting research topic. One factor, which has been ignored in studying elevated CO_2 effects on tea plants, is the volatile emission. Plants emit multiple volatile compounds upon insect and pathogen attacks. The volatile compounds not only impart characteristic aroma to tea as a quality parameter but also serve as defense signals and media for plant-plant communication as well as plant-insect interactions (Zhao et al., 2019). Therefore, this issue is expected to address in future studies.

When CO_2 concentration increases in the atmosphere, it not only influences plants but also other animals including insects and pests. Elevated CO_2 can influence the virulence, aggressiveness, growth, development, fecundity, fitness, and

survival of pests and pathogens by altering host physiology (Kazan, 2018). Therefore, it is highly likely that insects and pathogens may also evolve adaptive strategies in response to elevated CO_2. However, studies revealing elevated CO_2 effects on insect herbivory and pathogen infections rarely used elevated CO_2-adapted insects and pathogens. Thus, it is important to consider this issue to better understand the response of tea plants to pests and pathogens. Moreover, genotype screening in response to elevated CO_2 is necessary to develop tea cultivars resilient to climate change.

Plant β-carbonic anhydrases (βCAs) are important CO_2 sensing and metabolizing proteins that catalyze the interconversion between CO_2 and HCO_3^- (DiMario et al., 2017; Kazan, 2018). However, the role of βCAs in tea plants under elevated CO_2 remains far from being substantiated. Multiple hormones and their interaction mediates plant response to biotic and abiotic factors. Although a few studies have highlighted a role for gibberellins, abscisic acid, brassinosteroids, and SA in tea quality, the role of hormones and signaling molecules in elevated CO_2-induced changes in tea quality remains largely unknown. In addition, major transcription factors, such as, MYB, WRKY, bHLH, and WD40 that regulate the biosynthesis of terpenoids and flavonoids in other plants, should be taken into account to explore the mechanisms of elevated CO_2-induced regulation of tea quality.

In summary, existing literature suggests that elevated CO_2 promotes tea yield and quality, but it attenuates tea plant resistance to some insects and pathogens, which poses a serious threat to future tea production systems. Tea quality is a complex perception of multiple factors, which is believed to be improved under elevated CO_2 as tea plants grown under elevated CO_2 accumulate high levels of catechins and theanine, and low level of caffeine in tea leaves. It appears that decreased caffeine accumulation under elevated CO_2 is one of the main reasons of attenuated defense response in tea plants. However, there are some technical limitations in the studies relating to elevated CO_2 effects on tea plants, which should be taken into account to design more realistic experiments to better understand the responses of tea plants to elevated CO_2 in the future.

AUTHOR CONTRIBUTIONS

GA and XL conceived and designed the manuscript, and wrote the draft manuscript. GA, XL, AL, and SC reviewed and edited the manuscript. All authors have read and approved the manuscript.

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REVIEW
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Combating Climate Change in the Kenyan Tea Industry

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Climate change triggered by global warming poses a major threat to agricultural systems globally. This phenomenon is characterized by emergence of pests and diseases, extreme weather events, such as prolonged drought, high intensity rains, hailstones and frosts, which are becoming more frequent ultimately impacting negatively to agricultural production including rain-fed tea cultivation. Kenya is predominantly an agricultural based economy, with the tea sector generating about 26% of the total export earnings and about 4% gross domestic product (GDP). In the recent years, however, the country has witnessed unstable trends in tea production associated with climate driven stresses. Toward mitigation and adaptation of climate change, multiple approaches for impact assessment, intensity prediction and adaptation have been advanced in the Kenyan tea sub-sector. Further, pressure on tea breeders to release improved climate-compatible cultivars for the rapidly deteriorating environment has resulted in the adoption of a multi-targeted approach seeking to understand the complex molecular regulatory networks associated with biotic and abiotic stresses adaptation and tolerance in tea. Genetic modeling, a powerful tool that assists in breeding process, has also been adopted for selection of tea cultivars for optimal performance under varying climatic conditions. A range of physiological and biochemical responses known to counteract the effects of environmental stresses in most plants that include lowering the rates of cellular growth and net photosynthesis, stomatal closure, and the accumulation of organic solutes such as sugar alcohols, or osmolytes have been used to support breeding programs through screening of new tea cultivars suitable for changing environment. This review describes simulation models combined with high resolution climate change scenarios required to quantify the relative importance of climate change on tea production. In addition, both biodiversity and ecosystem based approaches are described as a part of an overall adaptation strategy to mitigate adverse effects of climate change on tea in Kenya and gaps highlighted for urgent investigations.

Keywords: *Camellia*, modeling, breeding, molecular, physiology

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INTRODUCTION

Human activity is driving significant changes in global and regional climate systems through enhanced greenhouse effects (Intergovernmental Panel on Climate Change, 2014). Global climate models predict that these changes will alter both mean climate parameters and the frequency and magnitude of extreme meteorological events that may include heat waves, severe storm events and

drought (Semenov and Halford, 2009). Such changes may have significant destabilizing effects, decoupling existing relationships between species, altering species distributions and challenging current management regimes. Understanding and predicting the impacts of climate change on agricultural ecosystem processes is thus critical.

Tea, *Camellia sinensis* (L.) O Kuntze, originated from areas of monsoon climates with a warm, wet summer and a cool, dry (or less wet) winter. However, with dispersal the plant is now grown in conditions which range from Mediterranean-type climates to the hot humid tropics (Carr, 1972). It is an economically important crop, extensively consumed as non-alcoholic beverage across the globe. It is profoundly known for its taste, flavor, aroma and medicinal properties attributed to rich beneficial secondary metabolites (Jin et al., 2018). As a perennial plant, tea encounters a large number of environmental stresses throughout its life span. The minimum annual rainfall generally considered sufficient for the successful cultivation of tea varies between 1150 and 1400 mm per year (Carr, 1972). In most tea growing areas, well distributed rainfall (150 mm per month) ensures continuous crop production. A positive correlation between the integrated measure of air temperature and the rate of shoot extension has been reported. Minimum air temperature required to support shoot growth is about 13–14°C, with optimum range of 18–30°C. Excessive daytime maximum temperature beyond 30°C is known to restrict shoot growth rate, whereas freezing night temperature followed by a rapid rise in day temperature (night frost) leads to leaf scorching (Eden, 1965). Relatively higher day temperatures as compared to night temperature, leaf temperature below 35°C and soil temperature between 20 and 25°C are considered optimum for tea growth (Carr, 2010a,b). Also long photoperiods are essential for maximum yield (Barua, 1969). Studies across the tea growing regions have revealed that the weather is becoming more erratic and less predictable: more hot days, reduced number of rainy days and discernible decline in the annual hours of sunshine (Esham and Garforth, 2013; Bore and Nyabundi, 2016; Han et al., 2016; Ochieng et al., 2016; Papalexiou et al., 2018).

Kenya is predominantly an agricultural based economy. Tea was reportedly introduced in the country by the Caine brothers who imported dark-leafed "Manipuri" hybrid seeds from Assam in 1904 and 1905 to establish a plantation at Limuru, Central Kenya (Matheson, 1950). In 1912, Chinary (var. *sinensis*) seeds were imported from Sri-Lanka to establish a plantation of tea with high quality and yield (Matheson, 1950). Planting expanded rapidly from 1924 following advice on the use of quality seeds from the light colored leaf Assam or Manipuri types for drought resistance (Greenway, 1945). In the year 2018, Kenya produced 493 Million Kg earning the country over Kshs. 140 Billion in foreign exchange. This represents about 26% of the total export earnings, and about 4% gross domestic product (GDP) (Wachira, 2002; Kamunya et al., 2012; Azapagic et al., 2016). The country has more than 232,742 hectares of tea (International Tea Committee, 2018) spread in 18 counties and due to the low level of mechanization involved in cultivation, it offers direct and/or indirect employment to over 10% of the population. Further, because the industry is largely rural based, it contributes to both the local rural economies and reduces rural-urban migration (Wachira, 2002). Sustainability of the industry is thus crucial to the country's socio-economic well-being and development. Being a rainfed plantation crop in Kenya, tea depends greatly on weather for optimal growth. The plant is grown in high altitude areas East and West of the Great Rift Valley, between 1400 and 2700 m amsl, where rainfall ranges between 1800 and 2500 mm annually. Evidence suggests a negative impact of global warming on production and quality of tea, especially with regards to temperature rise, unpredictable rainfall trends and increasing frequency of extreme weather events such as hail storms, drought and frost (**Figure 1**; Boehm et al., 2016; Ahmed et al., 2018, 2019). Studies have documented that stress, especially drought, account for 14–20% loss in yield and 6–19% plant mortality (Ng'etich et al., 2001; Cheruiyot et al., 2007). Multiple environmental parameters are known to impact tea quality, although the directionality and magnitude is not clear likely due to variations in various factors such as cultivar, environment and management conditions (Ahmed et al., 2019). Under such circumstances, tea production is vulnerable to the predicted climate change effects and, subsequently, greater economic, social, and environmental problems. There is need for scientific and community-based adaptation and mitigation strategies. Adoption of a multi-targeted approaches that seek to understand the complex physiological, biochemical and molecular regulatory networks associated with stress response will ensure sustainability of the tea sector. These necessitate intense research to improve tea production under diverse stress conditions.

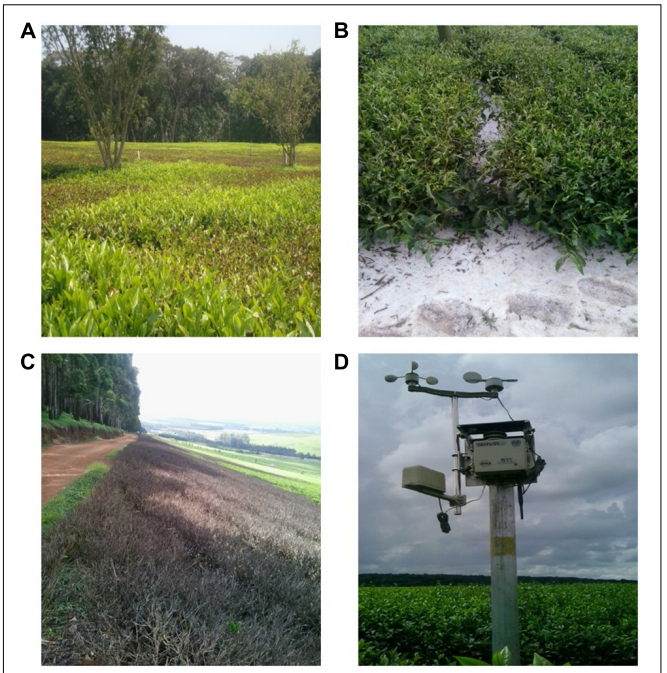

FIGURE 1 | (A) Effect of frost in tea plantation. **(B)** Severe hailstone damage resulting in suspension of tea plucking for several months. **(C)** Effect of planting eucalyptus trees near tea fields. **(D)** Wireless sensor network (WSN) that assist in timely weather data deployment.

IMPACT OF CLIMATE CHANGE ON TEA PRODUCTION

With climate change, it is expected that the main tea growing areas will experience an increase in the length of dry seasons per year, warmer temperatures and/or extreme rainfall intensity (Wijeratne, 1996; Trejo-Calzada and O'Connell, 2005; Eitzinger et al., 2011). Climate data collected at KALRO-TRI for over 58 years, indicate an annual temperature rise of 0.016°C per year while annual rainfall decreased by 4.82 mm per year over the same period (Cheserek et al., 2015). This has led to continued increase in soil water deficit (SWD) over time. On an annual basis, a large SWD, especially in January, February and March is reported leading to significant oscillations in tea production annually (Bore, 2008).

Simulation models provide the best known approach for integrating our understanding of complex plant processes that are influenced by weather and other environmental factors. Models are useful in guiding the direction of fundamental research by providing quantitative predictions and highlighting gaps in our knowledge (Semenov and Halford, 2009). Their use in assessing the impact of climate change and identifying potential future threats in plant sciences has been extensively reported (Sinclair and Muchow, 2001; Porter and Semenov, 2005; Passioura, 2007). Simulation models combined with high resolution climate change scenarios have been used to quantify the regions that could be suitable for economic production of tea in Kenya by the year 2075 (**Figure 2**; Bore and Nyabundi, 2016). Maps generated in a GIS environment using climate data from the Kenya Meteorological Services predicted that the mean air temperature for the region would increase by about 2% by 2025 and by 11% by 2075, if no action is taken. Distribution of areas suitable for tea cultivation within the current growing areas in Kenya will decrease. This is attributed to rainfall distribution and not amounts of rainfall received. The rise in mean air temperatures beyond the threshold of 23.5°C might also occur. Further, suitability of tea growing areas is expected to decline by 22.5% by the year 2075 while a suitability increase of 8% is expected by 2025. In order to boost the adaptation and performance of tea, a key strategy would be first to understand the mechanisms involved in stress tolerance, and then use appropriate tools and breed for stress tolerance.

Considering the established positive influence of temperature on tea production, it is imperative to conduct the economics of supplying water to tea fields during drought to reap from the enterprise (Cheserek et al., 2015). The irrigation or fertigation possibilities in tea had been documented in earlier experiments conducted in Sri Lanka (Wijeratne et al., 2007), India (Panda et al., 2003) and East Africa (Carr, 1972, 2010a,b). Timing of irrigation in tea fields has also been determined with inherent benefits (Carr, 1972; Othieno, 1978). This paves way for simulations into the projected temperature scenarios to reveal the potential yield levels. The divide between the cooler and already warm places could also support regional specific temperature thresholds for the crop hence support breeding activities for site specific cultivars with better heat stress tolerance.

Recent works were driven principally by the emergence of improved cultivars which had poor rooting system hence subject to water stress problems. Relations between young grafted teas with water stress had been done (Bore et al., 2010). How plants recover from drought event has also been determined (Muoki et al., 2012) as well as response of composite tea to progressive drought (Bore et al., 2010). A remarkable breakthrough in tea plant and drought relations was established revealing the threshold moisture content below which tea plant succumbs (Cheruiyot et al., 2008). Deeper understanding of the relationships could be achieved via controlled carbon dioxide (CO_2) enrichment experiments such as Free Air Carbon Enrichment (FACE). There is need for development of cultivars which are not only tolerant to heat stress but equally adaptable to higher CO_2 levels in the atmosphere (Wijeratne et al., 2007). A significant increase in concentrations of total catechins, other polyphenols and amino acids have been reported elsewhere with elevated carbon dioxide, while caffeine levels decrease (Li et al., 2017). Studies on carbon enrichment in tea cultivars grown in Kenya have, however, not been quantified though clear increase in temperature as CO_2 rises has been established.

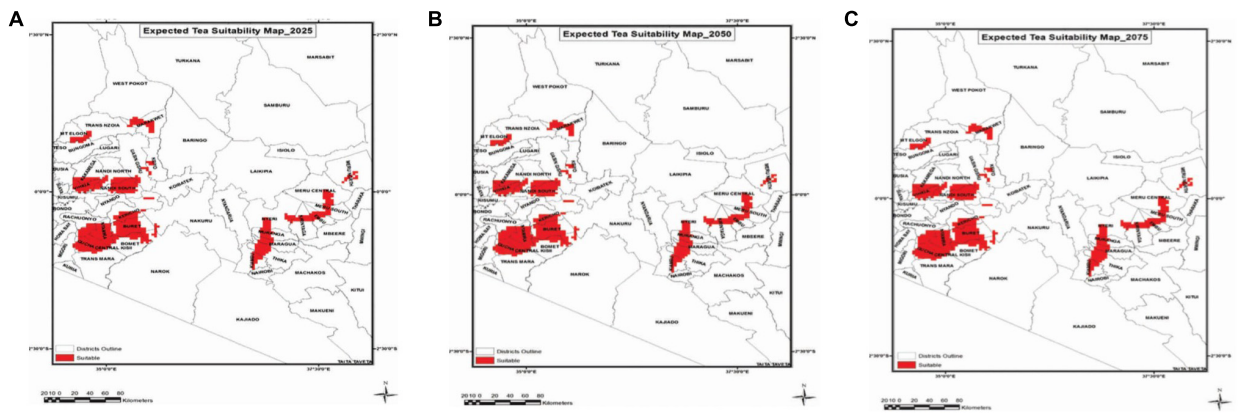

FIGURE 2 | Projected suitable areas for tea growing in Kenya. **(A)** 2025, **(B)** 2050, and **(C)** 2075. Source: Adapted from Bore and Nyabundi (2016).

TABLE 1 | An overview of various breeding strategies employed to improve tolerance of tea to adverse environmental factors in Kenya.

Techniques/approaches	Target trait	References
Physiological characterization		
Relative water content	Drought tolerance	Maritim et al., 2015; Cheruiyot et al., 2007
Shoot water potential	Drought tolerance	Maritim et al., 2015; Cheruiyot et al., 2007
Gas exchange measurement	Drought tolerance	Maritim et al., 2015; Cheruiyot et al., 2007
Genotype (G) × Environment (E) interaction	Yield	Wachira et al., 2002
Biochemical characterization		
Total Polyphenols	Drought tolerance	Cheruiyot et al., 2007
Amino acids (Proline)	Drought tolerance	Maritim et al., 2015
Amines (Glycinebetaine)	Drought tolerance	Maritim et al., 2015
Epicatechin and Epigallocatechin	Drought tolerance	Cheruiyot et al., 2008
Physiological:Biochemical characterization		
Short-time Withering Assessment of Probability for Drought Tolerance	Drought tolerance	Nyarukowa et al., 2016
Combining ability	Drought tolerance, Quality and Yield	Kamunya et al., 2009
Molecular approaches		
Linkage analysis and QTL mapping (RAPD, AFLP and SSR)	Yield	Kamunya et al., 2010
Linkage analysis and QTL mapping (DArT)	Drought tolerance and quality	Koech et al., 2018
Bulked segregant analysis (BSA)	Yield	Kamunya et al., 2010
Genomics	Drought tolerance and black tea quality	Koech et al., 2019
Suppression subtractive hybridization	Drought tolerance	Muoki et al., 2012
Transcriptomics	Drought tolerance	Maritim et al., 2016

RESPONSE OF TEA TO CLIMATE CHANGE

Plant responses to stress are dynamic and complex. This is often manifested by its physiological and biochemical reactions, which can provide a basis for screening for and selection of individual varieties and germplasm resistant to stress factors. Such responses include stomatal closure, repression of cell growth and photosynthesis, accumulation of organic osmolytes, and activation of respiration (Muoki et al., 2012; Maritim et al., 2015). Several studies have reported the effects of stress on critical components present in tea and corresponding synthetic genes. The present section focuses on providing an overview of the physiological, biochemical and molecular mechanisms of stress response and tolerance in tea (**Table 1**). This will provide theoretical knowledge for development of climate-resilient tea cultivars as the parameters described can be used as stress index for screening and clonal selection.

Physiological Responses

Climate change induced stresses affect plant systems such as photosynthesis, respiration and water retaining capacity. Tea plants exhibit C_3 mechanism of photosynthesis, a key process affected by water deficits, via decreased CO_2 diffusion to the chloroplast leading to metabolic constraints (Tezara et al., 2002; De Costa et al., 2007; Pinheiro and Chaves, 2011). Relative impact of such limitations varies with the occurrence and intensity of stress. Rate of photosynthesis in tea increases up to an illuminance (photon flux density) of about 1000 μmol m^{-2} s^{-1} and then remains relatively constant (Smith et al., 1993), while the optimum leaf temperature for photosynthesis in tea is about 25–30°C (Smith et al., 1993; Mohotti and Lawlor, 2002; Barman et al., 2008). Under stress condition, the photosynthetic machinery of the tea plant are damaged, hence limiting the stomatal conductance of the leaves and eventually leading to a significant decline in net photosynthesis and respiration rate. Using drought resistant and susceptible tea cultivars, several studies have reported a significant difference in photosynthesis and respiration rate following reduction in soil moisture content (Netto et al., 2010; Lin et al., 2014; Maritim et al., 2015).

Tea has a critical xylem water potential value of −0.7 to −0.8 megapascal (MPa) in relation to potential SWD and saturation deficits of the air (Carr, 2010b). Previous studies have highlighted key physiological responses in relation to water deficit in tea (Cheruiyot et al., 2007; Maritim et al., 2015). Relative water content (RWC) is one of the most important measures of plant water status when plants are exposed to drought and heat stress. It reflects the degree of plants water status, retaining or regulation capacity (Anjum et al., 2011). RWC varies according to genotypes, with resistant genotypes maintaining higher RWC compared to susceptible ones (Maritim et al., 2015). Furthermore, a method for Short-time Withering Assessment of Probability for Drought Tolerance (SWAPDT) validated by targeted metabolomics for predicting the drought tolerance (DT) in tea was developed (Nyarukowa et al., 2016). The method relies on the percent RWC of tea leaves after 5 h under withering conditions. Based on metabolite profiles, drought tolerant tea cultivars differed from drought susceptible tea cultivars providing a basis for selection of new drought tolerant tea cultivars that may lead to improvement of

crop productivity, amidst challenges imposed by drought due to climate change.

Biochemical Responses

As water is being removed from the cell, osmotic potential is reduced due to the effect of solute concentration (Yamada et al., 2005). However, if during the course of cellular water loss solutes are actively accumulated, osmotic potential would be reduced beyond the rate dictated by the mere effect of concentration. These involve the accumulation of organic compounds such as amino acids (e.g., proline), quaternary and other amines (e.g., glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g., mannitol, trehalose, and galactinol). Proline is widely studied because of its considerable role in stabilizing sub-cellular structures, scavenging free radicals, and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007). In tea, proline accumulation under stress is significantly correlated with stress tolerance, and its concentration has been shown to be higher in stress-tolerant than in stress-sensitive plants (Chakraborty et al., 2002; Maritim et al., 2015). However, its use as a drought index is cultivar dependent. Nevertheless, stresses beyond tolerance levels will induce oxidative damage due to intensive production of reactive oxygen species (ROS) (Smirnoff, 1993). Glycinebetaine has also been reported to increase under stress condition (Maritim et al., 2015). Furthermore, tolerant cultivars have been reported to maintain higher polyphenol content at low SWC suggesting that cultivars with more stable polyphenols are more tolerant to water stress (Cheruiyot et al., 2007). Phenolic compounds can thus be useful indicators of DT in tea and will hasten the development of better-adapted cultivars to water-stress environments.

Genomic Responses

Rapid progress in molecular breeding in tea is attributable to advances in genomics technologies, especially DNA sequencing, leading to publication of two draft genomes (Xia et al., 2017; Wei et al., 2018). In Kenya, the approach has been integrated into tea improvement programs. Muoki et al. (2012) used subtracted cDNA libraries from irrigated and drought stressed plants of a tolerant cultivar to understand the molecular responses of tea to abiotic stresses, especially drought. With progressive drought, genes related to chaperones, cell rescue/defense and cellular transport categories exhibited an early up-regulation in tolerant as compared to the susceptible variety. Dysfunction of enzymes and proteins usually accompanies abiotic stresses. Plants induce the expression of chaperones to ensure protein stabilization and cellular homeostasis during stress (Wang et al., 2004). Maintenance of proteins in their functional conformations and prevention of aggregation of non-native proteins is particularly important for cell survival under stress (Muoki et al., 2012). Molecular chaperones function in the stabilization of proteins and membranes, and assist protein refolding under stress conditions (Wang et al., 2003). In addition, Maritim et al. (2016) reported a significant upregulation of drought-related genes such as heat shock proteins (HSP70), superoxide dismutase (SOD), gene catalase (CAT), ascorbate peroxidase (APX), calmodulin-like protein (Cam7) and galactinol synthase (Gols4) in drought tolerant as compared to drought sensitive tea cultivars. Further, three major enzymes, namely transferases, hydrolases and oxidoreductases are involved in flavonoid biosynthesis, alkaloid biosynthesis, ATPase family proteins related to abiotic/biotic stress response have been identified (Koech et al., 2019). However, plants have evolved various antioxidative systems to keep the levels of ROS under control (Mittler, 2002). ROS are capable of unrestricted oxidation of various cellular components and can damage cell membranes and macromolecules. Many abiotic stresses directly or indirectly affect the synthesis, concentration, metabolism, transport and storage of important carbohydrates in plants. Soluble sugars are known to act as potential signals interacting with light, nitrogen and abiotic stress to regulate plant growth and development (Cramer et al., 2011). Overall, the level of soluble sugars increases with progressive drought stress in tea, wherein drought tolerant cultivars maintained higher levels as compared to the susceptible cultivars (Damayanthi et al., 2010). This finding indicated the ability of the tolerant cultivars to withstand drought by osmotic adjustments. Data generated from these studies provide critical resource for development of markers that can be used for selection of climate resilient tea cultivars.

BREEDING AND SELECTION

Conventional Approach

Sustainability and profitability of the tea industry depends primarily on the availability of desired planting materials. Most of the genetic improvement and the substantial increase in tea yields realized this far is brought about by conventional breeding through selection for hybrid vigor, though the process has continued to evolve over the years (**Figure 3**). Tea breeding essentially consists of four phases; generation of genetic variability, selection of useful genotypes and comparative tests to demonstrate the superiority of the selected genotypes. A fourth phase that involves exposing pre-released and promising clones to multiple sites (genotype-environment interaction) for stability and adaptability is always the final phase in plant improvement programs (Wachira et al., 2002; Kamunya et al., 2010). It is worthwhile to note that TRI has developed over 1,000 improved cultivars, out of which 58 cultivars have been selected for consistent superiority in yield and quality and released for commercial exploitation. Fourteen of these clones are capable of yielding between 5,000 and 8,000 kg of made tea per hectare per year. These yield levels are some of the highest in the world and are in the magnitude of three times the average yields of unimproved tea.

Approaches involving intravarietal and interspecific hybridizations have also been tapped as means of introducing desirable traits (Kamunya et al., 2012). The approach is facilitated by the availability of diverse genetic collection, standardized vegetative propagation procedures, continuous germplasm enrichment through material transfers between

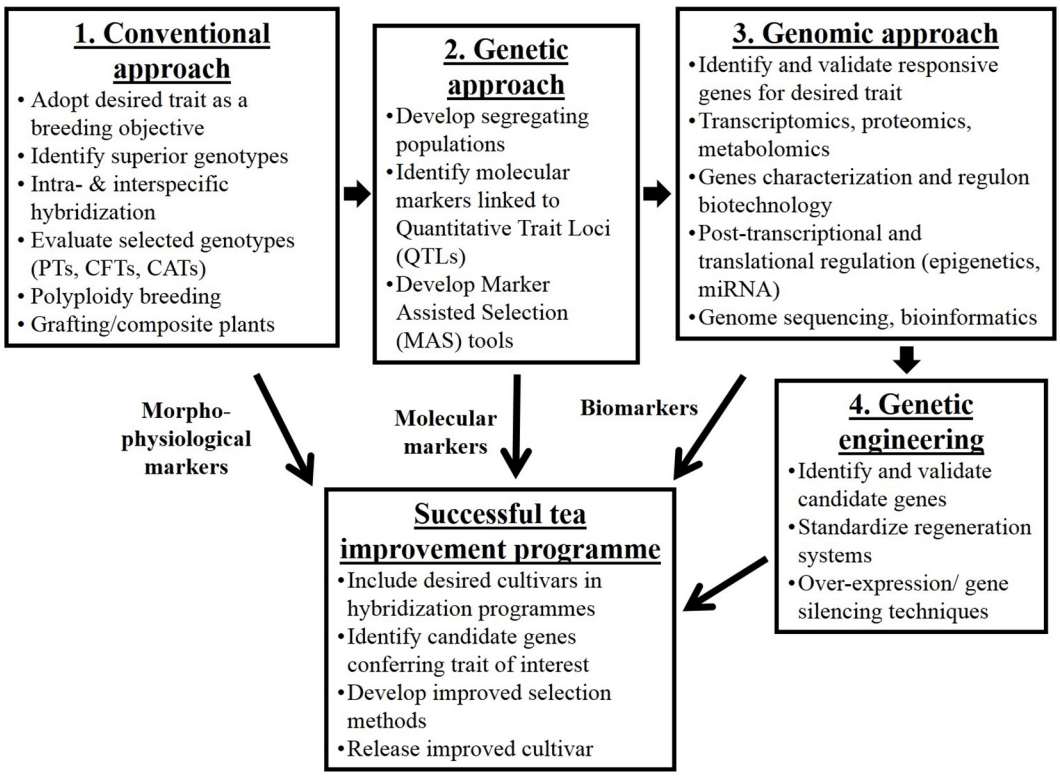

FIGURE 3 | Schematic diagram on tea improvement strategies and techniques that have evolved over a period of time. Arrows in between boxes **1**, **2**, **3**, and **4** show the evolutionary time scale of the development of the strategies. CATs, clonal adaptability trials; CFTs, clonal field trials; MAS, marker assisted selection; PTs, progeny trials; QTLs, quantitative trait loci.

research institutions and the comparatively low operational costs involved. A remarkable achievement of conventional breeding was the transition from pioneer seedling tea plantations to the adoption of modern high-yielding vegetatively propagated cultivars. This led to a drastic increase in tea production globally in the mid-20th century. The technique reduced the juvenile period to as short as 6 months from the protracted 3 years for seed raised tea plants (Wachira, 2000). Given the financial considerations associated with clonal teas, farmers are now uprooting and replacing the old and diverse seedling tea plantations with a few improved clones. As clones represent instantly fixed genotypes, the practice means over-reliance on a limited number of cultivars, implying that on-farm diversity is minimizing and the risks posed by co-evolving challenges associated with climate change is increasing. Another emanating problem is that most tea breeding programs rely heavily on a few clonal parents as donors of desired genes, thereby manifesting the potential danger of mono-cropping (Wachira, 2002). For instance, 67% of released varieties in Kenya share the same female parent, cultivar TRFK 6/8 which is susceptible to root knot nematodes.

As water resources for agriculture become more limiting, the need to develop drought tolerant cultivars is increasingly gaining importance. The ability of plants to tolerate changes in extremes of abiotic stress conditions is a complex and coordinated response, involving hundreds of genes. These responses are also affected by interactions between the different environmental factors and the developmental stage of the plant. Breeding involves genetic alteration or modification of organisms through natural or human-imposed mutations or crosses. This process has continued to evolve in tea over the years. A foundation in conventional breeding has contributed significantly to tea improvement. This involves the identification of stress tolerant parents (**Table 2**), intra- or interspecific hybridization, establishment of progeny trials (PTs), clonal field trials (CFTs), and clonal adaptability trials (CATs).

Attempts to improve stress tolerance in tea through conventional breeding programs have, however, met limited success, partially attributed to the robust breeding programs and improved crop husbandry (Kamunya et al., 2010, 2012). However, due to the lack of sufficient genetic information about genes that govern this complex trait and its component secondary traits, progress in tea improvement has been slow. Research has shown that DT varies considerably between tea cultivars (Ng'etich et al., 2001; Cheruiyot et al., 2008; Carr, 2010a,b; Kamunya et al., 2010), which further suggests the need for investigating the genetic architecture and adaptive responses of tea to drought. Limitations in conventional breeding coupled with advances in molecular breeding have unveiled a new era in tea breeding.

TABLE 2 | Breeding stocks and their expected genetic contribution in breeding program.

High yield potential	High quality potential	Pest tolerance/ resistance	Drought tolerance	High soil pH tolerance	Cold tolerance	Genetic study
TRFK 31/8	TRFK 6/8[9]	TRFK 7/9[3]	TRFCA SFS150	EPK TN14-3	EPK TN14-3	TRFK 12/2[1]
TRFK 303/577[8]	GW Ejulu-L	TRFK 57/15[3]	TRFK 303/577[8]	NDT Tai	TRFCA SFS150	TRFK K-Purple
TRFK 301/4	EPK TN 15-23	AHP SC31/37[3]			EPK C12	TRFK 31/30[2]
TRFK 301/5		AHP S15/10[3]			NRIT Yabukita[6]	TRFK 311/287[2]
EPK C12		EPK TN14-3[5]			NRIT Yutakamidori[6]	TRFK 382/1[7]
BBLK 35		TRFK 303/1199[3]				TRFK 382/2[7]
AHP S15/10[9]		TRFK 54/40[4]				TRFK 386/2[7]
AHP SC12/28[9]		TRFCA SFS150[3]				TRFK 371/1[7]
AHP SC31/37		AHP CG28U864[4]				TRFK 306[10]
AHP CG28V929[9]		TRFK 301/1[4]				Wild *Camellia* spp.
AHP CG28U864		TRFK L/16[4]				

[1]Non fermenter; [2]tetraploid; [3]resistant to red crevice mite [4]susceptible to scales; [5]preferred but highly tolerant to red crevice mite; [6]green tea varieties-low catechin content; [7]triploid; [8]susceptible to root knot nematodes; [9]very susceptible to water stress; [10]anthocyanin tea cultivar.

From Conventional to Molecular Breeding

Understanding the genetics of how organisms adapt to changing environment is crucial for the adaptability of a genotype (Chinnusamy and Zhu, 2009). Due to the limitations associated with conventional breeding approaches, other means of genetic improvement are being explored. Availability of molecular tools arising from the development of molecular markers manifested a significant advancement in crop improvement in the 1980s. Different marker systems such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), sequence tagged sites (STS), single-strand conformation polymorphism (SSCP), inter simple sequence repeat (ISSR), simple sequence repeat (SSR) or microsatellite, Diversity Arrays Technology (DArT) microarray and chloroplast DNA (cpDNA) have been developed and applied in tea breeding (Wachira et al., 2001; Mondal et al., 2004; Chen et al., 2007; Sharma et al., 2009; Wambulwa et al., 2016a,b, 2017; Koech et al., 2018, 2019). These markers have been applied in genetic studies relating to assessment of genetic diversity and germplasm characterization, genotype identification and fingerprinting, estimation of genetic distances between populations, assessment of mating systems, detection of quantitative trait loci (QTLs), and marker-assisted selection (MAS) in tea (Wachira et al., 1995, 1997; Paul et al., 1997; Hackett et al., 2000; Muoki et al., 2007; Kamunya et al., 2010; Koech et al., 2018, 2019).

Most attributes of agricultural importance frequently manipulated by plant breeders (e.g., size, shape, yield, quality, tolerance to abiotic, and sometimes biotic stresses) display a quantitative mode of inheritance and normally exhibit continuous variation (Collard et al., 2005). Continuous variation in a phenotype can be explained by the independent actions of many distinct genetic factors, each having small effects on the overall phenotype. Detection of QTL controlling complex traits followed by selection has become a common approach for selection in crop plants. QTLs or linkage mapping aims at identifying genomic regions that could be useful to analyze genetics of complex traits (Stapley et al., 2010). QTLs are typically mapped by crossing parental varieties contrasting for the trait of interest to generate a mapping population which are then scored for phenotypes and genotyped so as to identify the parts of the genome that improve the trait and the genome regions that influence component trait linked to the main trait. Once achieved, targeting of genomic regions for varietal improvement could be possible through MAS, thereby shortening the development and release of elite varieties for commercialization (Hackett et al., 2000; Kamunya et al., 2010). The approach is helpful in tea where conventional breeding technique takes over 20 years to develop an improved cultivar. Integration of molecular markers in breeding and clonal selection would also help in reducing the number of clones/seedlings for field testing (Kamunya et al., 2010).

The first genetic linkage maps for tea was constructed using RAPD and AFLP markers and covered 1349.7 cM with an average distance of 11.7 cM (Hackett et al., 2000). In addition, QTLs for yield, DT, quality traits [percent total polyphenols (%TP)], fermentability (FERM), theaflavins (TF), thearubigins (TR), and pubescence (PUB) has been reported (Kamunya et al., 2010). Here, bulk segregant analysis followed by complete genotyping identified 260 RAPD and AFLP informative markers. Of these, 100 markers showing 1:1 segregation, were used to generate a linkage map with 30 (19 maternal and 11 paternal) linkage groups spanning 1411.5 cM with mean interval of 14.1 cM between loci. On the basis of the map, QTL analysis was done on data over two sites. A total of 64 putative QTLs for various traits across different sites were detected. More recently, phenotypic data for two segregating tea populations was used to identify QTL influencing tea biochemical and drought stress traits based on a consensus genetic map constructed using the DArTseq platform (Koech et al., 2018). The map consisted of 15 linkage groups from the two populations comprised 261 F1 clonal progeny and spanned 1260.1 cM with a mean interval of 1.1 cM between markers. Both interval and multiple QTL mapping revealed a total of 47 putative QTL in the 15 LGs associated with tea quality and percent RWC at a significant genome-wide threshold. These markers contribute

greatly to adoption of MAS for DT and tea quality improvement. However, positional cloning of genes controlling important traits in tree species is difficult (Stirling et al., 2001), partly due to the complexity of gene networks and interactions among or between genetic elements and the environment (Ribaut and Ragot, 2007). Such limitations can be overcome by adopting new approaches that exclude the need to map QTLs.

FUTURE PROSPECTS

Great progress has been made in assessment of the relationship between tea productivity and climate change. In order to anticipate the effects of climate change on tea and provide scientists with necessary knowledge and tools, multidisciplinary approaches should be embraced. The approaches outlined below are recommended:

(a) It would be important to quantify the long term response of the tea plant to elevated CO_2 concentrations so as to understand the link between carbon supply and plant growth. The extensive use of artificial environments such as the free air CO_2 enrichment (FACE) technology can help examine the magnitude of elevated CO_2 on tea yield and quality at the level of the ecosystem.

(b) Invest in alternative breeding approaches such as mutation breeding for increased genetic variability. This should be followed by standardizing selection procedures which attempt to identify useful genotypes.

(c) Studies have shown that the response of plants to a combination of stresses is unique and cannot be directly extrapolated from the response of plant to each of the different stresses applied individually. Further, simultaneous occurrence of several stresses enhances the intensity of lethality to crop as compared to that imposed by a single stress. Nevertheless, little is known about the molecular mechanisms underlying the acclimation of tea to a combination of different stresses. Systems biology approach facilitate a multi-targeted approach for understanding complex molecular regulatory networks associated with stress adaptation and tolerance. The approach can help overcome limitations associated with morphological, biochemical and molecular adaptation of the plants to stress. Tolerance to a combination of different stress conditions, particularly those that mimic the field environment, should be the focus of future research programs aimed at developing improved varieties and plants with enhanced tolerance to naturally occurring environmental conditions.

(d) Establish multi-stakeholders collaborations aimed at developing sustainable adaptation strategies for management of climate risks associated with climate change in the tea industry.

AUTHOR CONTRIBUTIONS

All authors wrote and revised the manuscript.

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METHODS
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A Proposed Method for Simultaneous Measurement of Cuticular Transpiration From Different Leaf Surfaces in *Camellia sinensis*

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The plant cuticle is the major barrier that limits unrestricted water loss and hence plays a critical role in plant drought tolerance. Due to the presence of stomata on the leaf abaxial surface, it is technically challenging to measure abaxial cuticular transpiration. Most of the existing reports were only focused on leaf astomatous adaxial surface, and few data are available regarding abaxial cuticular transpiration. Developing a method that can measure cuticular transpiration from both leaf surfaces simultaneously will improve our understanding about leaf transpiration barrier organization. Here, we developed a new method that enabled the simultaneous measurement of cuticular transpiration rates from the adaxial and abaxial surfaces. The proposed method combined multi-step leaf pretreatments including water equilibration under dark and ABA treatment to close stomata, as well as gum arabic or vaseline application to remove or seal the epicuticular wax layer. Mathematical formulas were established and used to calculate the transpiration rates of individual leaf surfaces from observed experimental data. This method facilitates the simultaneous quantification of cuticular transpiration from adaxial and abaxial leaf surfaces. By applying this method, we demonstrated that the adaxial intracuticular waxes and the abaxial epicuticular waxes constitute the major transpiration barriers in *Camellia sinensis*. Wax analysis indicated that adaxial intracuticular waxes had higher coverage of very long chain fatty acids, 1-alkanol esters, and glycols, which may be attributed to its higher transpiration barrier than that of the abaxial intracuticular waxes.

Keywords: cuticle, cuticular transpiration, epicuticular waxes, intracuticular waxes, adaxial, abaxial, gum arabic, vaseline

Abbreviations: ABA, abscisic acid; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; SEM, scanning electron microscope; TEM, transmission electron microscope; VLCFAs, very long chain fatty acids.

INTRODUCTION

Global climate change is projected to cause large-scale fluctuations in precipitation patterns, including more frequent incurrences of extreme drought conditions coupled with rising temperature (Scott et al., 2019; https://climate.nasa.gov/effects/). Understanding how plants respond to drought is essential for the development of new germplasms better adapted to the changing environment. Plants have two major pathways for water loss: under normal conditions, stomata transpiration accounts for most of the water loss of plants; under drought conditions, cuticular transpiration becomes the major route for water loss (Holmgren et al., 1965). Thus, cuticular transpiration is tightly associated with plant drought tolerance (Schuster et al., 2017). The plant cuticle covers outer epidermal surface of terrestrial plant species (Ingram and Nawrath, 2017); it minimizes UV irradiance and pollutant entry/retention, defends against pathogens and herbivores, and prevents organ fusion (Jenks et al., 1994; Eigenbrode and Espelie, 1995; Kerstiens, 1996; Chen et al., 2003; Long et al., 2003). However, the principal function of the plant cuticle is to protect leaves, fruits, and other aboveground organs from uncontrolled water loss (Sieber et al., 2000). These diverse functions of the plant cuticle are provided by its complex and heterogeneous chemical and physical nature (Guzmán et al., 2014a). The cutin, cutan, and polysaccharides form polymer matrix as the cuticle backbone (Nawrath, 2006; Domínguez et al., 2011), and cuticular waxes deposit into polymer matrix. Depending on the deposition position, cuticular waxes can be divided into epicuticular waxes (EWs) and intracuticular waxes (IWs). The EWs exist as a thin layer on cuticle's outer surface; in contrast, the IWs are embedded into cutin/cutan polymer matrix (Jetter et al., 2000). Thus, the EWs can be efficiently stripped off by adhesives such as gum arabic, while IWs are resistant to such treatment (Jetter et al., 2000). Cuticular waxes are complex mixtures of aliphatic (mainly long-chain fatty acids, alcohols, alkanes, aldehydes, esters, and ketones) and aromatic compounds (Jetter et al., 2006). In *Camellia sinensis*, leaf waxes are constituted of 13 chemical classes, including esters, glycols, terpenoids, fatty acids, and their derivatives (Zhu et al., 2018). The cuticle can be divided into three different layers from the external to the internal surface, including the EW layer, the cuticle proper (CP), and the cuticular layer (CL; Jeffree, 2006). It has been traditionally assumed that cutin/cutan and waxes are present in the CP and CL, while polysaccharides are restricted to the CL (Jeffree, 2006). Guzmán et al. (2014a,b, 2016) reported that cellulose and pectins were found on both CP and CL layers. Based on these new findings, Fernández et al. (2016, 2017) proposed that the cuticle may be interpreted as a modified cell wall region which contains additional lipids.

The plant cuticle covers both the adaxial and abaxial leaf surfaces, while stomata are usually present on the abaxial surface. There are lots of interests to investigate cuticular transpiration barrier properties, and several methods have been developed during the past half century. A widely used method is applying microbalance to measure water weight loss from excised leaf. Later, a transpiration chamber measurement method was developed (Becker et al., 1986) and provides more controlled conditions and reproducible results. This method can only be applied with isolated cuticular membranes (CMs) and thus the results may not reflect the transpiration property *in planta*. Different detection techniques have also been developed, including electrolysis cell (Keidel, 1959), microbalance (Schönherr and Lendzian, 1981), moisture sensor, and ^{3}H-labeled water in combination with scintillation counter (Schreiber et al., 2001), and the last method offered high precision and sensitivity. CMs isolation method by enzyme digestion was also developed (Schönherr and Riederer, 1986); CMs were isolated from diverse plant species and their transpiration was measured (Becker et al., 1986).

To investigate the cuticular transpiration barrier organization, EWs were selectively stripped off by adhesives such as nitrocellulose, cellulose acetate, or gum arabic (Haas and Rentschler, 1984; Riederer and Schönherr, 1984; Silcox and Holloway, 1986; Jetter and Schäffer, 2001). These studies demonstrated that each adhesive has its own advantages and concerns in terms of convenience, efficiency, and cross-contamination. The transpiration changes after EW removal indirectly reflect the contribution of EWs to the leaf transpiration barrier and directly reflect the contribution of IWs to the leaf transpiration barrier.

The presence of stomata on the leaf abaxial surface makes it technically challenging to measure abaxial cuticular transpiration. It is not surprising that most reports only used astomatous cuticles (Becker et al., 1986; Vogg et al., 2004) and leaf abaxial cuticles were deliberately neglected. To face this challenge, Šantrůček et al. (2004) developed a method that is based on the manipulation of water vapor diffusivity in different types of gases; this method allowed measuring water permeability through stomatous CMs, but only applicable to isolated CMs. Here, we developed a new method to measure cuticular transpiration from the intact leaf. This method can differentiate adaxial transpiration from abaxial transpiration. By applying this method, we demonstrated that adaxial IWs and abaxial EWs constitute the major transpiration barrier in *C. sinensis* cv *Fuyun 6*.

MATERIALS AND METHODS

Reagents

Gum arabic and abscisic acid (ABA) were purchased from Solarbio (Beijing, China), and vaseline was obtained from Borui (Quanzhou, China).

Plant Material

Camellia sinensis cv *Fuyun 6* was grown in a tea garden at Fujian Agriculture and Forestry University (Fuzhou, China; 119.2°E, 26.1°N), and the fourth leaf was used for cuticular transpiration and waxes measurement.

Leaf EW Removal With Gum Arabic

The fourth leaf from *C. sinensis* cv *Fuyun 6* does not have trichome on either side of the leaf surfaces and was used to localize leaf cuticular transpiration barrier. Gum arabic was

selected to remove EWs due to its water solubility, thus avoiding the potential side effects of organic solvents on wax extraction. Before the experiment, gum arabic was extracted seven times with hot chloroform to remove any soluble lipids. A 90% (w/w) aqueous solution of delipidated gum arabic was evenly applied onto leaf surface (∼0.1 ml cm^{-2}) with a small soft paintbrush. After 1 h, a dry and stable polymer film was formed, which was peeled off to strip off EWs.

Leaf Surface Vaseline Sealing

Vaseline was evenly applied onto leaf surface (∼3 mg cm^{-2}) with a small soft paintbrush.

Stomatal Aperture Measurement

Pieces of abaxial epidermal cell layer were peeled off by forceps, mounted onto glass slide with 0.9% NaCl solution, immediately observed under microscope to measure stomatal aperture.

Leaf Transpiration Measurement

New shoots from clonally propagated *C. sinensis* cv *Fuyun 6* were harvested at the stage of 1 bud with 5–6 leaves at 6:00 pm; the cut ends of the shoots were immersed in fresh tap water and kept in the dark overnight for equilibration. Next day, leaf abaxial surfaces were evenly sprayed with 50 μM ABA, kept in the dark for 1 h, and excess water was gently blotted dry by a soft tissue. Gum arabic or vaseline application was then applied as described above. After completing these pretreatment steps, the fourth leaf was gently removed from each shoot; the excision site on leaf stem was immediately sealed by vaseline. Each leaf was photographed, leaf area (A) was calculated by Image J software, and the initial water saturated fresh weight (W_i) was recorded; leaves were placed in a controlled dark room (25°C, 45% humidity), and leaf weight was recorded hourly in a balance (JA5003, Liangping, Shanghai, China) for a total 6 h ($W_{t1,\ 2,\ldots\ 6}$). Each treatment group included 4–6 leaves. At the end of experiment, leaves were dried in 80°C for 24 h, and then the dry weight (W_d) for individual leaf was recorded.

Leaf water loss is given by formula (I):

(I) Leaf water loss = $(W_i - W_t)/A$
W_i and W_t represent initial water-saturated leaf weight and leaf weight at t hour post-excision, respectively; A is projected leaf area.

Leaf transpiration rate is given by formula (II):

(II) Leaf transpiration rate = $(W_t - W_{t+1})/A$
W_t, W_{t+1} represent leaf weight at t and t + 1 hour post-excision, respectively.

Leaf relative water deficit (RWD) is given by formula (III) (Burghardt (2003):

(III) $RWD = [1- (W_t - W_d)/(W_i - W_d)] \times 100\%$

To calculate transpiration rates from the adaxial and abaxial surfaces as well as different cuticle layers, six different treatment groups were prepared simultaneously, including:

(1) Control. The total leaf transpiration rate (T) is given by formula (IV):

(IV) $T = T_{Ad} + T_{Ab}$
T, T_{Ad}, and T_{Ab} represent total leaf transpiration rate, adaxial transpiration rate, and abaxial transpiration rate, respectively.

(2) Adaxial surface was sealed with vaseline (Ad/Vas). The observed total transpiration rate ($T_{Ad/Vas}$) is given by the formula (V):

(V) $T_{Ad/Vas} = k \times T_{Ad} + T_{Ab}$
k is vaseline diffusion coefficient factor for water vapor.

(3) Abaxial surface was sealed with vaseline (Ab/Vas). The observed total transpiration rate ($T_{Ab/Vas}$) is given by formula (VI):

(VI) $T_{Ab/Vas} = T_{Ad} + k \times T_{Ab}$

(4) Both leaf surfaces were sealed with vaseline (Ad/Vas:Ab/Vas), the observed total transpiration rate ($T_{Ad/Vas:Ab/Vas}$) is given by formula (VII):

(VII) $T_{Ad/Vas::Ab/Vas} = k \times (T_{Ad} + T_{Ab})$
k is parameter reflecting the physio-chemical nature of vaseline and affected by vaseline film thickness and uniformity. During our experiments, care was taken to ensure that vaseline was evenly applied on both leaf surfaces, and we assume that the vaseline diffusion coefficient factor from both leaf surfaces is identical.

(5) Adaxial EW layer was removed by gum arabic while abaxial surface was sealed with vaseline (-EW_{Ad}:Ab/Vas). The observed total transpiration rate ($T_{-EWAd:Ab/Vas}$) is given by formula (VIII):

(VIII) $T_{-EWAd:Ab/Vas} = T_{Ad/intra} + k \times T_{Ab}$.
$T_{Ad/intra}$ represents the transpiration rate from the adaxial IWs.

(6) Abaxial EW layer was removed by gum arabic while adaxial surface was sealed with vaseline (-EW_{Ab}:Ad/Vas). The observed total transpiration rate ($T_{-EWAb:Ad/Vas}$) is given by formula (IX):

(IX) $T_{-EWAb:Ad/Vas} = T_{Ab/intra} + k \times T_{ad}$.
$T_{Ab/intra}$ represents the transpiration rate from the abaxial IWs.

Adaxial cuticular transpiration can be regarded as the same as T_{Ad}. However, due to the possible existence of residual stomatal transpiration from the abaxial leaf surface (T_{Ab_s}), abaxial cuticular transpiration (T_{Ab_c}) should be lower than T_{Ab}. Their relation is given by formula (X):

(X) $T_{Ab} = T_{Ab\_c} + T_{Ab\_s}$

Wax Sampling

The shoots with concurrent bud break were selected, and the fourth leaf from each shoot was harvested. Twenty leaves were randomly selected and pooled together as one biological replicate;

four biological replicates were used. EWs were removed by gum arabic; the film was collected into a glass tube containing 21 ml of chloroform:water (2:1, v/v), and 75 μg of *n*-tetracosane (Sigma-Aldrich, St. Louis, United States) was added as internal standard. After vigorous agitation and phase separation, the organic phase was transferred into a new glass tube. Extraction was repeated with another 4.5 ml of extraction buffer. The organic phases were combined and evaporated under CentriVap Console (Labconco, KS, United States). The adaxial EWs were firstly removed and then abaxial EWs were isolated from the same batch of leaves.

After EW removal from both leaf surfaces by gum arabic, the leaves were still physically intact, and were used to extract IWs. The adaxial IWs were rinsed five times by chloroform; the elution was collected into a glass beaker. Abaxial IWs were isolated in the same manner. The elution was dried down by CentriVap Console to recover IWs.

Adaxial and Abaxial IW Analysis

Before analysis, hydroxyl groups in wax lipids were converted to trimethylsilyl derivatives by reacting with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (GC grade BSTFA, Aldrich) containing 1% trimethylchlorosilane (Aldrich) in pyridine (Aldrich, 99.8%, anhydrous) and the reaction was maintained at 70°C for 1 h. Each sample was divided into two parts and analyzed by GC-MS (GCMS-QP2010 Ultra, Shimadzu, Japan) and GC-FID (GC-2010 plus, Shimadzu, Japan), respectively. GC-MS and GC-FID were equipped with the same type of capillary column (DB-1, 30 m × 0.25 mm × 0.25 μm, Agilent, California, United States). Helium was used as carrier gas at a constant flow of 1.2 ml min^{-1} and 1.7 ml min^{-1} for GC-MS and GC-FID, respectively. The flow rates for hydrogen, nitrogen, and zero air were 40, 30, and 400 ml min^{-1}, respectively. Oven temperature was programmed at 70°C, raised by 10°C min^{-1} to 200°C, held for 2 min, raised by 3°C min^{-1} to 320°C, and held for 20 min. The MS detector setting was as follows: EI-70 eV, ionization source temperature 230°C. Individual wax component was identified by comparing its mass spectra with those of authentic standards and literature data. FID data were used to quantify wax amount by normalizing peak areas from individual wax homologs against that of the internal standard.

Scanning Electron Microscopy (SEM)

EWs from half of the leaf were stripped by gum arabic; the other half was left untreated as control. Samples were air dried at room temperature. Before observation, small pieces of samples were fixed to aluminum sample holders, freeze dried (HCP-2 critical point dryer, Hitachi, Japan), sputtered with a thin layer of gold (IB5 ion coater, Eiko, Japan), and then observed under scanning electron microscope (SEM) (JEM-6380LV, JEOL, Japan).

Transmission Electron Microscopy (TEM)

Samples were cut into small pieces (2 mm × 4 mm) and fixed in 5% (v/v) glutaraldehyde solution overnight in 4°C. Samples were rinsed three times with 0.1 M PBS buffer (pH 7.2), post fixed with 1% (w/v) osmium tetroxide at 4°C for 2–2.5 h, rinsed three times with PBS buffer, and then dehydrated through 30% (v/v) and 50% (v/v) ethanol for 15 min. Samples were stained with saturated uranyl acetate overnight, and then rinsed with 70% (v/v) ethanol several times to remove unbound uranyl acetate. Samples were dehydrated in 90% (v/v) and 100% ethanol for 17 min, and dehydrated once more in 100% ethanol for 17 min. Samples were treated sequentially with acetone:ethanol (1:3, v/v), acetone:ethanol (1:1, v/v), acetone:ethanol (3:1, v/v), and 100% acetone for 17 min. The dehydration step was repeated in 100% acetone. Samples were infiltrated through a graded acetone/Epon/Spurr's epoxy resin and polymerized at 70°C for 24 h (Zhu et al., 2018). Samples were sectioned at 70 nm thickness using an ultra 35° diatome diamond knife; the thin sections were collected onto 200-mesh copper thin bar grids and observed under transmission electron microscope (TEM) (HT7700, Hitachi, Japan).

Statistical Analysis

Mean and standard error were calculated with ANOVA within standard Excel software package. Probabilities for significance were calculated using Student's *t* test.

RESULTS

Gum Arabic Can Selectively Remove EWs

The adaxial and abaxial leaf surfaces looked flat (**Figures 1A,B**). The mechanical removal of EWs by gum arabic was visualized under SEM and TEM. The native leaf adaxial surface had an irregular coverage with EW crystalloids. When gum arabic was carefully applied onto half of the leaf (**Figure 1C**, right half), leaving another half untreated (**Figure 1C**, left half), a clear border between treated and untreated halves of the cuticle was visible (**Figure 1C**, dashed line). After gum arabic treatment, EW crystals completely disappeared and cuticle exhibited a very smooth appearance (**Figure 1C**, right half). The native abaxial leaf surface showed ridges and grooves and spread with rod-like crystalloids (**Figure 1D**). After gum arabic stripping, abaxial EW crystals almost completely disappeared and the cuticle exhibited a smooth appearance (**Figure 1E**). Stomata kept intact after gum arabic stripping (**Figures 1D,E**). The cross section of the cuticle before and after gum arabic stripping also was visualized by TEM. Without gum arabic treatment, the outmost cuticle exhibited a rough appearance (**Figures 1F–H**) and became very smooth after gum arabic treatment (**Figures 1G–I**). The IW was not affected by gum arabic treatment (**Figures 1F–I**). By comparing the cuticle thickness difference before and after gum arabic treatment, the thickness of the adaxial and abaxial EWs was estimated to be 0.21 ± 0.03 μm and 0.15 ± 0.02 μm, respectively. Since acetone and ethanol may remove some of the EWs during sample preparation, the actual EW thickness could be larger than the observed value. These observations demonstrated that gum arabic can selectively remove EWs from tea leaves without affecting IWs.

Gum Arabic Treatment Did Not Disrupt Stomatal Closure

To test whether gum arabic treatment on leaf abaxial surface affected stomatal closure, leaves were divided into two groups:

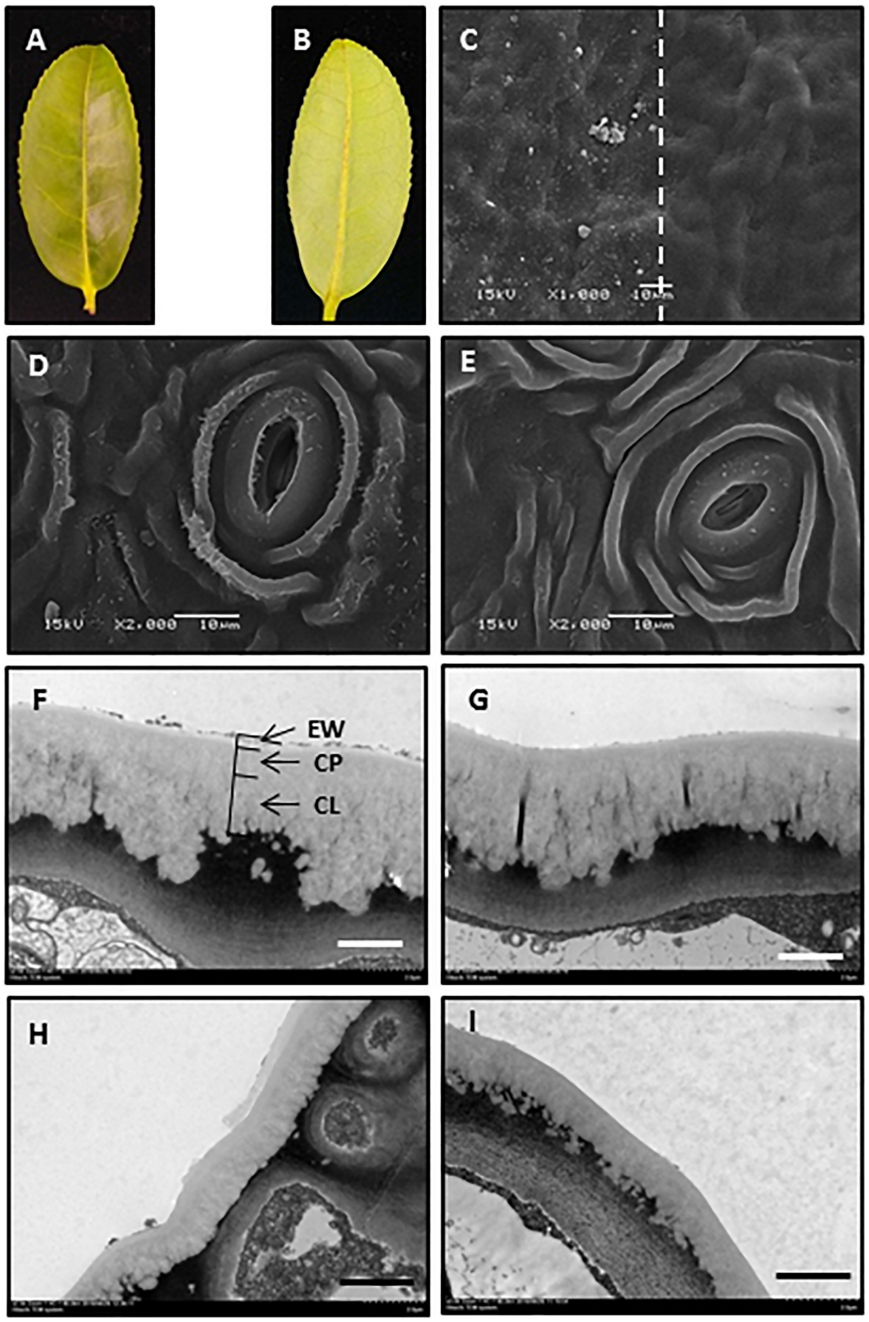

FIGURE 1 | Gum arabic effectively removed epicuticular waxes without affecting intracuticular waxes. **(A,B)** Adaxial and abaxial surfaces of the fourth tea leaf. **(C)** Scanning electron micrograph of adaxial leaf surface before (left) and after (right) gum arabic treatment. **(D,E)** Scanning electron micrograph of abaxial leaf surface before and after gum arabic treatment, respectively. **(F,G)** Transmission electron micrograph of adaxial leaf surface before and after gum arabic treatment, respectively. **(H,I)** Transmission electron micrograph of abaxial leaf surface before and after gum arabic treatment, respectively. Bar = 10.0 μm for **(C–E)**; bar = 2.0 μm for **(F–I)**.

one group pretreated with ABA (ABA treatment group), and the others left untreated (ABA treatment control group). Then, each group was divided into two subgroups; one subgroup had the leaf abaxial surfaces stripped by gum arabic (gum arabic treatment group) while the second subgroup was left untreated as gum arabic treatment control, and water loss was measured in both cases. If gum arabic treatment disrupted stomata closure, one would expect that after gum arabic treatment, water loss from ABA pretreated leaves would become indistinguishable from the leaves without ABA pretreatment. When leaves were not stripped by gum arabic, water loss from ABA pretreated leaves was lower compared to leaves without ABA treatment,

but there was not statistically significant difference at each time point. These observations indicated that ABA application had minor influence on leaf water loss when leaves were fully water equilibrated, and ABA is not sufficient to close stomata under this scenario. After gum arabic stripping, water loss was significantly increased regardless of the pretreatment with ABA or not. However, water loss from ABA-pretreated leaves was significantly lower compared with leaves without ABA treatment (**Figure 2**); thus, gum arabic stripping did not disrupt stomata closure.

Vaseline Effectively Reduced Leaf Water Loss

To test the moisture reduction efficiency of vaseline, vaseline was evenly applied to either the adaxial or abaxial surfaces or both surfaces, and leaf water losses were quantified over a 6-h period. We found that vaseline coating on adaxial surface, abaxial surface, and both leaf surfaces significantly reduced water loss by 15%, 56%, and 77%, respectively (**Figure 3**). These data demonstrated that more water was lost through abaxial surface than that of adaxial surface, and vaseline application can effectively reduce leaf water loss.

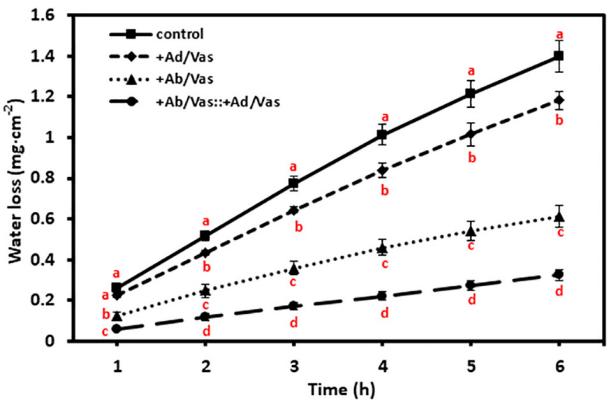

FIGURE 3 | Vaseline application reduced leaf water transpiration. +Ad/Vas, leaf adaxial surface sealed with vaseline; +Ab/Vas, leaf abaxial surface sealed with vaseline; +Ab/Vas: +Ad/Vas, both leaf surfaces sealed with vaseline. Data were expressed as means ± standard error (n = 5). Statistical analysis was performed among different treatments at the same time point, and different letters at the same time point indicate statistically significant ($p < 0.05$).

Adaxial IWs and Abaxial EWs Are Respectively the Major Leaf Transpiration Barriers

To investigate tea leaf transpiration barrier organization, the EWs from the adaxial and abaxial surfaces were removed by gum arabic stripping. When the adaxial EWs were removed, the water loss showed small and statistically insignificant increase compared to untreated control (**Figures 4A,B**). Thus, our observation in tea leaves was in accordance with previous results from other plant species (Zeisler and Schreiber, 2016). However, when the abaxial EWs were removed, the water loss was significantly increased compared to untreated control (**Figures 4A,B**). When both adaxial and abaxial EWs were removed simultaneously, the water loss was not significantly different from leaves with abaxial EWs removal only (**Figures 4A,B**). These observations demonstrated that abaxial EWs were the major transpiration barrier while the adaxial IWs constituted another transpiration barrier.

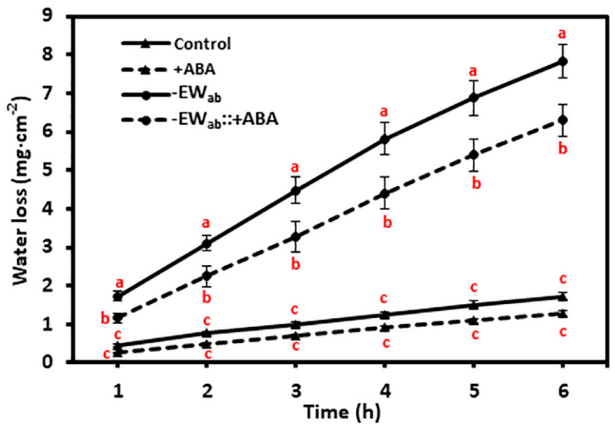

FIGURE 2 | Gum arabic treatment on abaxial surface did not affect stomata closure. Control, leaves not treated with ABA and gum arabic; + ABA, leaves treated with ABA only; -EW$_{ab}$, leaves without ABA pretreatment but treated with gum arabic; -EW$_{ab}$: + ABA, leaves treated with ABA and gum arabic. Data are expressed as means ± standard error (n = 5). Statistical analysis was performed among different treatments at same time point, and different letters at the same time point indicate statistically significant ($p < 0.05$).

Transpiration Rate Measurement of Adaxial and Abaxial Leaf Surfaces

To measure water transpiration rate from individual leaf surfaces, six different treatments were conducted simultaneously as detailed in the "Materials and Methods" section. In order to calibrate cuticular transpiration, it is essential to estimate residual stomatal transpiration. Two data sets were used for this purpose, including control (leaves without gum arabic and vaseline treatment) and leaves with adaxial surface coated by vaseline. The transpiration rates of the control leaves were kept constant during the first 4 h post-excision, and then showed a small but significant drop at 5 h post-excision (**Figure 5A**). To identify the source responsible for this transpiration decline, stomata aperture was measured before ABA pretreatment, 0 h, and 5 h post-excision. We found that stomata aperture was reduced 34% by ABA treatment compared with untreated control and was further reduced 59% at 5 h post-excision (**Figures 5B,C**). After excision, leaf water continuously evaporated, which would reduce the overall leaf water content (Clarke et al., 1991). To investigate how the decline of leaf water content affect stomatal transpiration and cuticular transpiration, leaf transpiration rates and corresponding relative water deficit (RWD) were calculated over 10 h post-excision. We found that for the first 4 h post-excision, the decline in transpiration rates were correlated with RWD increase; starting at 5 h post-excision, the transpiration rate was kept constant regardless of RWD increase (**Figure 5D**). Burghardt and Riederer (2003) also reported that after complete

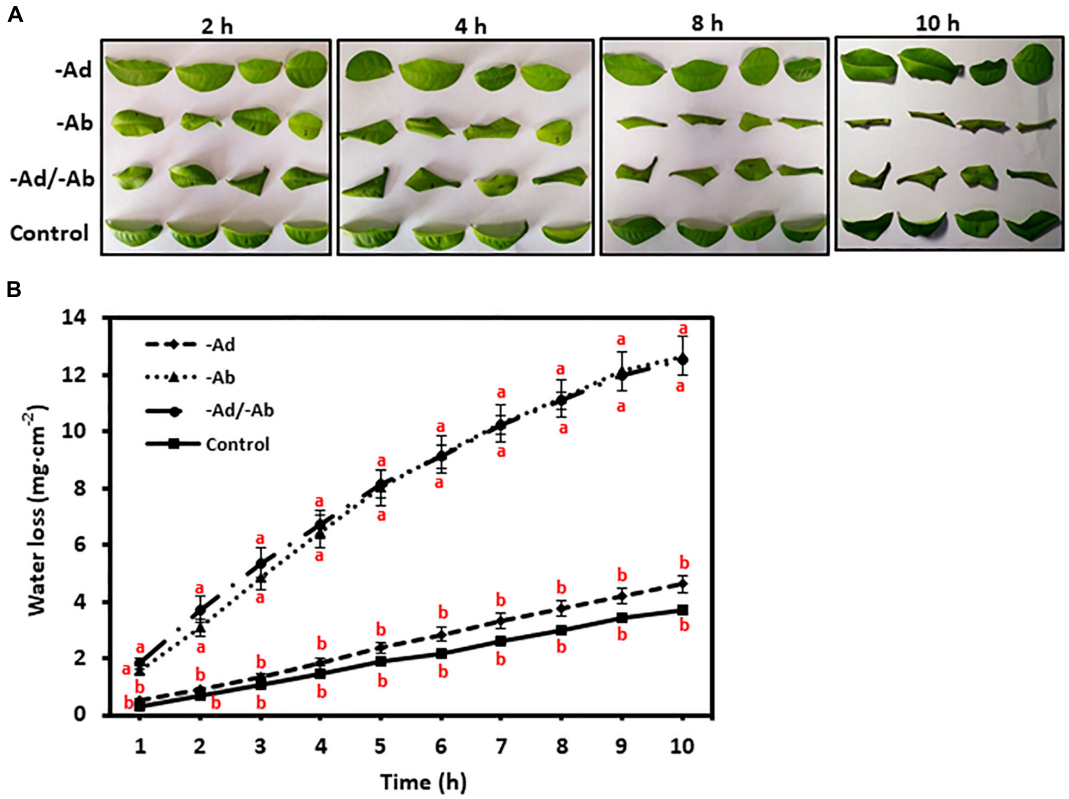

FIGURE 4 | Adaxial and abaxial epicuticular waxes had different contribution to leaf transpiration barrier. -Ad, adaxial epicuticular waxes were removed by gum Arabic; -Ab, abaxial epicuticular waxes were removed by gum Arabic; -Ad/-Ab, epicuticular waxes from both surfaces were removed by gum arabic. Data are expressed as means ± standard error (n = 5). Statistical analysis was performed among different treatments at same time point, and different letters at same time point indicate statistically significant ($p < 0.05$). **(A)** Leaf morphological changes after epicuticular wax removal by gum arabic; **(B)** Leaf water loss was affected by the epicuticular wax removal.

stomatal closure, a constant minimum transpiration rate was maintained over a wide range of water deficits. These data suggest that stomata closure was responsible for the transpiration drop at 5 h post-excision (**Figure 5A**), while cuticular transpiration was not affected by the reduction of leaf water content. Thus, the transpiration difference between the 5th h and the 1st h can be used to estimate residual stomatal transpiration (T_{Ab_s}); this led to the estimation of residual stomatal transpiration rate as 0.06 ± 0.008 mg h^{-1} cm^{-2}, which account for 22% of total leaf transpiration. When the adaxial leaf surface was sealed by vaseline, the residual stomatal transpiration accounted for 28% of the observed total leaf transpiration. When the leaf abaxial surface was sealed by vaseline, the observed transpiration rate was reduced by 0.13–0.15 mg. h^{-1} cm^{-2} compared to that of control leaves. Residual stomatal transpiration may be largely eliminated by vaseline coating on the abaxial surface; abaxial cuticular transpiration was thus reduced in the range of 0.07–0.09 mg h^{-1} cm^{-2} by vaseline coating (**Figure 5A**).

When the abaxial EWs were removed by gum arabic and the adaxial surface was sealed by vaseline, the observed transpiration rate drop was observed at 4 h post-excision (**Figure 5A**), and the transpiration difference between the 4th h and the 1st h was 0.042 ± 0.006 mg h^{-1} cm^{-2}, which was close to the value calculated from control leaves (0.06 ± 0.008 mg h^{-1} cm^{-2}). These data further demonstrated that gum arabic stripping on leaf abaxial surface did not affect stomata closure.

Based on the observed transpiration rates and the estimated residual stomatal transpiration rates, the transpiration rates from adaxial surface, adaxial IWs, abaxial surface, abaxial cuticle, and abaxial IWs were calculated according to their mathematical relationships in formula IV–IX (**Table 1**). Since leaf water content was continuously decreasing after excision, the transpiration rate at the first hour post-excision would offer a more accurate estimation. For comparison, the hourly transpiration rates during the 6-h post-excision are listed (**Table 1**). Vaseline diffusion coefficient factor (k), which is expressed as the ratio of transpiration with both leaf surfaces sealed by vaseline to that of control leaves, gradually increased from 0.21 to 0.29 during the 6-h post-excision; this mild increase mainly resulted from the gradual decrease of transpiration rate in the control (**Figure 5A**). The transpiration rates from either leaf surfaces or IWs showed general downward trends (**Table 1**); this was attributed to the k increase during post-excision. The transpiration rate from abaxial surface (T_{Ab}) was about 1-fold higher than that of adaxial surface (T_{Ad}), residual stomatal transpiration (T_{Ab_S}) accounted for one third of abaxial transpiration (T_{Ab}), and the remaining

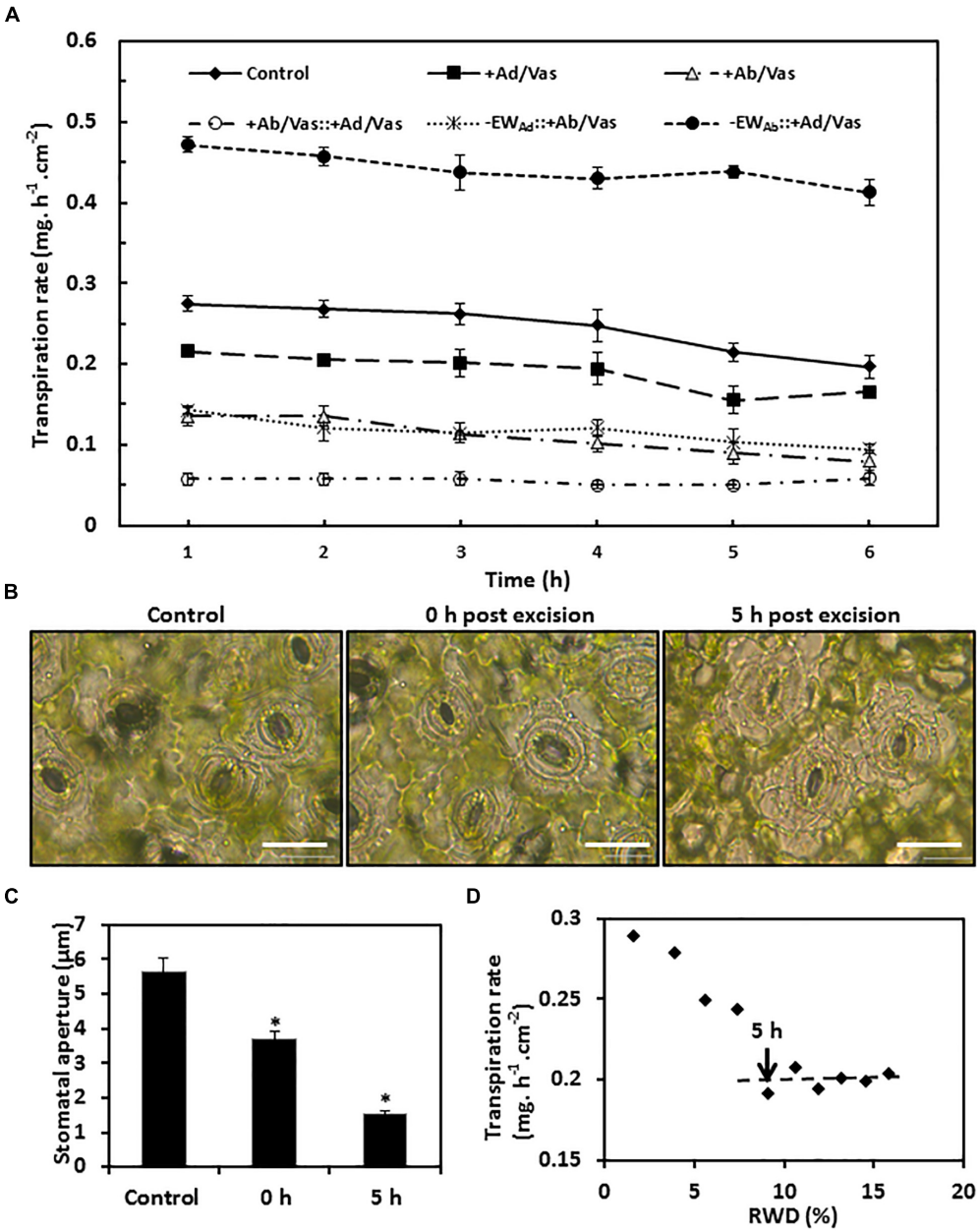

FIGURE 5 | Observed transpiration rate measurement and stomata changes during post-excision. **(A)** Observed transpiration rates during 6 h post-excision. +Ad/Vas, leaf adaxial surface sealed with vaseline; +Ab/Vas, leaf abaxial surface sealed with vaseline; +Ab/Vas: +Ad/Vas, both leaf surfaces sealed with vaseline; -EW_{Ad}: +Ab/Vas, adaxial epicuticular waxes removed by gum arabic and abaxial surface sealed with vaseline; -EW_{Ab}: +Ad/Vas, abaxial epicuticular waxes removed by gum arabic and adaxial surface sealed with vaseline. Data were expressed as mean ± standard error (n = 6). Statistical analysis was performed among different treatments at the same time point, and different letters at the same time point indicate statistically significant ($p < 0.05$). **(B)** Stomata changes before ABA treatment (control), and post-excision (0 h and 5 h). Abaxial epidermal cell layer was removed, mounted onto glass slide, and immersed in 0.9% NaCl solution, then immediately observed under microscope. Bar = 20 μm. **(C)** Stomata aperture before ABA treatment, and post-excision (0 and 5 h). Data were given as mean ± standard error (n = 20). Asterisks indicate statistically significant at 0.01 levels. **(D)** The regression curve between leaf transpiration rates and relative water deficit (RWD) during 10 h post-excision.

two thirds should be attributed to abaxial cuticular transpiration (T_{Ab_C}). After excluding residual stomatal transpiration, the abaxial cuticular transpiration rate was still about 50% higher than that of adaxial surface. When adaxial EWs were removed by gum arabic, the transpiration rate from adaxial IW ($T_{Ad/intra}$) remained almost unchanged as that of control leaves (T_{Ad}), indicating that adaxial EWs did not contribute much to the transpiration barrier. However, when abaxial EWs were removed by gum arabic, the abaxial IW transpiration rate ($T_{Ab/intra\_c}$) was more than onefold higher than that of control leaves (T_{Ab_C}), and

TABLE 1 | Water transpiration rates from different leaf sides and different cuticle layers.

	K	T_{Ad} (mg $h^{-1}.cm^{-2}$)	T_{Ab} (mg $h^{-1}.cm^{-2}$)	T_{Ab-C} (mg $h^{-1}.cm^{-2}$)	$T_{Ad/intra}$ (mg $h^{-1}.cm^{-2}$)	$T_{Ab/intra}$ (mg $h^{-1}.cm^{-2}$)	$T_{Ab/intra-C}$ (mg $h^{-1}.cm^{-2}$)
1 h	0.206 ± 0.019	0.094 ± 0.011^{e}	0.199 ± 0.006^{c}	0.142 ± 0.002^{d}	0.104 ± 0.002^{e}	0.452 ± 0.008^{a}	0.413 ± 0.004^{b}
2 h	0.211 ± 0.022	0.095 ± 0.012^{e}	0.190 ± 0.005^{c}	0.133 ± 0.003^{d}	0.097 ± 0.005^{e}	0.437 ± 0.009^{a}	0.398 ± 0.003^{b}
3 h	0.224 ± 0.035	0.069 ± 0.003^{d}	0.198 ± 0.017^{b}	0.141 ± 0.013^{c}	0.077 ± 0.010^{d}	0.422 ± 0.022^{a}	0.383 ± 0.017^{a}
4 h	0.213 ± 0.036	0.061 ± 0.007^{d}	0.189 ± 0.023^{b}	0.132 ± 0.019^{c}	0.087 ± 0.005^{d}	0.417 ± 0.012^{a}	0.378 ± 0.006^{a}
5 h	0.238 ± 0.031	0.053 ± 0.010^{e}	0.154 ± 0.017^{c}	0.096 ± 0.013^{d}	0.078 ± 0.013^{de}	0.426 ± 0.006^{a}	0.387 ± 0.004^{b}
6 h	0.293 ± 0.034	0.029 ± 0.014^{e}	0.172 ± 0.005^{c}	0.115 ± 0.008^{d}	0.048 ± 0.006^{e}	0.405 ± 0.012^{a}	0.365 ± 0.006^{b}

K: Vaseline diffusion coefficient; T_{Ad}: adaxial cuticular transpiration rate; T_{Ab}: abaxial transpiration rate; T_{Ab-C}: abaxial cuticular transpiration rate; $T_{Ad/intra}$: adaxial intracuticular transpiration rate; $T_{Ab/intra}$: abaxial intra transpiration rate; $T_{Ab/intra-C}$: abaxial intracuticular transpiration rate. Data are given as means ± standard error (n = 6). The different letters in the same row indicate significant difference among treatments at the 0.05 level.

was threefolds higher than that of adaxial IW (**Table 1**). These data further demonstrated that the adaxial IWs and the abaxial EWs were the major leaf transpiration barriers, while adaxial EWs and abaxial IWs contributed minor to the cuticular transpiration barriers in *C. sinensis*.

Adaxial and Abaxial IW Compositional Analysis

To understand the chemical base of the transpiration difference, the composition of adaxial and abaxial IWs was analyzed. We found that the adaxial IWs showed higher coverage in very long chain fatty acids (VLCFAs), 1-alkanol esters, and glycols than that of abaxial IWs. Interestingly, caffeine was detected from the cuticle, and more enriched in the abaxial IW than the adaxial IWs (**Figure 6A**). The coverage of triterpenoids and steroids were similar between adaxial and abaxial IWs (**Figures 6A,D**).

The chain length distributions were also compared. Adaxial IWs were more enriched with longer-chain fatty acids (C24–C28), alkanes (C29, C31, and C35), 1-alkanols (C26 and C28), and glycol esters (C18 and C21) compared with abaxial IWs (**Figures 6A,C**).

The total glycol content of adaxial IWs was significantly higher than that of abaxial IWs, and mostly contained C20 and small portions of C16 and C22 molecules (**Figure 6D**). For triterpenoids and steroids, although their total contents were similar (**Figure 6A**), individual terpenoid component still showed large variations between adaxial and abaxial IWs (**Figure 6E**).

DISCUSSION

The Assumptions, Limitations, and Advantages of the Proposed Method

The goal of this study is to measure transpiration rates from excised leaf; thus, the method of choice has to meet the following requirements: (1) the method should be able to specifically and consistently strip off EWs without affecting IWs; (2) the method should be non-destructive to the excised leaf. Jetter and Schäffer (2001) have demonstrated that gum arabic can specifically and consistently remove EWs by comparing with the most selective method that employs frozen glycerol as cryo-adhesive. Zeisler and Schreiber (2016) independently confirmed gum arabic's specificity and consistency for EW removal. Jetter and Riederer (2016) applied gum arabic to remove EWs from eight plant species and located the transpiration barriers. We performed SEM and TEM observation to demonstrate its applicability to tea leaves; our data shown in **Figure 1** were in accordance with previous reports (Jetter and Schäffer, 2001; Jetter and Riederer, 2016; Zeisler and Schreiber, 2016). Based on these data, we believe that gum arabic is the best fit for this research objective. Compared to other adhesives that often require organic solvents, gum arabic can be easily dissolved in water and thus avoid the possible side effects of organic solvent on IWs.

Several alternative methods in the literature have been used to remove EWs; they are either cryo-adhesive-based or polymer-based mechanical wax removal. Glycerol or water was commonly used as cryo-adhesive. Due to its destructive nature, cryo-adhesive-based wax removal method does not fit the described method. For polymer-based mechanical wax removal, collodion, cellulose acetate, and gum arabic were widely used (Haas and Rentschler, 1984; Silcox and Holloway, 1986; Jetter and Schäffer, 2001). Zeisler and Schreiber (2016) demonstrated that the organic solvent acetone used to dissolve cellulose acetate affected the transpiration barrier; thus, cellulose acetate was out of our consideration. Interestingly, the authors demonstrated that collodion and gum arabic are equally efficient to remove EWs; in addition, the collodion solvent (diethyl ether:ethanol) did not show side effects on IWs. These observations suggested that collodion could be an alternative EW removal agent. Compared to gum arabic, collodion shows several advantages for EW removal: (1) it can be conveniently applied to the CM surface; (2) solvent evaporation and the polymer film formation are fast (∼30 s) and thus can speed up the experiment and save time; (3) it does not damage CMs. In contrast, the water used to dissolve gum arabic evaporates much slower and takes about 1 h to form polymer film. In addition, gum arabic is not suitable to remove EWs from CMs. Although collodion has been demonstrated to be working well to remove EWs from the adaxial surface (Zeisler and Schreiber, 2016), it remains unclear if it is applicable to remove abaxial EWs; the presence of stomata on abaxial surface may affect collodion's applicability on this surface.

Vaseline is a mixture of semi-solid non-polar hydrophobic hydrocarbon and insoluble in water, and it possesses excellent coating properties and is widely used as an ingredient in skin lotions and cosmetics by reducing skin moisture loss. In this

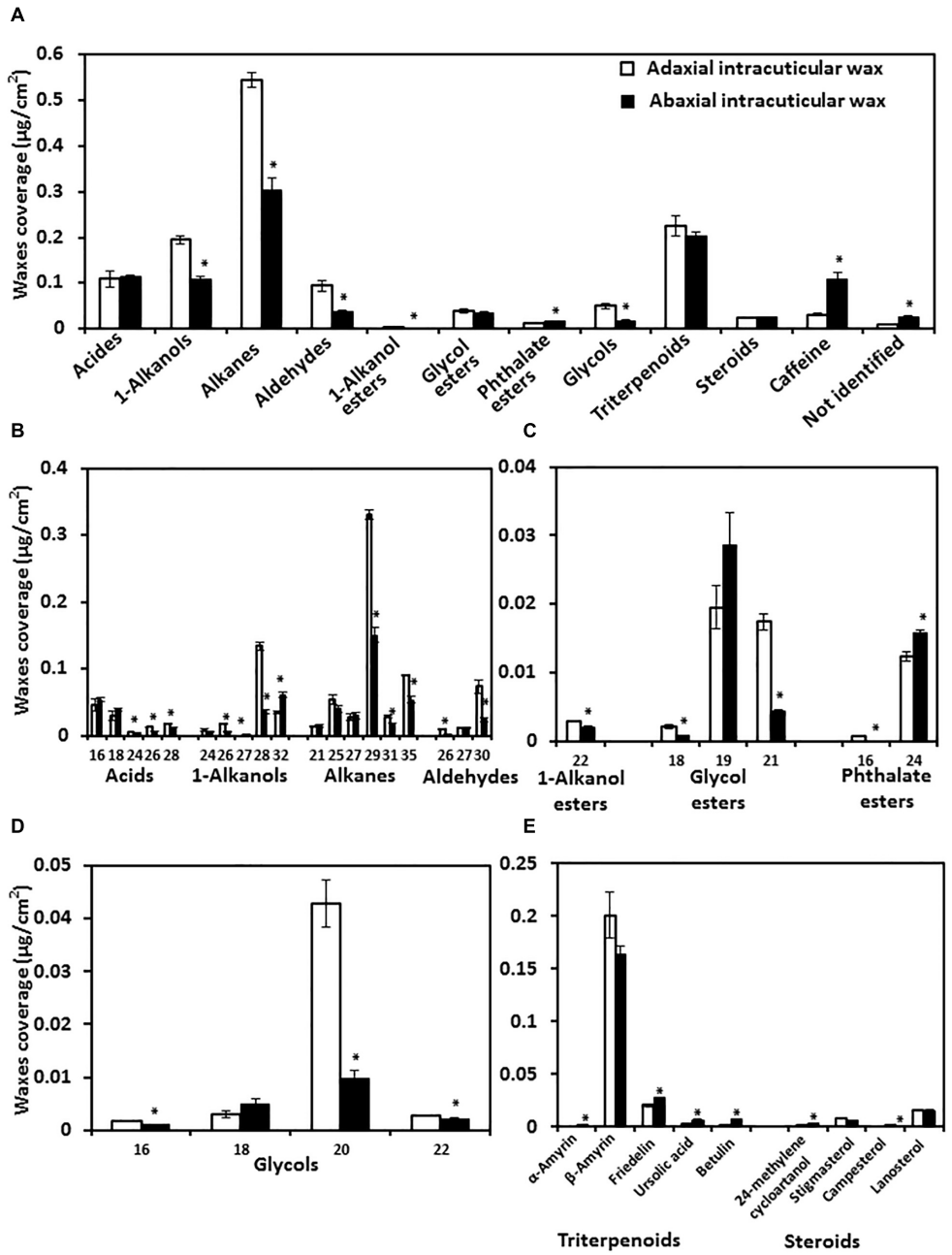

FIGURE 6 | Wax lipid composition and chain length distribution of adaxial and abaxial intracuticular waxes. **(A)** Intracuticular wax compositions. **(B)** Chain length distributions of fatty acids and their derivatives. **(C)** Chain length distributions of esters. **(D)** Chain length distributions of glycols. **(E)** Chain length distributions of triterpenoids and steroids. Data were expressed as mean ± standard error ($n = 4$). *Statistically significant ($p < 0.05$).

study, we tested the effectiveness of vaseline in reducing leaf water loss. We found that vaseline application can reduce more than 70% leaf water loss (**Figure 3**). Thus, vaseline diffusion coefficient factor (k) was introduced in formula IV to XI to consider this leakage. k reflects the proportion of water loss through the applied vaseline film and should be affected by the thickness and uniformity of the vaseline film. During our experiment, care was taken to ensure that vaseline was evenly coated on both surfaces, and reproducible k was obtained in our experiments. As we mentioned above, k essentially reflects the physicochemical characteristics of the vaseline itself regardless where it is applied; theoretically, k should be stable once a consistent film was applied. However, during the 6-h post-excision, k increased from 0.21 to 0.29 (**Table 1**); this mild increase is mainly due to the way how it is calculated. We demonstrated that the gradual decrease of transpiration rates from the control leaves rather than from

sealed leaves is responsible for the variation of k, the gradual decrease of transpiration rates from the control could be driven by the gradual stomata closure during post-excision (**Figure 5A**). As the data showed in **Table 1**, slight variation of k during post-excision had a large influence on the calculation of transpiration rates; we believe that the k value of the first hour should be used, and the transpiration rates at the first hour post-excision should be more accurate. One unresolved question is that if vaseline may interact with waxes, cutin and other lipids present in the cuticle even dissolve and diffuse in the cuticle due to its non-polar nature, these interactions consequently could alter cuticle structure and functionality. Although we can't exclude these possibilities, considering that the transpiration rates from vaseline-coated leaves were kept constant during post-excision (**Figure 5A**), we tend to believe that vaseline application did not significantly interfere this proposed method.

The proposed method has two major limitations: (1) It does not apply to the leaves covered with trichomes, since gum arabic application will damage trichomes and cause uncontrolled water loss. In *C. sinensis* cv *Fuyun 6*, trichomes are present on the abaxial surfaces of tender leaves and shed off with leaf maturation. Thus, in this study, the mature fourth leaf was used. (2) It requires multi-step simultaneous leaf pretreatments.

Compared to other transpiration measurement methods, the proposed method has two advantages: (1) intact leaves can be directly used for transpiration rate measurement, and the obtained results are more relevant to leaf transpiration *in planta*. In contrast, other reported methods require the isolation of intact CMs, which is labor-intensive and time-consuming. (2) The proposed method can obtain transpiration rates from both leaf surfaces at the same time, and residual stomatal transpiration can also be estimated. This will offer us an integrated image about leaf transpiration.

Residual Stomatal Transpiration Calculation

Accurate estimation of residual stomatal transpiration is essential for the calculation of abaxial cuticular transpiration, which raise the question if gum arabic stripping on abaxial surface affects stomatal closing. Here, three lines of evidences suggested that this is not the case: (1) stomata structure was kept intact after gum arabic stripping (**Figures 1D,E**); (2) gum arabic stripping to leaf abaxial surface did not affect ABA-promoted stomata closure (**Figure 2**); (3) the residual stomata transpiration rate from gum arabic-stripped leaves was similar to that of control leaves (**Figure 5A**).

Generally, stomata show variable response to ABA-promoted closing; this will lead to relatively large variations in leaf transpiration measurement. However, our transpiration data presented in **Figures 2** and **3** showed small standard error bars; this may be attributed to the multi-step pretreatment procedures: (1) leaves were fully water equilibrated before experiment; it is well known that leaf water contents have a large influence on stomata transpiration; (2) leaves at the same developmental stage (the fourth leaf) were used; it is well-established that tender leaf and mature leaf show large variations in leaf water contents and transpiration rates; (3) the leaves were dark adapted overnight before ABA treatment; it is well known that dark promotes stomata closure. These data highlight the importance of the multi-step pretreatment procedures described in this proposed method.

If ABA treatment can make full stomata closure, then the 1st h transpiration difference between control leaves and ABA-treated leaves would accurately represent the residual stomatal transpiration. However, 50 μM ABA treatment did not make stomata fully close (**Figures 5B,C**). In another pretrial, 100 μM ABA was applied, but stomata aperture was similar to 50 μM ABA treatment. These observations suggested that for fully water equilibrated leaves, stomata remain partially open even after overnight dark adaptation; ABA application alone cannot make stomata close fully. However, at 5-h post-excision, stomata were mostly closed (**Figures 5B,C**); this is likely promoted by the decreasing leaf turgor pressure and the enhanced ABA synthesis during post-excision. Thus, the transpiration difference between 1st h and 5th h post-excision of the control leaf can be used to calculate stomatal transpiration. Considering that, even without ABA pretreatment, stomata still come to close fully at some time point of post-excision, this raises the question about the necessity of ABA pretreatment. We tend to believe that it is not plausible and not necessary through ABA application to obtain maximal reduction in stomatal transpiration, but ABA treatment can reduce sample variations; thus, it is helpful to obtain consistent reduction in stomatal transpiration. It is anticipated that the transpiration rate at 5th h post-excision could be lower than that of 1st h post-excision. Hence, the actual residual stomatal transpiration could be overestimated by this proposed method; accordingly, the abaxial cuticular transpiration was underestimated. Even so, the abaxial cuticular transpiration rate was still 50% higher than the adaxial cuticular transpiration rate (**Table 1**). Thereby, our main conclusions should not be affected much by the overestimation of residual stomatal estimation. The stomatal transpiration estimation in the proposed method assumed that the residual stomatal transpiration after stomata closure is fully negligible. Burghardt and Riederer (2003) observed that the minimum conductance of *Hedera helix* was threefold higher than the cuticular permeance; in contrast, the minimum conductance from the other four plant species was similar to their cuticular permeance. Based on these data, the authors suggested that the residual stomatal transpiration after full stomata closure was negligible for most plant species except *H. helix*. However, from the stated methods, the cuticular permeance was measured from adaxial cuticle, while the minimum conductance was measured from both adaxial and abaxial surfaces. An alternative explanation why minimum conductance of *H. helix* was threefold higher than the cuticular permeance could be due to its abaxial cuticular conductance much higher than that of its adaxial cuticular conductance; this will lead to a higher average value (so called "minimum conductance" by authors) than the adaxial cuticular permeance (so called "cuticular permeance" by authors). To further support this explanation, Šantrůček et al. (2004) demonstrated that for *H. helix* leaves, abaxial cuticular transpiration was about 11 times higher than

that of the adaxial cuticular transpiration. These data suggested that after full stomata closure, residual stomata transpiration was negligible for all five plant species tested, and this likely is true for tea leaves. Thus, stomatal transpiration can be reliably estimated by using the transpiration difference before and after full stomata closure of control leaves.

Tea Leaf Adaxial and Abaxial Surfaces Show Different Transpiration Barrier Organization

Zeisler and Schreiber (2016) and Zeisler et al. (2018) studied 10 different plant species including tea tree and concluded that the adaxial EWs were not the main cuticular transpiration barrier, and our data were in accordance with that conclusion. In addition, unlike the adaxial EWs, the abaxial EWs contributed significantly to the cuticular transpiration barrier (**Figures 4**, **5**). The adaxial EWs stripped off by gum arabic was only slightly higher than that of abaxial EWs (**Supplementary Table S1**), suggesting that gum arabic had similar efficiencies in removing EWs in adaxial as in abaxial surfaces; the lower effect on transpiration induced by gum arabic when applied to adaxial surface was unlikely due to less EWs removed compared to abaxial surfaces. At the tissue level, adaxial IWs and abaxial EWs constituted the major leaf transpiration barriers, while adaxial EWs and abaxial IWs contribute negligibly to leaf transpiration barrier. We also demonstrated that abaxial cuticular transpiration rate was 50% higher than adaxial cuticular transpiration rate (**Table 1**); this was in accordance with the report of Šantrůček et al. (2004) even though different methods were applied by these two studies.

Relationships Between Wax Composition and Cuticular Transpiration

Cuticle is a highly heterogeneous structure and formed by an array of compounds with different physicochemical properties (Fernández et al., 2017). Khayet and Fernández (2012) demonstrated that alkanes are fully apolar molecules, whereas molecules containing oxygen in their functional groups (e.g., alcohols, acids, ketones, or ester bonds) had some degree of polarity and H-bonding interactions. Thus, it is anticipated that wax composition would affect cuticular transpiration properties. After comparing transpiration in combination with wax analysis from eight different plant species, Jetter and Riederer (2016) concluded that transpiration barrier is associated mainly with VLCFA derivatives. Here, we found that transpiration rate from abaxial IWs was threefold higher than that of adaxial IWs (**Table 1**); this provided an independent system to reexamine that conclusion (Jetter and Riederer, 2016). We found that some compounds from adaxial IWs, including VLCFAs (1-alkanols, alkanes, and aldehydes), 1-alkanol esters, and glycols, showed higher contents than that of abaxial IWs (**Figure 6A**). Thus, our data from tea leaves were in accordance to that conclusion (Jetter and Riederer, 2016). Currently, there are few integrative studies on the internal nature of cuticle (Viougeas et al., 1995; Yeats et al., 2012); it remains largely unknown if there are potential cross-links and/or molecular assemblies between cuticular chemical constituents (Kunst and Samuels, 2009). In addition, given the major structural and chemical variability of plant cuticles, more research was still needed before a valid general cuticle model could be established for all species or organs (Fernández et al., 2017). Interestingly, caffeine was detected from tea IWs as well as EWs, and more enriched from abaxial IWs compared to adaxial IWs. Currently, it remains unclear if caffeine has any contribution to cuticle barrier properties. Previous studies demonstrated that caffeine has potent antipathogenic and antiherbivore activities (Uefuji et al., 2005; Kim and Sano, 2008; Wang et al., 2016), suggesting that caffeine is potentially involved in cuticle's defense against pathogens and herbivores rather than a contributor to transpiration barrier.

CONCLUSION

We established a new method that allows simultaneous measurement of cuticular transpiration rates from individual leaf surface. By applying this method, we demonstrated that the adaxial IWs and the abaxial EWs constitute the major cuticular transpiration barriers; the abaxial cuticular transpiration rate was 50% higher than that of adaxial surface. These new findings would facilitate the identification of key factors that delineate leaf barrier properties.

DATA AVAILABILITY STATEMENT

All data generated or analyzed for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YZ and XC carried out experiments and prepared SEM and TEM samples. YZ analyzed the wax lipids with ZD, WZ, and ZC. CC provided the study materials. MC and WS designed the experiments. MC, YZ, and ZC wrote the manuscript. AD helped with manuscript editing. All authors contributed to manuscript revision, and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00420/full#supplementary-material

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Changes in Tea Plant Secondary Metabolite Profiles as a Function of Leafhopper Density and Damage

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Insect herbivores have dramatic effects on the chemical composition of plants. Many of these induced metabolites contribute to the quality (e.g., flavor, human health benefits) of specialty crops such as the tea plant (*Camellia sinensis*). Induced chemical changes are often studied by comparing plants damaged and undamaged by herbivores. However, when herbivory is quantitative, the relationship between herbivore pressure and induction can be linearly or non-linearly density dependent or density independent, and induction may only occur after some threshold of herbivory. The shape of this relationship can vary among metabolites within plants. The tea green leafhopper (*Empoasca onukii*) can be a widespread pest on tea, but some tea farmers take advantage of leafhopper-induced metabolites in order to produce high-quality "bug-bitten" teas such as Eastern Beauty oolong. To understand the effects of increasing leafhopper density on tea metabolites important for quality, we conducted a manipulative experiment exposing tea plants to feeding by a range of *E. onukii* densities. After *E. onukii* feeding, we measured volatile and non-volatile metabolites, and quantified percent damaged leaf area from scanned leaf images. *E. onukii* density had a highly significant effect on volatile production, while the effect of leaf damage was only marginally significant. The volatiles most responsive to leafhopper density were mainly terpenes that increased in concentration monotonically with density, while the volatiles most responsive to leaf damage were primarily fatty acid derivatives and volatile phenylpropanoids/benzenoids. In contrast, damage (percent leaf area damaged), but not leafhopper density, significantly reduced total polyphenols, epigallocatechin gallate (EGCG), and theobromine concentrations in a dose-dependent manner. The shape of induced responses varied among metabolites with some changing linearly with herbivore pressure and some responding only after a threshold in herbivore pressure with a threshold around 0.6 insects/leaf being common. This study illustrates the importance

of measuring a diversity of metabolites over a range of herbivory to fully understand the effects of herbivores on induced metabolites. Our study also shows that any increases in leafhopper density associated with climate warming, could have dramatic effects on secondary metabolites and tea quality.

Keywords: *Camellia sinensis*, *Empoasca onukii*, secondary metabolites, herbivory, induced responses, plant VOCs, catechins, crop quality

INTRODUCTION

Insect herbivores have the potential to induce chemical changes in the plants they feed on, with notable implications for ecological interactions and crop quality in agricultural systems. Induced plant responses to herbivores include increased production of secondary metabolites that reduce herbivore feeding or fitness (Karban et al., 1997), or upregulation of indirect defenses such as volatile organic compounds or extrafloral nectar that attract natural enemies (Arimura et al., 2005). As global environmental change impacts herbivore populations (Berggren et al., 2009; Bebber et al., 2013), it is also likely to impact induced plant responses including the production of secondary metabolites (DeLucia et al., 2012).

Most studies elucidating induced plant responses to herbivores have drawn conclusions from comparisons of plants with no damage to plants with damage at a single level. However, plants growing in the wild or agricultural settings are exposed to a range of herbivory. Several studies have found variation in the relationship between the extent of herbivore damage and plant responses induced by this damage. For example, Underwood (2000) found a threshold in the amount of damage required to induce resistance in soybean. This threshold varied among soybean genotypes with cultivars responding at lower or higher levels of herbivory and one cultivar that did not show induced resistance even at 90% leaf damage.

Furthermore, the relationship between herbivore pressure and secondary metabolite concentration often varies among individual compounds resulting in a change in metabolite profiles with increasing herbivory (Horiuchi et al., 2003; Shiojiri et al., 2010; Cai et al., 2012, 2014). For example, Shiojiri et al. (2010) demonstrated that volatile compounds induced by diamondback moth (*Plutella xylostella*) larvae feeding on cabbage varied in direction and magnitude of induction. Specifically, the metabolite sabinene was produced at a higher concentration with 5% damage compared to 15 and 30% damage while emission of (*Z*)-3-hexenyl acetate was induced only at 30% damage. Similarly, Horiuchi et al. (2003) identified only 5 out of 10 compounds induced by *Tetranychus urticae* as being induced in a density-dependent manner (increasing concentration with increasing herbivore density). By investigating the impact of a range of herbivore densities on multiple classes of metabolites, we can develop a comprehensive understanding of the impact of herbivores on plant chemistry.

Herbivore density-dependent changes in plant chemistry have an important role in ecological interactions. For example, herbivores may respond to induced volatile profiles as ecological cues for conspecific density, being attracted to a volatile profile emitted by plants with low or moderate infestations, but repelled by a volatile profile produced by heavily infested plants (Horiuchi et al., 2003; Robert et al., 2012). Plant volatile profiles also serve as a signal of host density for parasitoids (Geervliet et al., 1998; Girling et al., 2011) and even hyperparasitoids (Cusumano et al., 2019). In agricultural systems, the quality of many crops is highly dependent on secondary metabolite profiles that impact flavor and nutritional quality for humans (Ahmed et al., 2014; Scott and Orians, 2018; Wüst, 2018; Johnson et al., 2019). Reductions in crop quality have important economic consequences for farmers that can outweigh reductions in yield (Kawasaki and Uchida, 2016). In integrated pest management, an economic threshold is the density of a pest at which some treatment will result in an economic return mostly based on crop yield (Higley and Pedigo, 1996). Understanding the relationship between herbivory, secondary metabolite induction, and crop quality would lead to more comprehensive economic thresholds for herbivory compared to taking only yield into account.

Tea is a globally important specialty crop produced from young leaves of the tea plant, *Camellia sinensis* (L.) O. Kuntze. Tea quality is important for consumers and farmer livelihoods (Ahmed et al., 2014; Boehm et al., 2019) and is influenced not only by the total concentration of secondary metabolites in tea, but also by the relative proportion of different metabolites (metabolite profile) (Zeng et al., 2019). Tea secondary metabolites are known to vary as a function of multiple environmental and management factors (Ahmed et al., 2019) including plant genotype (Cherotich et al., 2013; Chen et al., 2018; Mu et al., 2018), shade (Sano et al., 2018), elevation (Han et al., 2017; Kfoury et al., 2018b), drought (Scott et al., 2019), precipitation (Ahmed et al., 2014; Kowalsick et al., 2014), temperature (Lee et al., 2010), model of agricultural production (Ahmed et al., 2013; Han et al., 2018), microbes (Singh et al., 2010), and numerous pest insects (Scott and Orians, 2018). These environmental factors are shifting with global change including climate change and are impacting tea quality (Ahmed et al., 2019).

The tea green leafhopper (*Empoasca onukii* Matsuda) is a major pest on tea (Mao et al., 2014). *Empoasca onukii* are cell rupture feeders and inject a watery saliva containing a suite of digestive and oxidative enzymes into plant tissues while feeding (Backus et al., 2005; Jin et al., 2012). Leafhopper feeding on tea causes a set of symptoms known as "hopperburn," including yellowing (chlorosis), thickening of leaves, occasionally necrosis at leaf margins, and sometimes leaf abscission. In Taiwan and Southern China, *E. onukii* can reduce tea yields by 15–20% (Fu et al., 2014). Leafhopper damage also induces the production of volatiles by tea plants (Cai et al., 2014), with implications for crop quality including flavor. In fact, farmers in some regions

take advantage of changes in volatiles induced by *E. onukii* to improve the aroma of processed tea by maintaining specific levels of leafhopper herbivory in their tea production systems (Kawakami et al., 1995; Cho et al., 2007; Mei et al., 2017; Scott and Orians, 2018). This style of "bug-bitten" tea originates in Taiwan with a tea known as Eastern Beauty oolong (Scott and Orians, 2018). In a growth chamber study, Cai et al. (2014) investigated the effects of low and high densities of *E. onukii* on tea volatile metabolites and found that higher densities were associated with higher volatile emission.

Leafhoppers such as *E. onukii* are expected to increase their population densities with climate change (Masters et al., 1998; Bale et al., 2002; Baker et al., 2015). *Empoasca onukii* currently has 9–15 overlapping generations per year in China and Taiwan and 5–8 generations per year in more temperate Japan (Fu et al., 2014). Although it is known that *E. onukii* induces changes in tea plant chemistry, the effects of a range of *E. onukii* densities on the induction of volatiles is unknown, and studies on leafhopper induction of both volatile and non-volatile metabolites are lacking. It is also unclear how these responses translate to studies in the field. This study seeks to address the aforementioned research gaps.

In this study, we manipulated leafhopper density on potted tea plants grown outdoors to determine the impact of leafhopper herbivory on the concentrations of volatile and non-volatile secondary metabolites that determine tea quality. In addition to the impact on tea quality, the relationship between leafhopper damage and secondary metabolite concentrations is important for ecological interactions, such as attracting natural enemies (Gao et al., 2004).

MATERIALS AND METHODS

Leafhopper Collection

Tea plants are commonly attacked by a single species of leafhopper, *Empoasca onukii* Matsuda (Mao et al., 2014). Leafhoppers were reared from eggs collected from tea fields and supplemented with nymphs aspirated from tea leaves in the field. *Empoasca onukii* lays its eggs under the epidermis of young tea stems, and eggs were found on field plants using a portable version of the Simplified Leafhopper Egg Detection by Autofluorescence (SLEDA) method described by Herrmann and Böll (2004). Under the light of a blue LED flashlight, leafhopper eggs fluoresce green and chlorophyll fluoresces red. By wearing goggles that block blue light (Ultra-spec 2000 S0360X, Uvex, Fürth, Germany), leafhopper eggs in the tea shoots are visible as small green dots (**Supplementary Figure S1**). Shoots with eggs were cut and placed in hydrated floral foam inside of a mesh bug dorm (MegaView Science, Taichung, Taiwan). First instar nymphs were found on the tea shoots in the bug dorm within a week. This lab-hatched population was supplemented with leafhopper nymphs aspirated from tea plants in the field. Leafhoppers were fed by replacing the tea shoots in the floral foam regularly. Leafhopper eggs and nymphs were collected from several tea cultivars at Shanfu Tea Company in Shaxian, Fujian Province, China.

Experimental Approach

In the summer of 2017, we assessed the effect of leafhopper density on plant chemical responses using 2-year-old potted tea plants of the Qing Xin Da Mao (青心大冇) cultivar at the Shanfu Tea Company in Shaxian, Fujian Province, China. Each pot contained 3–4 plants that were propagated from cuttings and grown outdoors. Qing Xin Da Mao was chosen because it is a popular cultivar for production of Eastern Beauty oolong (Perin, 2019). On June 19, 20 pots were individually covered with mesh fabric bags (<0.25 mm mesh) after removing any visible insects. On June 25, leafhopper nymphs (2nd–4th instars) from the lab colony were introduced to the potted plants in the mesh bags based on randomly assigned density treatments. Leafhopper density treatments were chosen based on preliminary surveys of leafhopper density in tea fields (unpublished). Leafhopper density on Qing Xin Da Mao plants in a field at Shanfu tea company was 0.24 ± 0.12 leafhoppers per young leaf. The leafhopper density in the fields was considered low according to the farm manager, so our treatments were 0, 0.5, 1, 1.5, or 2.0 insects per young leaf, which bracketed the field density. The potted tea plants had an average of 12.7 young leaves and the number of leafhoppers added ranged from 0 to 30. Because leafhoppers can die during transfer (personal observation), and leafhopper nymphs may have hatched from eggs already present on the plants, we used the final density of leafhoppers at the end of the experiment for all analyses. We terminated the experiment after 4 days (June 29), when plants in high density treatments showed symptoms of hopperburn. Four days is well known to be sufficient to observe induced chemical responses in tea plants (Cai et al., 2014; Li et al., 2018; Liao et al., 2019). At the end of the experiment (June 29) we sampled volatiles and collected leaves for non-volatile analysis.

Volatiles were sampled for 2 h from a representative leaf in each pot by direct contact sorptive extraction (DCSE) (Kfoury et al., 2017). We performed DCSE by placing a polydimethylsiloxane (PDMS) coated magnetic stir bar (Twister® brand, Gerstel, Mülheim an der Ruhr, Germany) on the abaxial surface of a leaf and holding it in place with two small (∼2.5 mm dia.) neodymium magnets on the adaxial surface of the leaf. During DCSE sampling, volatile compounds from the headspace and leaf surface are absorbed by the PDMS (Kfoury et al., 2017). This method was preferred over headspace methods because it allowed us to easily sample all plants in the study simultaneously with minimal equipment in the field. A field blank was collected by attaching a PDMS stir bar to a metal binder clip attached to a wooden stake at a height and distance from other plants similar to the PDMS stir bars on leaves. After 2 h, we removed the PDMS stir bars from leaves, sealed them in vials and shipped them to Tufts University for analysis. After sampling volatiles, the leaf sampled by DCSE was marked using a permanent marker on the petiole, and we harvested all young leaves and scanned the abaxial surfaces on a flatbed scanner (Perfection V19, EPSON, Suwa, Japan). Because no drying oven was available at this field site, we microwaved the leaves on medium power for 2 min to stop enzymatic activity, and then continued to microwave 1 min at a time, cooling the samples to room temperature between bouts, until dry (Ahmed, 2011).

Image Analysis of Visible Leaf Damage

In addition to measuring the final density of leafhoppers at the end of the experiment we quantified visible leaf damage (stippling and browned tissue) as determined through pixel classification by machine learning. Using the scanned images of leaves, we created image files of individual leaves with petioles and any leaf folds or dark shadows manually edited out. Estimation of percent damaged leaf area was accomplished with the Trainable Weka Segmentation (TWS) plugin in FIJI (Schindelin et al., 2012; Schneider et al., 2012; Arganda-carreras et al., 2017). The TWS plugin is a machine learning tool that performs supervised pixel classification. To train the classifier, a random subset of 30 leaves was chosen and regions of interest were selected on each leaf representing damaged leaf area, undamaged leaf area, and background. Training features included the original image (hue, saturation, and brightness) as well as the Hessian, Sobel, variance, minimum, median, anisotropic, and bilateral filters provided in the plugin settings. The minimum and maximum sigmas were 2 and 16, respectively (Arganda-carreras et al., 2017). The training process was iterative and once we were satisfied with its performance on the training set, it was applied to a test set of 10 images to evaluate classifier performance. This test produced a Kappa coefficient of 0.998, indicating high accuracy. The final classifier was applied to all leaf images and numerical results were extracted using a custom script. Percent damage was calculated as damaged/(damaged + undamaged). Mean percent damaged leaf area was calculated for each pot. Additionally, we recorded damage to the focal leaf sampled by DCSE for each pot.

One low density leafhopper pot exhibited unusually high leaf necrosis (13.7% mean leaf damage at 0.33 leafhoppers/young leaf). Visual inspection revealed that two leaves were nearly entirely necrotic — damage that was unlikely to be due to direct effects of leafhopper feeding. This pot was therefore excluded from the experiment. As a consequence, a total of 19 pots with varying densities and levels of damage were used for further analyses.

Plant Secondary Chemistry

Volatiles by Gas Chromatography-Mass Spectrometry

Analysis of plant volatiles was done using established techniques (Kfoury et al., 2017). Analyses were performed on an Agilent 6890/5975 GC-MS (Santa Clara, CA) fitted with a MultiPurpose autosampler (Gerstel) and a 30 m × 250 μm × 0.25 μm RXI-5MS column (Restek). Prior to analysis by gas chromatography-mass spectrometry (GC-MS), PDMS stir bars used in DCSE sampling were directly spiked with 1 μl of 10 μg/ml naphthalene-d_8 (Restek, Bellefonte, PA, United States) as an internal standard. A thermal desorption unit (TDU, Gerstel GmbH, Müllheim an der Ruhr, Germany) provided spitless transfer of volatiles from the PDMS stir bars into a programmable temperature vaporization inlet (CIS, Gerstel) held at −100°C. Under helium gas flow (50 ml/min), the TDU was held at 40°C for 0.7 min, then heated to 275°C at 600°C/min and held at 275°C for 3 min. After 0.1 min the CIS was heated to 275°C at 12°C/min and held for 5 min. The GC column was heated at 40°C for 1 min, then heated to 280°C at a rate of 5°C/min with constant flow of helium at 1.2 ml/min. The ion source and quadrupole temperatures were set at 230 and 150°C, respectively, and the MS scanned at 70 eV between m/z 40 and 350.

Peaks were identified by spectral deconvolution with a target/non-target approach using Ion Analytics (Gerstel) software, as described previously (Kowalsick et al., 2014; Robbat et al., 2017; Kfoury et al., 2018a), using a database of reference mass spectra of 634 compounds previously detected in tea leaf samples (Kfoury et al., 2018b; Scott et al., 2019). The retention index (RI) of each compound was calculated using a standard mix of C7–C30 n-alkanes (Sigma-Aldrich) and used to confirm peak identity.

Relative peak areas (RPA) were calculated using the peak area of the internal standard. The RPA of compounds found in the field blank were subtracted and any compound where RPA ≤ 0 was replaced by the value 100/IS, where IS is the peak area of the internal standard. Any compounds detected in 5 or fewer samples were excluded from further analyses. RPAs were then natural log-transformed to improve normality. We tested for multivariate outliers using the *HDoutliers* package (Wilkinson, 2018) and removed one outlier ($n = 18$ for GC-MS data).

Sample Preparation and Analysis of Non-volatile Metabolites

Leaf samples were pulverized in a ball mill (KLECO, Visalia, CA, United States) and extracted in triplicate by mixing 20 ± 2 mg of powdered leaf material with 1 ml of 80:15:5 acetonitrile:water:1M HCl (v/v/v) in 1.5 ml Eppendorf microcentrifuge tubes. The microcentrifuge tubes were vortexed for 1 min under maximum setting. After vortexing, we sonicated the microcentrifuge tubes for 30 min at 20°C. The microcentrifuge tubes were then centrifuged for a minute at 1,300 g. The supernatant was decanted, and syringe filtered (0.45 μm PTFE membrane). The extracts were then stored at −20°C. Extracts were thawed and diluted 1:24 (40 μl extract and 960 μl extraction solvent) for measuring total phenolics and 1:9 for analysis by liquid chromatography-mass spectrometry (LC-MS).

The Folin-Ciocalteau assay was used to measure the total phenolic content of the tea extract as mg/g gallic acid equivalent (Unachukwu et al., 2010). Five gallic acid standard solutions (0.5, 0.25, 0.0625, and 0.03125 mg/ml) were prepared in 80:15:5 acetonitrile:water:1M HCl using serial dilution. The Folin-Ciocalteau assay was carried out in triplicate in a 96 well microtiter plate following Appel et al. (2001). All samples had coefficients of variation less than 20%. Triplicate results were averaged before analysis.

Analysis of specific non-volatiles was done using well established techniques (Scott et al., 2019). These non-volatiles included 8 catechins, 3 methylxanthine alkaloids, and 1 amino acid. Extracts for LC-MS analysis were spiked with 20 μg/ml paraxanthine (Sigma-Aldrich, ∼98%) as an internal standard. Non-volatile separations and quantitation were performed on a 1260 Infinity II HPLC (Agilent Technologies, Santa Clara, CA, United States) consisting of a quaternary pump, a chilled autosampler (4°C), a temperature-controlled column compartment, and a diode array detector (DAD) coupled with

a 6120 quadrupole MS and electrospray ionization (ESI) source. 10 μl of sample was injected onto a superficially porous C18 column (150 mm × 3.0 mm i.d. × 2.7 mm d_p, Agilent Technologies), with temperature set to 32°C. The mobile phase consisted of 0.1% formic acid in water (v/v) (A), and methanol (B). Separation was performed using a gradient elution program as follows: 6–35.7% B (0–22 min), 35.7–100% B (22–23 min), and held at 100% B for 5 min, with a re-equilibration time of 7 min, at a flow rate of 0.5 ml/min. The ESI used a drying gas temperature of 350°C, a flow rate of 12.0 l/min, a nebulizer pressure of 55 psig, and a capillary voltage of 3 kV. Mass spectra were acquired in positive ionization mode from *m/z* 100–500 at a rate of 0.943 spectra/sec, with fragmentation voltage of 130 V.

For calibration standards, we purchased a green tea standard (Sigma-Aldrich) containing 100 μg/ml each of (+)-catechin, (–)-catechin-3-gallate, (–)-epicatechin, (–)-epicatechin-3-gallate, (–)-epigallocatechin-3-gallate, (–)-gallocatechin, (–)-gallocatechin-3-gallate, and caffeine dissolved in 80:15:5 acetonitrile:water:1M HCL. In addition, L-theanine, theobromine, theophylline, and epigallocatechin were purchased in powdered form and dissolved in the same solvent as the green tea standard. Calibration samples were spiked with 10 μg/ml of paraxanthine internal standard (a compound not found in tea) and injected under conditions identical to the tea samples. Correlation coefficients for calibration curves ranged from 0.982 to 0.999. Ion Analytics software was used to analyze data files. Target compounds were quantified based on the $[M + H]^+$ ion intensity, with compound identities confirmed using two qualifier fragment ions present at intensity ratios consistent (±20% relative intensity) with those observed in the standards, utilizing three ions total for detection. Triplicate measurements were averaged before statistical analysis.

Statistics

All statistical analyses were conducted in R version 3.5.3 (R Core Team., 2018). To test for an effect of leafhopper herbivory we used two proxies of herbivore pressure: the density of leafhoppers at the end of the feeding period (insects/young leaf) and visible leaf damage (described in image analysis, above). Leaf damage was averaged across all leaves for non-volatile analysis since leaves were pooled for sampling. For volatile analysis, we present results from both mean leaf damage as well as damage to the focal DCSE leaf. Percent leaf damage was natural log-transformed for analysis to improve normality.

The effects of herbivory on total polyphenols were analyzed with linear models, but because of collinearity of metabolites, multivariate methods were used for LC-MS and GC-MS data. Redundancy analysis (RDA) was conducted on auto-scaled data (RPA for GC-MS and concentration for LC-MS) with either leafhopper density or damage as a continuous predictor variable using the *vegan* and *RVAideMemoire* packages (Hervé et al., 2018; Oksanen et al., 2018) as described in Hervé et al. (2018) (GC-MS n = 18, LC-MS n = 19). Metabolites with significant correlations with the first (only) RDA axis were considered biomarkers. For each biomarker, and total polyphenols, we fit univariate models with either natural log-transformed RPA (GC-MS) or concentration (LC-MS and total polyphenols) as the response variable and herbivory proxy as the predictor variable. Linear and intercept-only (null) models were fit with the *lm* function and step and hinge functions were fit with the *chngpt* package (Fong et al., 2017). For each compound, the best fit model was selected using a model competition approach with AIC (Akaike's Information Criterion). Step function models were only considered significant when the 95% confidence interval for the change point did not overlap the minimum or maximum value for the predictor and when the change in intercepts was significantly different from zero.

RESULTS

Herbivory

Final leafhopper densities ranged from 0 to 1.14 insects/leaf (mean = 0.49, *SD* = 0.35) and mean percent damaged leaf area ranged from 0.57% to 11.58% (mean = 2.99, *SD* = 2.83) (**Figure 1**). There was a non-linear trend in the relationship between final leafhopper density and visible leaf damage (**Figure 1B**). Damage remained below 3% leaf area until about 0.6 insects/young leaf, when damage started to increase with increasing leafhopper density. Additionally, mean damage across all leaves and damage to the DCSE focal leaf were strongly correlated (Pearson's $r = 0.71$).

Volatiles

We found 155 volatile metabolites in total (**Supplementary Table S1**) with 76 compounds detected in all samples. Final leafhopper density explained 10.66% of the variation in volatile profile. There was a significant effect of final leafhopper density on the volatile profile [RDA, $F_{(1,\ 16)} = 1.1$, $p = 0.002$]. Step functions (a line with a slope of zero but a change in y-intercept at a threshold) best described the relationship between leafhopper density and natural log-transformed RPA for 12 of the biomarkers, linear models best described the relationship for 11 biomarkers, and an intercept-only model best explained the relationship for 12 of the biomarkers (**Table 1**). Linear and step function relationships for all biomarker compounds were positive (increasing RPA with increasing insect density) except for cyclopentanone. For compounds with linear relationships, slopes ranged from −2.22 to 3.94, and for compounds with step relationships the difference between intercepts ranged from 7.27 to 15.08. The compounds most strongly correlated with the RDA axis were *cis*-3-hexenyl butyrate, (*E*,*E*)-α-farnesene, sulcatone, (*Z*)-3-hexenyl hexenoate, unknown 3, (*E*)-β-ocimene, and three linalool oxides (**Figure 2** and **Table 1**).

We did not detect any significant relationship between natural log-transformed mean percent leaf damage and volatile profiles [RDA, $F_{(1,\ 16)} = 1.132$, $p = 0.259$]. However, the effect of focal leaf damage on volatile profile was marginally significant [RDA, $F_{(1,\ 16)} = 1.329$, $p = 0.076$] and explained 7.67% of the variation in volatiles. Linear relationships best described the shape of the relationship for 15 biomarkers, and intercept-only models best described the relationship for 7 biomarkers (**Table 2**). Most biomarkers had a positive relationship with focal leaf damage. Only isovaleric acid, *cis*-methyl dihydrojasmonate, and 1,2,4-trimethylbenzene showed a negative relationship with increasing

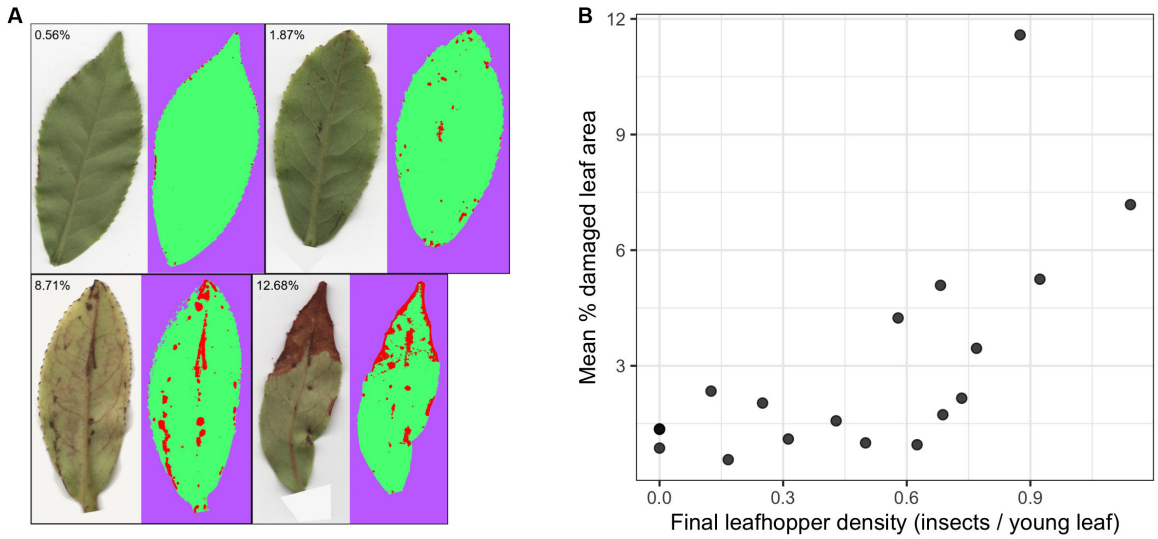

FIGURE 1 | The relationship between leafhopper density and visible leaf damage. **(A)** Representative leaf images highlighting the range and types of damage with results from WEKA segmentation where red, green, and purple represent pixels that have been classified as damaged, undamaged, and background, respectively. **(B)** Scatter plots show the relationship between leafhopper density and mean percent leaf damage where each point represents a pot (n = 19). There is a threshold type relationship between leafhopper density and leaf damage.

focal leaf damage. The compounds most strongly correlated with the RDA axis were 1-hexanol; (*Z*)-3-hexenyl hexanoate; *cis*-3-hexenyl isovalerate; benzyl alcohol; phenylethyl alcohol; *cis*-3-hexenyl butyrate; (*Z*)-2-hexenol; isovaleric acid; and diendiol I (**Figure 3**).

Non-volatiles

We did not detect a significant relationship between leafhopper density and total polyphenols (**Figure 4B**). However, for mean percent leaf damage, there was a significant negative linear relationship (ΔAIC $>$ 2 for other model comparisons and $p = 0.035$) (**Figure 4A**).

Final leafhopper density explained only 5.44% of the total variation in LC-MS compound catechins, methylxanthines, and L-theanine) concentrations and did not have a significant effect [RDA, $F_{(1,\ 16)} = 0.922$, $p = 0.449$]. Although not statistically significant, mean percent damaged leaf area explained 9.58% of the total variation in LC-MS compound concentrations [RDA, $F_{(1,\ 16)} = 1.696$, $p = 0.124$]. Most compounds had negative loadings along the one significant RDA axis, which corresponds to decreasing concentrations with increasing leaf damage. Epigallocatechin gallate (EGCG), theobromine, epicatechin gallate, and caffeine were identified as biomarkers. EGCG and theobromine had significant negative linear relationships with leaf damage (**Figure 5**).

DISCUSSION

Few studies of induced secondary metabolites subject plants to a range of herbivore pressure despite evidence that different levels of herbivory produce different responses in plants. Here we investigated the effects of two quantitative proxies of *E. onukii* herbivory on tea plants: insect density and visible leaf damage. Interestingly, leafhopper density was a better predictor of plant chemical responses than was leaf damage, indicating that tea plants respond to leafhoppers even before their feeding damage is visibly detectible. The relationship between the two proxies, density and leaf damage, is not linear, and there are different metabolites associated with each of these proxies. In addition, we found a threshold effect for some, but not all, metabolites in response to leafhopper density.

There were non-linear relationships between our two proxies for herbivory (density and damage) as well as in the induced responses of volatiles to leafhopper density. The underlying mechanism explaining the variation in induced responses to leafhopper density may be related to induction of plant hormones. Liao et al. (2019) exposed tea plants to feeding by *E. onukii* for either 48 or 96 h and found that low herbivore pressure (48 h) induced jasmonic acid (JA) and salicylic acid (SA) but not abscisic acid (ABA), while high herbivore pressure induced JA and SA to a similar degree but additionally strongly induced ABA. The variation in these hormonal responses could not be explained by intensity of mechanical wounding alone. Non-linear responses of plant hormones in response to increasing herbivore pressure may be underlying the mix of dose-dependent and threshold responses we observed in our experiment. Future studies looking to explain the relationship between herbivore pressure and induced metabolites should consider measuring hormonal responses of plants to a continuous range of herbivore pressures.

Estimating Herbivore Damage

Overall, our results suggest that increases in visible damage are not evident at low densities. We observed a non-linear

TABLE 1 | Biomarkers of leafhopper density in order of the strength of correlation to the RDA constrained axis.

Compound	Response type	Linear Slope	Change point	ΔIntercept	Aroma[a]	Biosynthesis
cis-3-Hexenyl butyrate	Linear	3.941	–	–	Wine, green	FA derivative[b]
(*E,E*)-α-Farnesene	Step	–	0.312	7.21	Woody, sweet, green, floral	Sesquiterpene[c]
Sulcatone	Linear	4.11	–	–	Citrus, green, musty, cheesy	Irregular terpene[c]
(3Z)-Hexenyl hexanoate	Step	–	0.579	2.651	Green, fruity, fatty, tropical	FA derivative[b]
unknown 3	Linear	2.293	–	–	–	–
(*E*)-β-Ocimene	Step	–	0.25	7.119	Citrus, green, terpene	Monoterpene[c]
trans-Dehydroxylinalool oxide	Step	–	0.625	2.297	Herbal, green, terpene	Monoterpene[d]
cis-Linalool oxide (pyranoid)	Step	–	0.429	2.456	Citrus, green	Monoterpene[d]
cis-Linalool oxide (furanoid)	Linear	3.65	–	–	Earthy, floral, sweet, woody	Monoterpene[d]
(*E,E*)-Allo-ocimene	Step	–	0.25	3.065	Terpenic, sweet, fresh, floral	Monoterpene[c]
Indole	Step	–	0.682	1.601	Concentrated = fecal, animal dilute = sweet, floral	Anthranilate[b]
Diendiol I (2,6-Dimethylocta-3,7-diene-2,6-diol)	Linear	3.673	–	–	–	Monoterpene[e]
Benzyl alcohol	Linear	2.276	–	–	Fruity, floral, sweet	VPB[b]
β-Myrcene	Step	–	0.625	1.101	Balsamic, must, spice	Monoterpene[c]
trans-α-Bergamotene	Step	–	0.429	2.5	Woody, warm, tea	Sesquiterpene[c]
cis-3-Hexenyl isovalerate	Step	–	0.579	1.853	Fresh, green, apple, fruity, tropical	FA derivative[c]
Heptanoic acid	Null	–	–	–	Cheesy, sour, rancid	FA[f]
Hexanoic acid	Null	–	–	–	Cheesy, fatty	FA[f]
(*Z*)-β-Ocimene	Linear	3.321	–	–	Citrus, herbal, floral	Monoterpene[c]
(*E*)-2-Hexenyl acetate	Linear	1.676	–	–	Sweet, apple skin, banana peel	FA derivative[b]
1-Non-anol	Linear	1.716	–	–	Fatty, green, orange	FA derivative[b]
Decanal	Null	–	–	–	Citrus, sweet, waxy	–
Cyclopentenone	Null	–	–	–	–	FA derivative[g]
trans-Linalool oxide (furanoid)	Null	–	–	–	Earthy, floral, sweet, woody	Monoterpene[d]
γ-Non-alactone	Step	–	0.579	1.606	Coconut, creamy, waxy, sweet	FA derivative[h]
γ-Butyrolactone	Null	–	–	–	Caramel, fatty, sweet	FA derivative[h]
unknown 2	Null	–	–	–	–	–
p-Xylene	Linear	2.198	–	–	Sweet	–
1-Octen-3-ol	Null	–	–	–	Mushroomy, green, earthy	FA derivative[h]
Benzyl nitrile	Null	–	–	–	–	VPB[i]
Octanoic acid	Null	–	–	–	Cheesy, fatty, waxy	FA[f]
Butylated hydroxytoluene	Null	–	–	–	Phenolic, camphor	–
Linalool	Null	–	–	–	Flower, lavender	Monoterpene[c]
Cyclopentanone	Linear	-2.219	–	–	Minty	FA derivative[g]
Coumaran	Step	–	0.769	2.088	–	–

For step function relationships, the change point, in units of leafhoppers per young leaf, and the difference in intercepts after and before the change point are reported. Biosynthetic pathways are reported when available from the literature (FA, fatty acid; VPB, volatile phenylpropanoid/benzenoid compounds derived from phenylalanine). [a]*The Good Scents Company, (2015),* [b]*Dudareva et al. (2013),* [c]*Knudsen et al. (2006),* [d]*Ho et al. (2015),* [e]*Zeng et al. (2019),* [f]*Kroumova et al. (1994),* [g]*Weber (2002),* [h]*Schwab et al. (2008),* [i]*Noge and Tamogami (2013).*

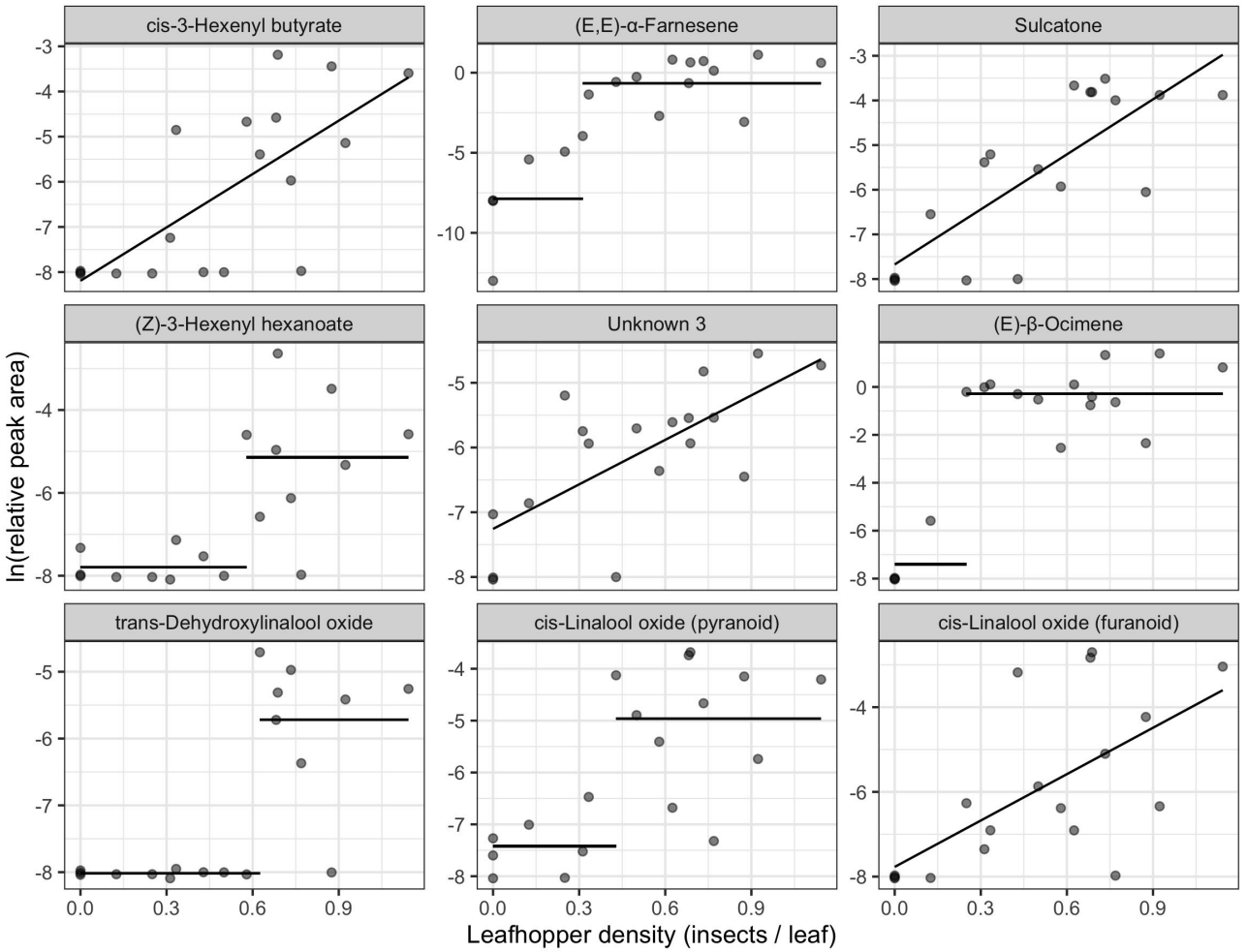

FIGURE 2 | Biomarkers of leafhopper density identified from redundancy analysis of natural log-transformed relative peak areas of compounds detected in tea samples (n = 18). The x-axis represents the number of leafhoppers per young leaf at the end of the feeding period. Panels are in order of correlation to the RDA axis from strongest to weakest going left to right, top to bottom. Lines show fitted values of the winning univariate models. Only the top 9 biomarkers are plotted here. See **Table 1** for a full list of biomarkers.

relationship between our two proxies for herbivory, with a threshold of about 0.6 leafhoppers per young leaf before seeing an increase in visible damage. *Empoasca* species are known to be plastic in their feeding mode, switching between "burning," which primarily targets leaf veins, and "stippling" which targets mesophyll (Backus et al., 2005; Jin et al., 2012). The non-linear relationship between insect density and visible damage could be a result of a density-dependent switch in feeding mode. A density-dependent switch in the response of the tea plant is also possible. For example, at low levels of herbivory there may be lower expression of PPO and therefore less browning of leafhopper feeding sites resulting in less visible feeding damage.

Herbivory and Volatile Chemistry

Leafhopper density and visible damage are associated with different chemical profiles. The top biomarkers of leafhopper density were largely terpenes, with some contribution by fatty acid derivatives, such as green leaf volatiles, and some volatile phenylpropanoids/benzenoids (VPBs) (**Table 1**). Many of the volatile biomarkers of leafhopper density were compounds detected in previous studies of leafhopper feeding on tea (Gohain et al., 2012; Cai et al., 2014; Zeng et al., 2019). The shape of the relationship between leafhopper density and compound concentration (RPA) varied among compounds with induction being linearly density-dependent for some compounds or being induced only after a minimum threshold in herbivory for others. When step-functions were significant for biomarkers of leafhopper density, the threshold of induction was most often between 0.5 and 0.7 leafhoppers per leaf (**Table 1**), corresponding to the threshold at which we see mean leaf damage rise above 3% (**Figure 1**). These threshold-induced volatiles may be associated with whatever change is happening to produce an increase in visible damage, whether it is a change in feeding mode or a change in plant metabolic response. We note that the exact shape of the relationship between RPA and herbivory for any given compound depends on data transformation and may be partly influenced by

TABLE 2 | Biomarkers of visible leafhopper damage in order of the strength of correlation to the RDA constrained axis.

Compound	Response type	Linear slope	Aroma[a]	Biosynthesis
1-Hexanol	Linear	0.869	Resin, flower, green	FA derivative[b]
(*Z*)-3-Hexenyl hexanoate	Linear	0.842	Green, fruity, fatty, tropical	FA derivative[b]
cis-3-Hexenyl isovalerate	Linear	0.778	Green, apple, tropical, pineapple	FA derivative[b]
Benzyl alcohol	Linear	0.554	Fruity, floral, sweet	VPB[b]
Phenethyl alcohol	Linear	0.897	Honey, spice, rose, lilac	VPB[b]
cis-3-Hexenyl butyrate	Linear	0.782	wine, green	FA derivative[b]
(*Z*)-2-Hexenol	Linear	0.688	Leaf, green, wine, fruit	FA derivative[b]
Isovaleric acid	Step	-0.708	Sour, cheesy, rancid	FA[c]
Diendiol I	Linear	0.698	–	Monoterpene[d]
cis-Linalool oxide (pyranoid)	Linear	0.553	Citrus, green	Monoterpene[e]
(3-hydroxy-2,4,4-trimethylpentyl) 2-methylpropanoate	Null	–	–	–
Benzothiazole	Null	–	Rubbery, sulfury, vegetal, gasoline	–
γ-Non-alactone	Null	–	Coconut, creamy, waxy, sweet	FA derivative[f]
Indole	Null	–	Concentrated = fecal, animal dilute = sweet, floral	Anthranilate[b]
Coumaran	Linear	0.386	–	VPB[g]
cis-Linalool oxide (furanoid)	Null	–	Earthy, floral, sweet, woody	Monoterpene[e]
trans-Linalool oxide (furanoid)	Null	–	Earthy, floral, sweet, woody	Monoterpene[e]
1,2,4-Trimethylbenzene	Linear	-0.169	Plastic	VPB[g]
o-Hydroxybiphenyl	Null	–	–	–
Methyl salicylate	Linear	0.292	Wintergreen	VPB[g]
cis-Methyl dihydrojasmonate	Linear	-0.461	Jasmine, floral, green	FA derivative[g]
Benzyl nitrile	Null	–	–	VPB[h]

Biosynthetic pathways are reported when available from the literature (FA, fatty acid; VPB, volatile phenylpropanoid/benzenoid compounds derived from phenylalanine). [a]*The Good Scents Company, (2015),* [b]*Dudareva et al. (2013),* [c]*Kroumova et al. (1994),* [d]*Zeng et al. (2019),* [e]*Ho et al. (2015),* [f]*Schwab et al. (2008),* [g]*Margl et al. (2005),* [h]*Noge and Tamogami (2013).*

the detection limits of the instrumentation. Regardless, the shape of the response of induced volatiles to leafhopper density varies among the entire suite of compounds analyzed, and this results in a change in volatile profile with increasing herbivore pressure.

Several of the compounds most strongly correlated with leafhopper density have been previously reported as being induced by insect feeding and serve as attractants for parasitoids or predators. Du et al. (1998) reported induction of (*E*)-β-ocimene, sulcatone (6-methyl-5-hepten-2-one), and linalool by pea aphids (*Acrothosiphon pisum*) feeding on broad bean (*Vicia faba*). Among the compounds they detected, sulcatone was most attractive to the parasitoid *Aphidius ervi*. They also reported that the concentration of (*E*)-β-ocimene was constant over 4 days of aphid feeding, while sulcatone, linalool, and other compounds increased in concentration with increased aphid feeding time. In our study, we see a similar pattern where (*E*)-β-ocimene is induced in a density-independent manner after a threshold of 0.25 leafhoppers per leaf, while sulcatone has a density-dependent relationship with leafhopper density (**Table 1** and **Figure 2**). Interestingly, sulcatone is an oxidation product of (*E*,*E*)-α-farnesene in apples (Lurie and Watkins, 2012), so it is fascinating that they were not both linearly correlated with damage. This may indicate that induction of (*E*,*E*)-α-farnesene is less dependent on leafhopper density than it's oxidation.

In contrast, our analysis of leaf damage revealed a different set of volatile biomarkers: largely fatty acid derivatives and VPBs, with a smaller contribution from terpenes (**Table 2**). Many of the volatile biomarkers related to visible leaf damage are commonly produced by plants as a result of mechanical damage to leaves by activation of the lipoxegenase (LOX) pathway including 1-hexanol, (*Z*)-3-hexenyl hexenoate, *cis*-3-hexenyl isovalerate, and (*Z*)-2-hexenol (Ho et al., 2015). It is likely that visible leaf damage is more closely associated to disruption of cell membranes compared to leafhopper density, which would explain why volatiles derived from free cell membrane lipids are correlated more strongly with this proxy for herbivory. In addition, the plant hormone methyl salicylate increases with increasing leaf damage in our study, consistent with the findings of Liao et al. (2019).

A biomarker of both leafhopper density and leaf damage, diendiol I, has been previously described as a compound induced in tea plants uniquely by *E. onukii* feeding (Zeng et al., 2019) and is a precursor to hotrienol, a compound formed during tea processing which is important for Eastern Beauty oolong flavor (Kawakami et al., 1995; Cho et al., 2007). Diendiol I increased linearly with increasing leafhopper density (and natural log-transformed leaf damage), although other volatile compounds that may impart off flavors also increased with leafhopper density. For example, sulcatone and *cis*-3-hexenyl butyrate increase at

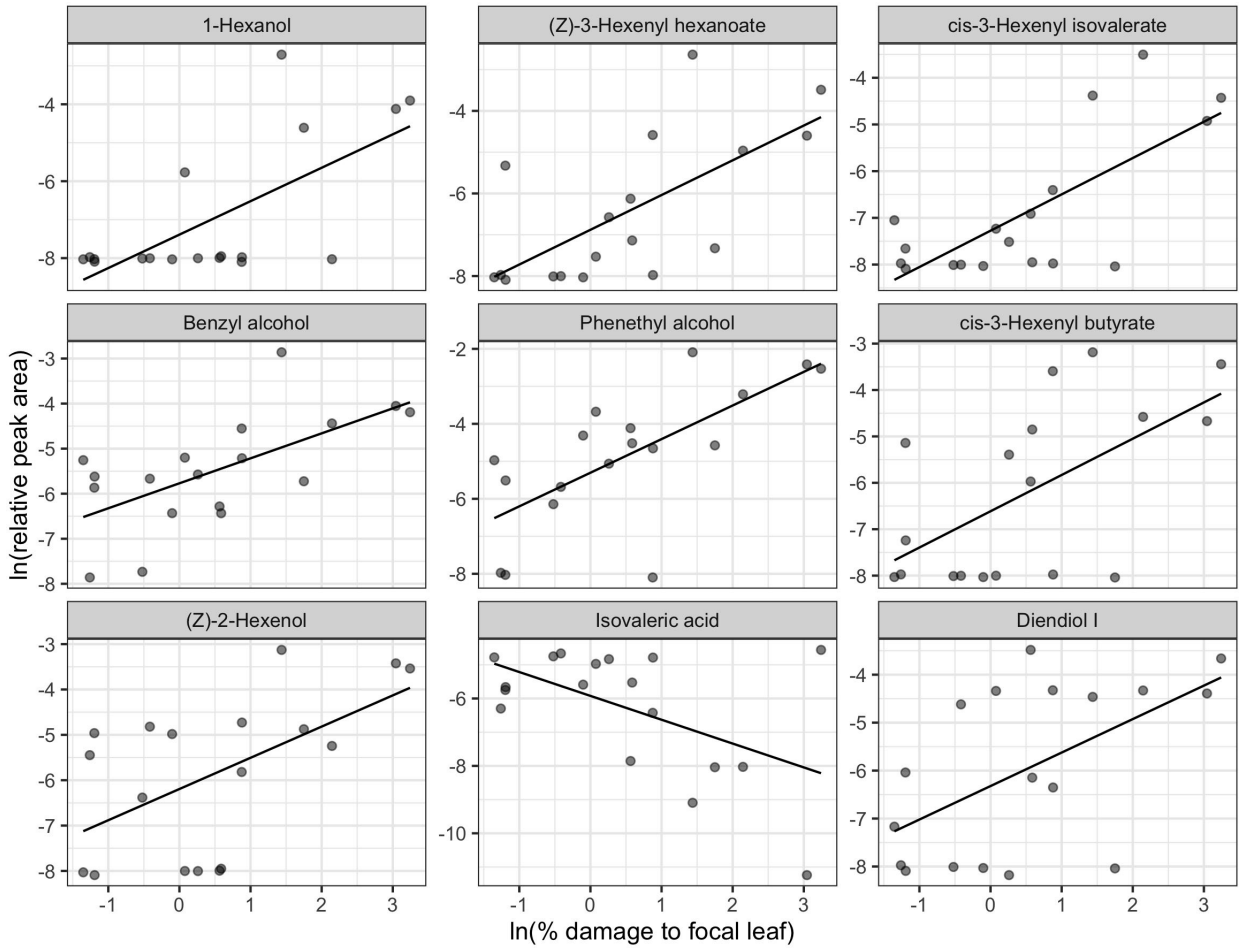

FIGURE 3 | Biomarkers of leaf damage identified from redundancy analysis of natural log-transformed relative peak areas of compounds detected in tea samples (n = 18). The x-axis represents the natural log-transformed percentage of pixels classified as damaged on the leaf used for volatile sampling by DCSE. Panels are in order of correlation to the RDA axis from strongest to weakest going left to right, top to bottom. Lines show fitted values of the winning univariate models. Only the top 9 biomarkers are plotted here. The full chemical name for diendiol I is 2,6-Dimethylocta-3,7-diene-2,6-diol. See **Table 2** for a full list of biomarkers.

a steeper slope than diendiol I and impart green, musty, and vegetative odors (The Good Scents Company,, 2015).

Although volatile profile undergoes a dramatic change during tea processing, these results from live plants in the field have implications for finished tea quality (Cho et al., 2007). In addition to inducing important chemical precursors (i.e., diendiol I), leafhopper herbivory may potentially prime tea leaves to respond differently to the stresses experienced during the oxidation phase of oolong tea processing (Zeng et al., 2019). In order to fully understand how environmental factors such as insect herbivory affect the quality of tea, more studies are needed that track tea metabolites in mature tea plants in the field, through processing, and in finished tea.

Because metabolites differ in their response to increasing leafhopper herbivory, and different proxies for herbivory are associated with different metabolites, volatile profiles change with increasing leafhopper herbivory in complex ways. This has implications for leafhoppers and their natural enemies which both use plant volatiles to locate suitable food (Gao et al., 2004; Xin et al., 2017). For example, future studies could examine how predator or parasitoid behavior changes with exposure to volatile profiles produced by tea plants above or below the threshold of leafhopper density we've observed.

Herbivory and Non-volatile Chemistry

In contrast to volatiles, variation in non-volatiles (including catechins and total polyphenols) was explained by visible damage rather than by leafhopper density. We detected a significant reduction in epigallocatechin gallate (EGCG) and total polyphenols with increasing leaf damage, but no relationship with leafhopper density, which is consistent with the prediction that visible damage is due to increased polyphenol oxidase (PPO) activity producing brown theaflavins and thearubigins from catechin precursors (Tounekti et al., 2013). Liao et al. (2019) observed an increase in PPO activity and an increase in theaflavins (the oxidation products of catechins) with *E. onukii* damage on tea, although they saw no decrease in catechin concentrations. In contrast to our results, Li et al. (2018) found

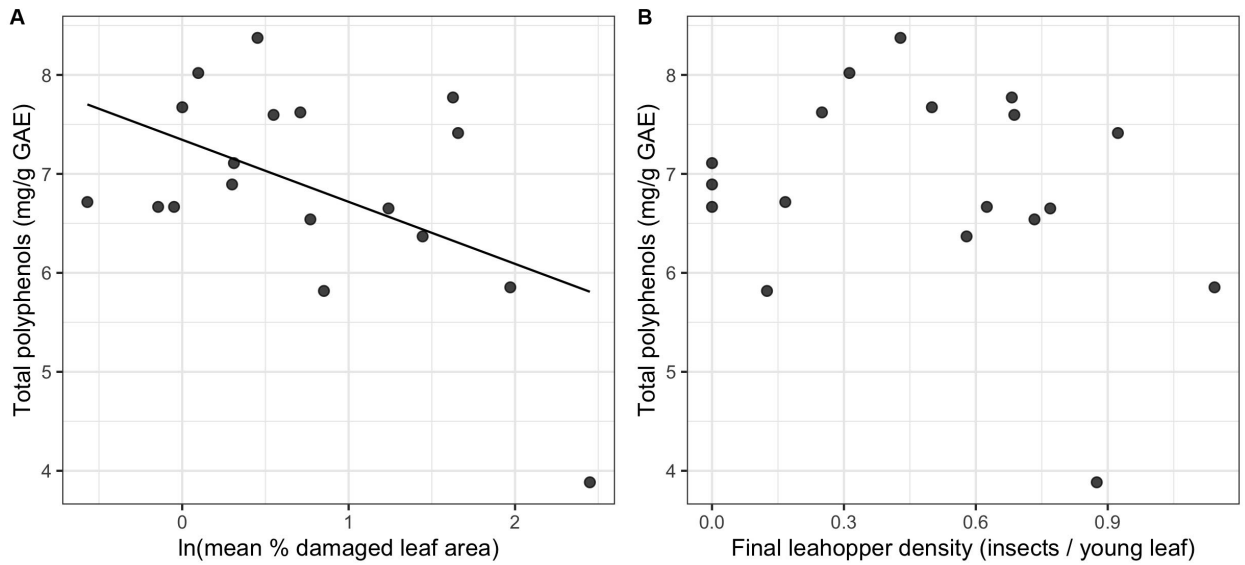

FIGURE 4 | Relationship between total polyphenols and two proxies of herbivore damage: natural log-transformed mean% damaged leaf area **(A)** and leafhopper density **(B)** (n = 19). Only the relationship between leaf damage and total polyphenols in 2017 was statistically significant.

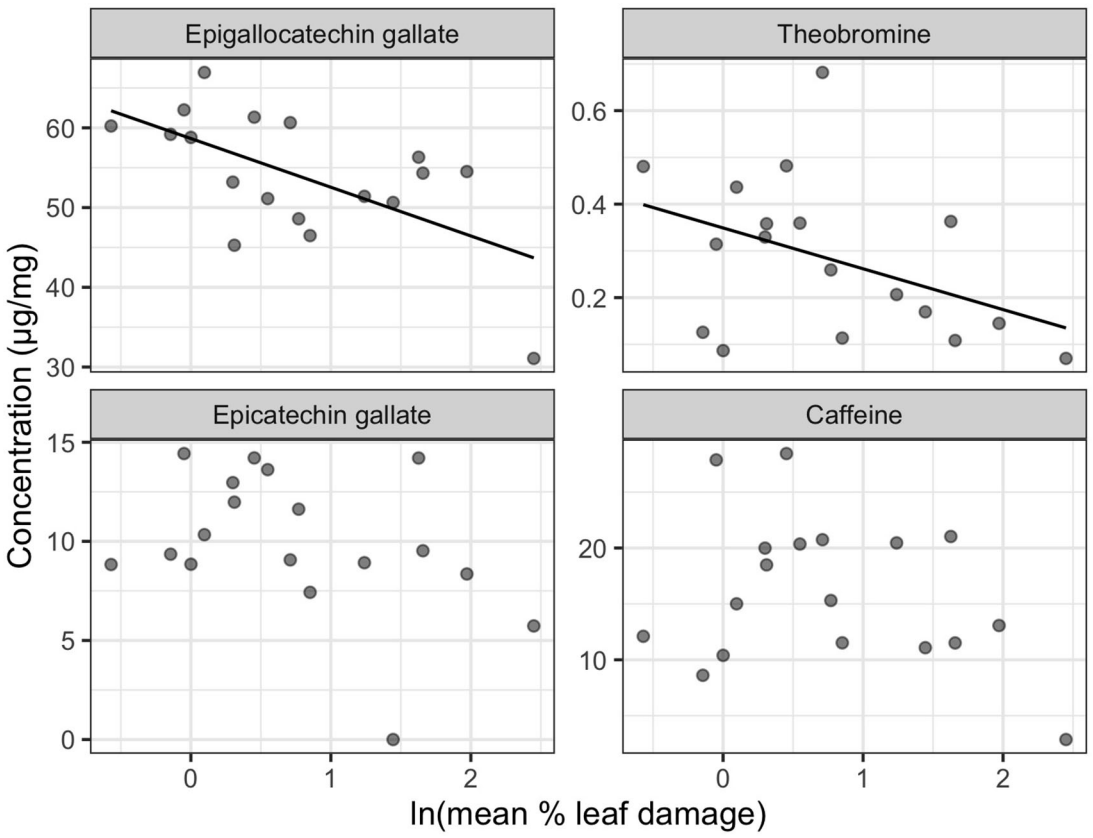

FIGURE 5 | Non-volatile biomarkers for leafhopper damage. Only the LC-MS compounds with significant correlations to the RDA constrained axis are plotted (n = 19). Only epigallocatechin gallate and theobromine had a significant univariate linear relationship with natural log-transformed mean percent leaf damage.

a quadratic relationship between total polyphenol content and *E. onukii* density with the highest polyphenol concentration at an intermediate level of herbivory corresponding to 2 insects per leaf. Theobromine, a caffeine precursor (Xia et al., 2017), also decreased in concentration with leafhopper damage in our study. Previous research showed an increase in caffeine synthase gene expression after leafhopper feeding, but no increase in caffeine concentration was detected (Yang et al., 2011; Liao et al., 2019). Theobromine concentrations were not measured in either of these studies, although it is possible that theobromine reduction is associated with increased caffeine synthase expression.

Decreases in polyphenols and EGCG may not be desirable for tea farmers, as these compounds are beneficial for health (Pon Velayutham, 2008). However, polyphenols can also contribute bitterness and astringency to tea, which may decrease quality from a flavor standpoint (Scott and Orians, 2018). In addition, the non-volatile and volatile metabolite profiles of tea changes with tea processing (Chengying et al., 2018; Jiang et al., 2019). So while our study shows that leafhopper herbivory has implications for finished tea quality, the exact effects of herbivore density and damage on taste, aroma, and health beneficial compounds have yet to be elucidated.

SUMMARY

We show that leafhoppers affect tea plant metabolites in complex ways as the intensity of herbivory increases. Although the effects of climate change on *E. onukii* are currently unknown, their population densities are likely to increase, resulting in changes in tea metabolites. As a highly multivoltine species, increases in the length of the growing season may allow for additional generations and an increase in numbers (Berggren et al., 2009; Reineke and Hauck, 2012). This, in turn, could have implications for the practice of taking advantage of natural levels of leafhopper herbivory to improve tea quality.

As importantly, the two methods used for quantifying herbivore pressure are associated with different lists of biomarkers, and thus suggest that plant responses to damage are complex. Thus, gathering data on multiple proxies for herbivore pressure can be key to elucidating plant responses to herbivory. For example, Lin et al. (1990) found that behavioral responses of beetles to plants damaged by conspecifics depended on the number of wounded cells in contact with healthy cells and not on the area of plant tissue removed by herbivory. Similarly, the density of herbivores, the area of damaged tissue, and feeding time (not manipulated in this study, but used as a dependent variable in many others) may be non-equivalent measures of herbivore pressure on plants. When possible, multiple proxies for herbivory can be measured and any disagreement in the results using these proxies should be viewed as an opportunity to better understand the underlying biological and methodological mechanisms that lead to contrasting results.

Finally, our results show that treating herbivory as continuous rather than categorical (e.g., control vs. damaged), has the potential to reveal important non-linear responses to herbivory. These non-linear responses may be ecologically relevant if they result in a different behavior of conspecifics or predators after a certain threshold of herbivory, and they may be economically relevant when induced secondary metabolites contribute to crop quality. Future studies of induced responses to herbivory should, at minimum, include more than one level of herbivory and ideally account for natural variation in herbivore feeding by measuring actual herbivory quantitatively.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the following Zenodo archive: http://doi.org/10.5281/zenodo.3614045.

AUTHOR CONTRIBUTIONS

ES, W-YH, J-PW, XL, and CO contributed to the conception and design of the study. SA, TG, SC, and JS provided input on study design and field site selection. W-YH and XL secured field sites and tea plants for the study. ES, J-PW, M-MG, and XL carried out the field experiments. NK, JM, AR, and AA performed the chemical analyses. ES and AA carried out the image analysis. ES wrote the manuscript with contributions from SA, NK, CO, XL, SC, TG, and JS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00636/full#supplementary-material

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