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Functional Analysis of the *Gibberellin 2-oxidase* Gene Family in Peach

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Peach (*Prunus persica* L. Batsch) trees grow vigorously and are subject to intense pruning during orchard cultivation. Reducing the levels of endogenous gibberellins (GAs) represents an effective method for controlling branch growth. Gibberellin 2-oxidases (GA2oxs) deactivate bioactive GAs, but little is known about the GA2ox gene family in peach. In this study, we identified seven *PpGA2ox* genes in the peach genome, which were clustered into three subgroups: C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I. Overexpressing representative genes from the three subgroups, *PpGA2ox-1*, *PpGA2ox-5*, and *PpGA2ox-2*, in tobacco resulted in dwarf plants with shorter stems and smaller leaves than the wild type. An analysis of the GA metabolic profiles of the transgenic plants showed that PpGA2ox-5 (a member of subgroup C_{19}-GA2ox-II) is simultaneously active against both C_{19}-GAs and C_{20}-GAs,which implied that C_{19}-GA2ox-II enzymes represent intermediates of C_{19}-GA2oxs and C_{20}-GA2oxs. Exogenous GA_3 treatment of shoot tips activated the expression of all seven *PpGA2ox* genes, with different response times: the *C_{19}-GA2ox* genes were transcriptionally activated more rapidly than the *C_{20}-GA2ox* genes. GA metabolic profile analysis suggested that C_{20}-GA2ox depletes GA levels more broadly than C_{19}-GA2ox. These results suggest that the *PpGA2ox* gene family is responsible for fine-tuning endogenous GA levels in peach. Our findings provide a theoretical basis for appropriately controlling the vigorous growth of peach trees.

Keywords: *Prunus persica*, peach, GA2ox, functional divergence, GA homeostasis

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INTRODUCTION

Peach (*Prunus persica* L.) is one of the most widely cultivated deciduous fruit trees worldwide. According to FAOSTAT data, the global cultivation area of peach and nectarine reached 1712.4 ha in 2018. Peach trees must be pruned to keep the tree size manageable and to facilitate harvesting, but most cultivated peach varieties grow vigorously, increasing the labor costs required for orchard management. One effective way to inhibit the rapid growth of peach branches is to treat the trees with paclobutrazol (PBZ), an inhibitor of gibberellin (GA) biosynthesis, indicating that endogenous GA is at least one of the phytohormones that promote the vigorous growth of peach branches.

The biosynthesis of GA has been studied in many plant species, and is catalyzed by seven enzymes: *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), GA 13-oxidase (GA13ox), GA 20-oxidase (GA20ox), and GA 3-oxidase (GA3ox) (Yamaguchi, 2008). Because GA homeostasis is crucial for proper plant growth and development, several mechanisms have evolved to reduce the levels of bioactive GAs in

plants (Varbanova et al., 2007; Gao et al., 2016). The main mechanism involves catabolism through the 2β-hydroxylation of active GA, a process catalyzed by GA 2-oxidase (GA2ox) (Rieu et al., 2008).

GA2oxs belong to the 2OG-Fe (II) oxygenase superfamily (Rieu et al., 2008). GA2oxs are encoded by small gene families, as revealed in plants such as *Arabidopsis thaliana* (Schomburg et al., 2003; Rieu et al., 2008), rice (Lo et al., 2008), grapevine (Giacomelli et al., 2013), and tomato (Serrani et al., 2007). In Arabidopsis, *GA2ox* genes are expressed throughout plant development and in different tissues (Rieu et al., 2008). The role of GA2oxs in reducing the level of bioactive GAs has been demonstrated in many species, including rice (Lo et al., 2008), poplar (Gou et al., 2011), spinach (Lee and Zeevaart, 2005), and switchgrass (Wuddineh et al., 2015).

The expression levels of *GA2ox* genes vary in response to environmental changes and phytohormones. In Arabidopsis, bioactive GA is deactivated in the hypocotyls of light-grown seedlings via the activation of *GA2ox1* transcription (Achard et al., 2007). Blue light-mediated hypocotyl elongation is promoted by repressing the transcription of *GA2ox1* and *GA2ox8* (Wang et al., 2011). Low temperature deactivates GA by activating the transcription of *GA2ox* genes in rice (Wang et al., 2018). *GA2ox6* genes are upregulated under water-limiting conditions (Dubois et al., 2013). *GA2ox7* is upregulated by high salinity, which results in lower levels of active GAs (Magome et al., 2008). Finally, *GA2oxs* are significantly upregulated in response to treatment with exogenous GA_3 (Thomas et al., 1999; Tan et al., 2018). Therefore, regulating the expression of *GA2ox* genes is a vital way to control the levels of endogenous GAs in response to environmental changes.

The biosynthesis of GA produces intermediates (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_{53}, GA_{44}, GA_{19}, and GA_{20}) in addition to bioactive GAs (GA_1 and GA_4). GAs are classified into different groups based on their number of carbon (C) atoms, including C_{20}-GAs (such as GA_{12}, GA_{15}, GA_{24}, GA_{53}, GA_{44}, and GA_{19}) and C_{19}-GAs (such as GA_9, GA_{20}, GA_1, and GA_4). GA2ox proteins are classified as C_{20}-GA2ox or C_{19}-GA2ox based on their preference for C_{20}-GA or C_{19}-GA substrates, respectively. Phylogenetic analysis of GA2ox proteins further divided the C_{19}-GA2ox group into two subgroups (Lee and Zeevaart, 2005; Lo et al., 2008; Wuddineh et al., 2015), suggesting that members of these two C_{19}-GA2ox subgroups do not have exactly the same functions.

In the current study, we identified seven *PpGA2ox* genes in the peach genome. Phylogenetic analysis clustered these genes into three subgroups: C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I. The overexpression of representative *PpGA2ox* genes from the three subgroups in tobacco resulted in a typical dwarf phenotype, with shorter stems and smaller leaves than the wild type. *PpGA2ox5*, a member of the C_{19}-GA2ox-II subgroup, encodes an enzyme with activity against C_{20}-GAs and C_{19}-GAs. Our findings suggest that C_{19}-GA2ox-II enzymes might be intermediate forms that arose during the divergence of the C_{20}-GA2ox and C_{19}-GA2ox lineages. Exogenous GA_3 treatment of peach branches activated the expression of all seven *PpGA2ox* genes but with different time courses. *PpGA2ox* subgroup C_{19}-GA2ox-I members were the earliest to be upregulated by this treatment, while *PpGA2ox* genes in subgroups C_{19}-GA2ox-II and C_{20}-GA2ox-I were activated later.

RESULTS

Identification of Seven *PpGA2ox* Genes in the Peach Genome

We identified seven putative *GA2ox* genes in the peach genome: *PpGA2ox1*, *PpGA2ox2*, *PpGA2ox3*, *PpGA2ox4*, *PpGA2ox5*, *PpGA2ox6*, and *PpGA2ox7*. These genes are distributed on Chromosomes 1, 3, and 4 (**Figure 1**). All *PpGA2ox* genes contain three exons and two introns, which is similar to the structure of *GA2ox* family members in Arabidopsis (Han and Zhu, 2011). *PpGA2ox1* and *PpGA2ox2* on Chromosome 1 share 26% sequence identity and have opposite gene orientations. *PpGA2ox4*, *PpGA2ox5*, *PpGA2ox6*, and *PpGA2ox7* are located on Chromosome 4. The pairs *PpGA2ox5-PpGA2ox6* (65% identity) and *PpGA2ox4-PpGA2ox7* (52% identity) share the highest sequence identity among the *GA2ox* genes (**Supplementary Table S1**). *PpGA2ox4, PpGA2ox6*, and *PpGA2ox7* are oriented in the same direction, while *PpGA2ox5* is oriented in the opposite direction. These results suggest that intricate patterns of gene duplication occurred during the evolution of the peach *GA2ox* gene family.

Analysis of the conserved domains in the encoded enzymes revealed that the PpGA2oxs belong to the 2OG-Fe (II) oxygenase superfamily. All seven PpGA2oxs contain conserved amino acid residues that are thought to bind Fe^{2+} and 2-oxoglutarate (**Supplementary Figure S1**).

Classification of the PpGA2oxs Into Three Subgroups: C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I

GA2oxs are usually active against C_{19}-GAs (such as GA_9, GA_{20}, GA_1, and GA_4) or C_{20}-GAs (such as GA_{12} and GA_{53}), placing them into the C_{19}-GA2ox or C_{20}-GA2ox class (Rieu et al., 2008). To predict the functions of the PpGA2oxs, we collected reported GA2ox sequences from different plants and constructed a phylogenetic tree of the GA2ox family (**Figure 2**). The GA2ox family members fell into three groups: C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I. The reported members of C_{19}-GA2ox-I and C_{19}-GA2ox-II use C_{19}-GAs as substrates, while the members in C_{20}-GA2ox-I are mainly active against C_{20}-GAs. PpGA2ox1 and PpGA2ox3 belong to subgroup C_{19}-GA2ox-I; PpGA2ox5 and PpGA2ox6 belong to subgroup C_{19}-GA2ox-II; and PpGA2ox2, PpGA2ox4, and PpGA2ox7 belong to subgroup C_{20}-GA2ox-I. This clustering pattern predicts that PpGA2ox1, PpGA2ox3, PpGA2ox5, and PpGA2ox6 are active against C_{19}-GAs, while PpGA2ox2, PpGA2ox4, and PpGA2ox7 are active against C_{20}-GAs.

To determine whether the three subgroup division is conserved in other species, the reported GA2ox families from rice, grapevine, peach, tomato, and cucumber were selected and

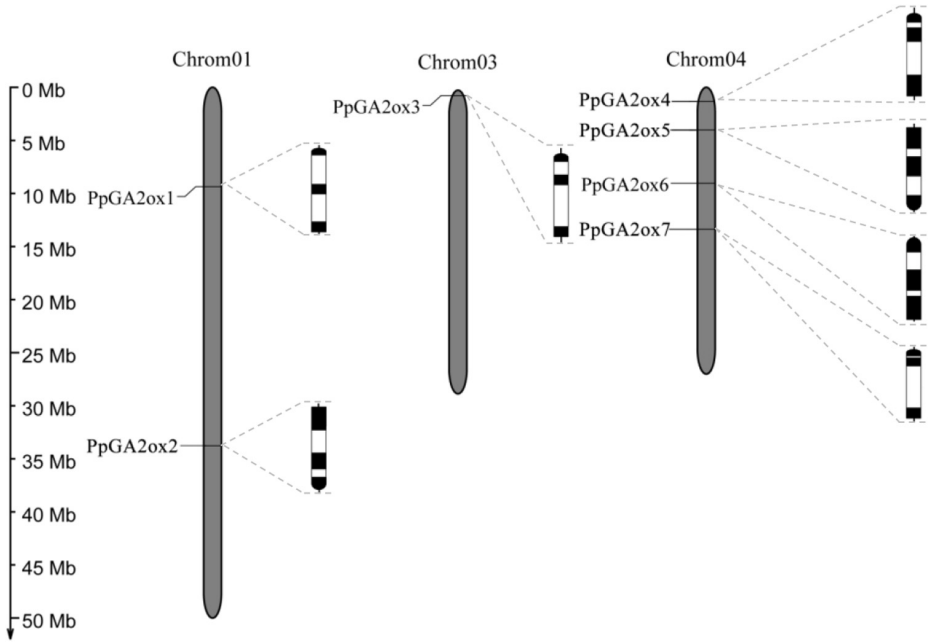

FIGURE 1 | Chromosomal locations and genomic structures of *PpGA2ox* genes. Black boxes represent exons and white boxes represent introns. The semi-circle black boxes indicate the direction of the end of gene transcription.

the number of *GA2ox* genes in each of the three subgroups was analyzed (**Table 1**). All species carry representatives of the C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I subgroups, suggesting that simultaneously possessing C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I genes is a highly conserved feature of both monocots and dicots.

Analysis of the Evolutionary Relationships Among C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I Genes

The functional divergence of genes is a common occurrence during evolution. We constructed a phylogenetic tree based on *GAox* genes from liverwort (*Marchantia polymorpha*), the bryophyte *Sphagnum fallax*, and the lycophyte *Selaginella moellendorffii* and *GA2ox* genes from the flowering plants rice (*Oryza sativa*), peach (*Prunus persica*), and *Arabidopsis thaliana* (**Supplementary Figure S2**). The first three species represent basal clades, with *Selaginella* representing the earliest vascular plants. All GAoxs from *M. polymorpha*, *Sph. fallax*, and *Sel. moellendorffii* clustered with the C_{20}-GA2oxs from monocots and dicots. This result implies that C_{20}-GA2ox is a basal clade of the GA2ox family that possesses more ancient gene functions.

Motif analysis of GA2ox, GA20ox, and GA3ox proteins uncovered an amino acid sequence in the N-termini of these proteins that is the signature motif of GA2oxs (Han and Zhu, 2011), pointing to a close relationship between the signature motif and the functions of GA2oxs. To identify the possible cause of the functional divergence within the *GA2ox* family, we analyzed the signature motifs of the proteins in the C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I subgroups (**Figure 3**). The most highly conserved amino acids in C_{19}-GA2ox-II proteins are the same as those in C_{19}-GA2ox-I proteins, except for tryptophan (W; at position 16 in the wider multiple sequence alignment), which is highly conserved among C_{20}-GA2ox-I proteins. This observation suggests that the C_{19}-GA2ox-II subgroup is intermediate between the C_{19}-GA2ox-I and C_{20}-GA2ox-I subgroups.

Transcriptional Profile of *PpGA2ox* Genes in Different Tissues

To investigate the expression profile of *PpGA2ox* genes in different tissues, we analyzed their transcript levels in six tissues (young fruit, flower bud, flower, young leaf, mature leaf, and shoot tip tissue) (**Figure 4**); *PpTEF2* was used for normalization. *PpGA2ox7* was expressed at the lowest level in all tissues analyzed compared to the six other *PpGA2ox* genes. *PpGA2ox1* was expressed at higher levels in flowers and sepals than in other tissues. *PpGA2ox2* and *PpGA2ox4* were expressed at higher levels in sepals compared to other tissues. *PpGA2ox1*, *PpGA2ox2*, and *PpGA2ox4* were expressed at relatively high levels in flower tissue. *PpGA2ox3* and *PpGA2ox5* were expressed at higher levels in young fruits compared to other tissues. *PpGA2ox6* was expressed at lower levels in shoot tips compared to other tissues.

Overexpressing *PpGA2ox* Genes in Tobacco Causes a Dwarf Phenotype

To clarify the function of a representative peach *GA2ox* gene from each of the three subgroups (C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I), we generated the *35S:PpGA2ox1*, *35S:PpGA2ox5*, and *35S:PpGA2ox2* constructs and introduced

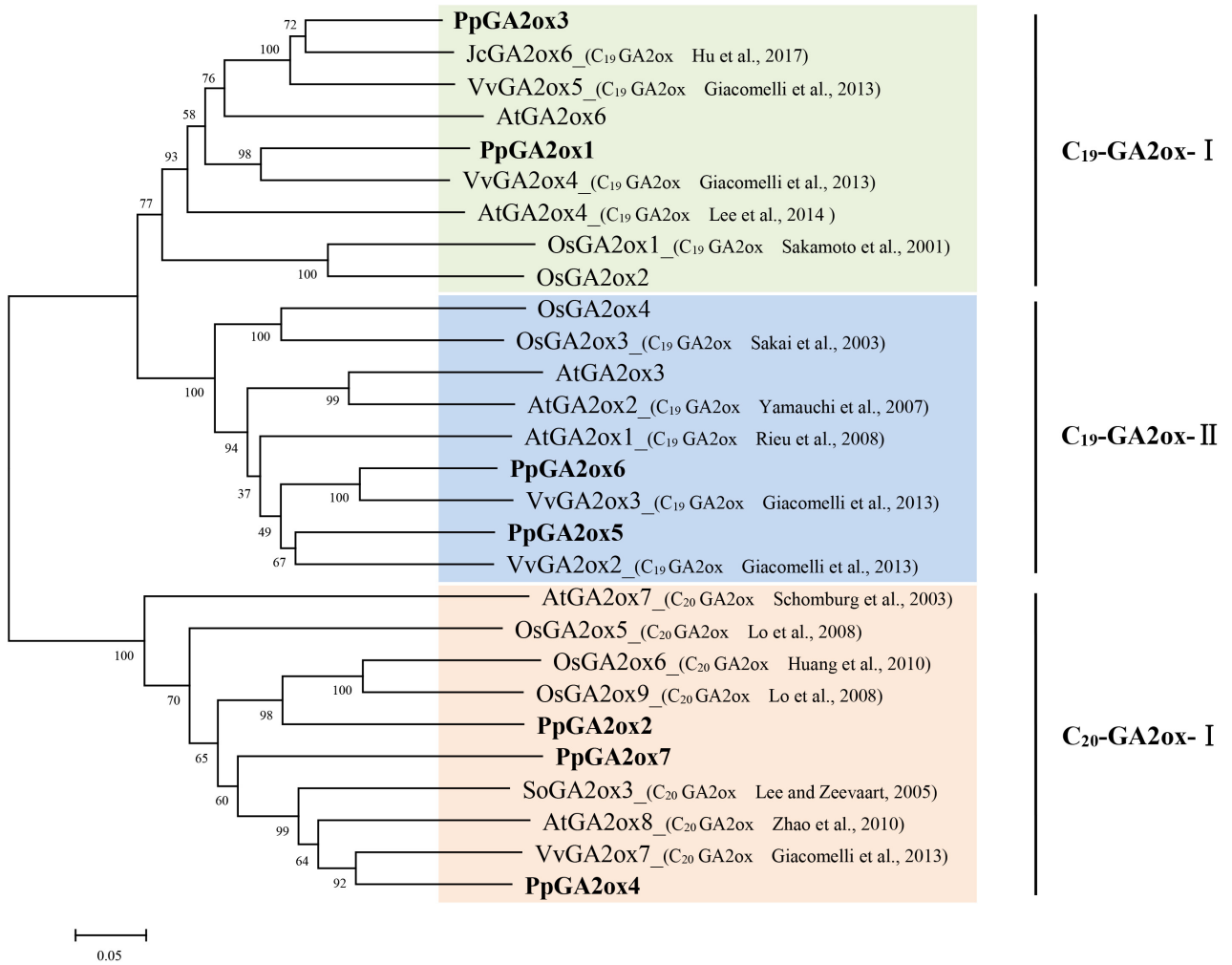

FIGURE 2 | Phylogenetic tree of the predicted amino acid sequences of the GA2oxs from different species using MEGA5.1 program. The amino acid sequences formed three subgroup (C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I) and highlighted in different colors. Gene accession numbers of the sequences used in this tree are listed in **Supplementary Table S2**.

them into tobacco by *Agrobacterium*-mediated transformation. For each construct, more than three independent transgenic tobacco lines were obtained, which were confirmed by PCR and qRT-PCR (**Supplementary Figure S3**). All transgenic plants exhibited dwarf phenotypes compared to the non-transgenic tobacco control (WT) (**Figure 5A**). The heights of the transgenic plants were no more than 30% that of the WT (**Figure 5B**). In addition, the mature leaves of the transgenic plants were crinkled and smaller than those of the WT (**Figures 5C,D**). These results suggest that PpGA2ox1, PpGA2ox5, and PpGA2ox2 negatively regulate plant height and strongly affect leaf development.

PpGA2ox5 Is Active Against Both C_{20}-GAs and C_{19}-GAs

Based on our phylogenetic analysis (**Figure 2**), we predicted that PpGA2ox1 and PpGA2ox5 would be active against C_{19}-GAs, while PpGA2ox2 would be active against C_{20}-GAs. To elucidate the different metabolic functions of these enzymes, we analyzed the responses of the PpGA2ox1-OE, PpGA2ox5-OE, and PpGA2ox2-OE tobacco lines to exogenous GA_1, a C_{19}-GA (**Figures 6A,B**). GA_3, which is resistant to enzyme-catalyzed degradation by GA2ox due to the presence of a double bond between C-2 and C-3 (Kanno et al., 2016), was used as a positive control. The wild type and all transgenic plants showed rapid growth after GA_3 treatment. By contrast, after GA_1 treatment, wild-type plants and plants overexpressing *PpGA2ox2* showed rapid growth, whereas plants overexpressing *PpGA2ox1* showed no significant difference in growth from the negative control (PpGA2ox1-OE with 0 mg/L GA_1 treatment). These results strongly suggest that PpGA2ox1 is a C_{19}-GA2ox and that PpGA2ox2 is a C_{20}-GA2ox. Interestingly, plants overexpressing *PpGA2ox5* showed an intermediate phenotype compared to the PpGA2ox1-OE and PpGA2ox2-OE lines. These results suggest that PpGA2ox5 is active against GA_1. However, PpGA2ox5 is less efficient than

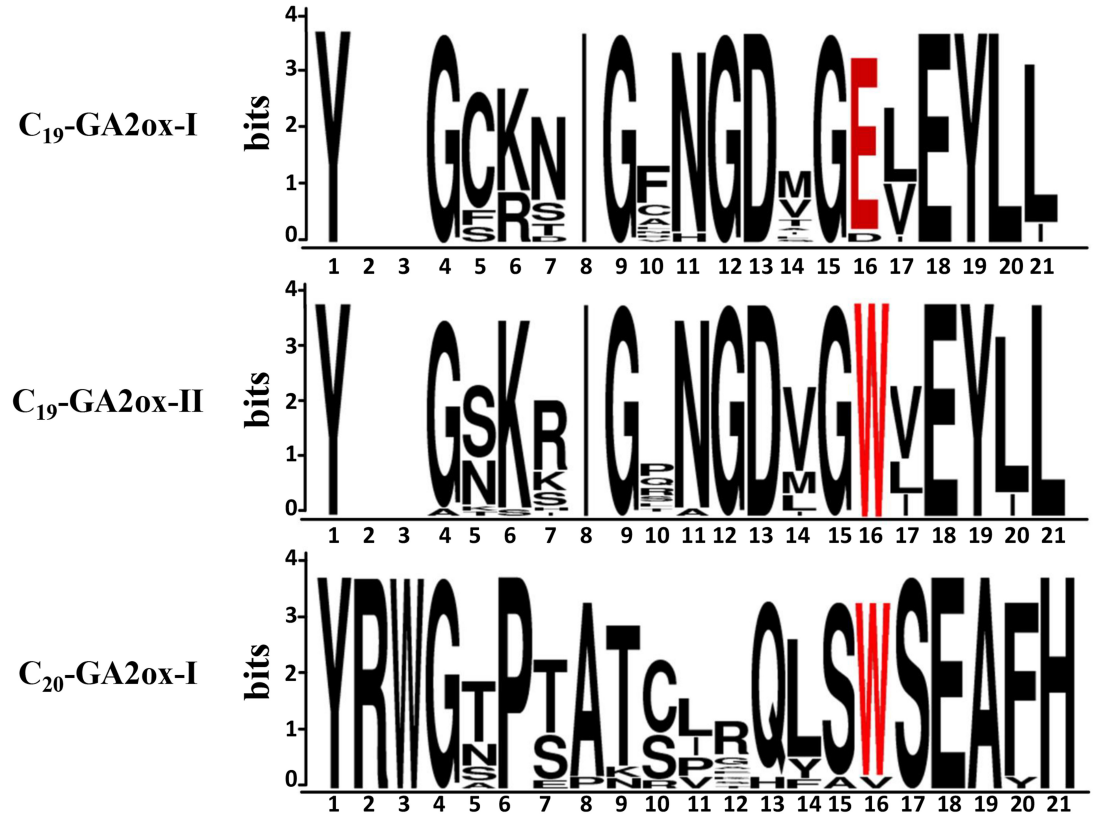

FIGURE 3 | Graphical representation of the signature motif of GA 2-oxidases generated by WebLogo. Letter size is proportional to the degree of amino acid conservation. The 16th amino acid was highlighted in red font.

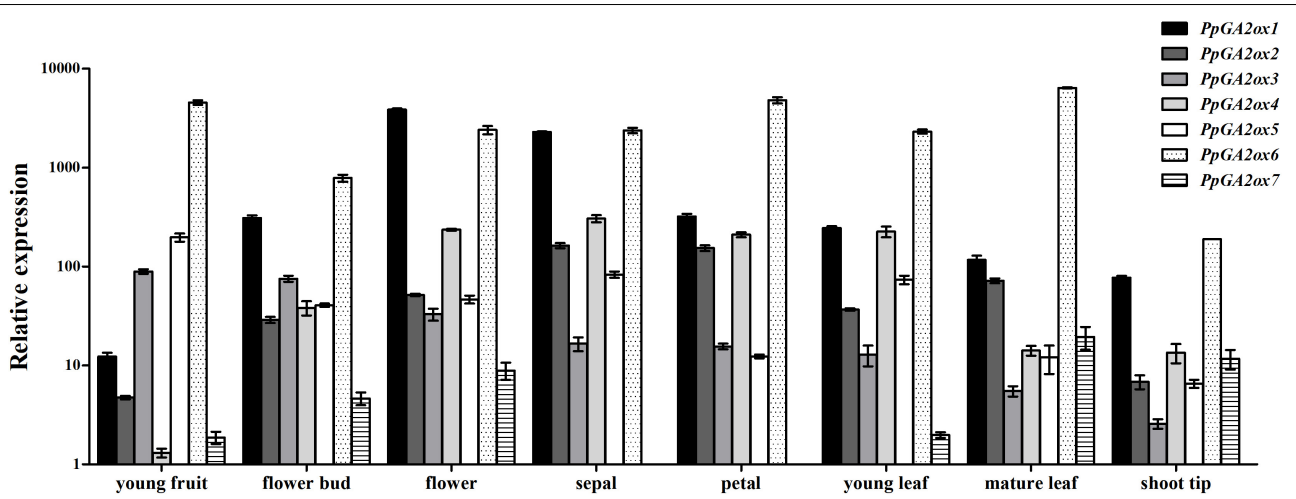

FIGURE 4 | Expression of *PpGA2ox* genes in organs harvested from 3 year old "QMH" peach trees. Total RNA was isolated and analyzed by qRT-PCR, and the PpTEF2 was used as a reference gene. Results are plotted as the ratio to the lowest detected level (PpGA2ox7 in sepal). Note that the *y*-axis is on a logarithmic scale. All data are means of three replicates with error bars indicating standard deviation.

PpGA2ox1, suggesting that PpGA2ox5 might not be a classical type of C_{19}-GA2ox.

To determine how PpGA2ox5 may differ from PpGA2ox1 and PpGA2ox2, we analyzed the GA metabolic profiles in shoot tips with young leaves from wild-type tobacco plants and transgenic lines overexpressing *PpGA2ox1*, *PpGA2ox2*, or *PpGA2ox5* (**Figure 6C**). We measured the contents of four C_{20}-GAs (GA_{15}, GA_{24}, GA_{44}, and GA_{19}) and seven C_{19}-GAs

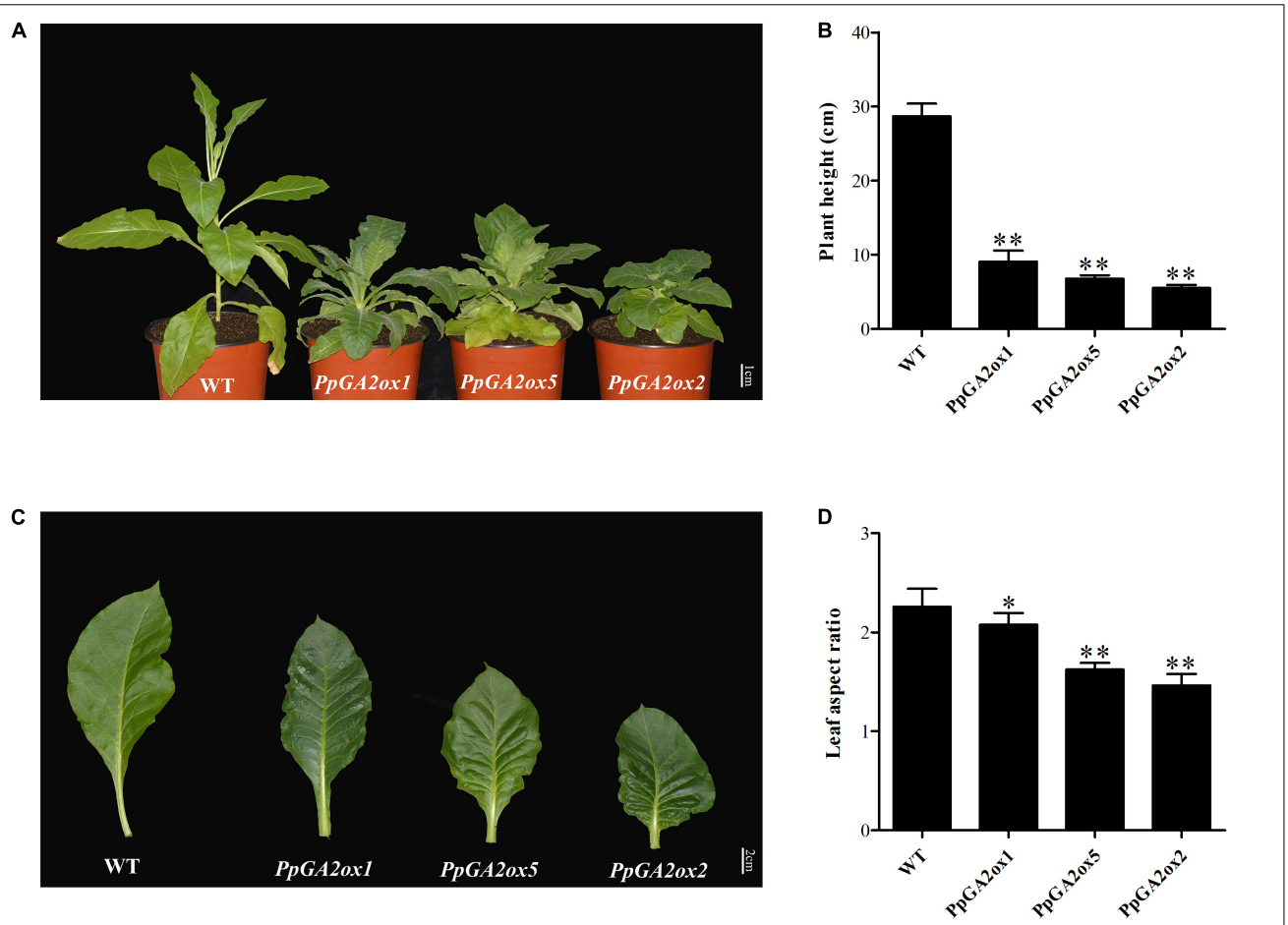

FIGURE 5 | Ectopic expression of *PpGA2ox1*, *PpGA2ox5*, and *PpGA2ox2* in tobacco induces dwarfism and smaller leaves. **(A)** Transgenic tobacco plants were grown in a climate chamber for 3 months. **(B)** Heights of the WT and transgenic tobacco plants. **(C)** Mature leaves of WT and transgenic tobacco. **(D)** Leaf aspect ratios of the WT and transgenic tobacco plants. The values are presented as the means ± standard deviation. *Significantly different from the wild type at $P < 0.05$. **Significantly different from the wild type at $P < 0.01$.

(GA_5, GA_8, GA_9, GA_{20}, GA_{29}, GA_{34}, and GA_{51}) using HPLC-MS/MS. The levels of the four C_{20}-GAs (GA_{15}, GA_{24}, GA_4, and GA_{19}) were higher in the PpGA2ox1-OE line and lower in the PpGA2ox5-OE and PpGA2ox2-OE lines vs. the wild type. GA_{24} and GA_{44} levels were significantly higher in the PpGA2ox1-OE line, whereas the levels of all four C_{20}-GAs were significantly lower in the PpGA2ox5-OE and PpGA2ox2-OE lines compared to the wild type. Only PpGA2ox2-OE plants showed significantly lower levels of both C_{19}-GAs (GA_9 and GA_{20}) compared to the wild type. GA_8 and GA_{29} levels were significantly higher in all transgenic plants compared to the wild type. GA_5 levels were significantly higher in all three transgenic lines vs. the wild type as well. Moreover, GA_5 contents were 30.3-fold higher in PpGA2ox5-OE than in the wild type. GA_{12}, GA_{110}, GA_{97}, GA_{51}, and GA_{34} were not detected in any of the plants. These results suggest that PpGA2ox5 is active not only against C_{19}-GAs, but also against C_{20}-GAs.

Surprisingly, there were no differences in GA_8 content among PpGA2ox1-OE, PpGA2ox5-OE, and PpGA2ox2-OE plants, but PpGA2ox1-OE plants showed higher activity against GA_1 compared to PpGA2ox5-OE and PpGA2ox2-OE. The main bioactive GA in tobacco is GA_4, whereas GA_1 levels are low in this plant (Xiao et al., 2016). This paradoxical result could be due to the different concentrations of GA_1 (endogenous vs. exogenous GA_1) in the plants.

C_{19}-GA 2-Oxidases Are Transcriptionally Activated More Rapidly Than C_{20}-GA 2-Oxidases After GA_3 Treatment

Increased levels of bioactive GA significantly activate the transcription of *GA2oxs* in Arabidopsis (Rieu et al., 2008). In the current study, overexpressing a C_{20}-GA2ox reduced the levels of GA_{15}, GA_{24}, GA_{44}, GA_{19}, GA_9, and GA_{20} in tobacco, while overexpressing a C_{19}-GA2ox promoted the accumulation of GA_{24} and GA_{44} (**Figure 6C**). These findings suggest that C_{20}-GA2oxs and C_{19}-GA2oxs play different roles in regulating the levels of bioactive GAs. We therefore investigated the responses of C_{20}-GA2ox and C_{19}-GA2ox genes in shoot tips to treatment with GA_3 for 0.5, 2, and 4 h (**Figure 7**). The

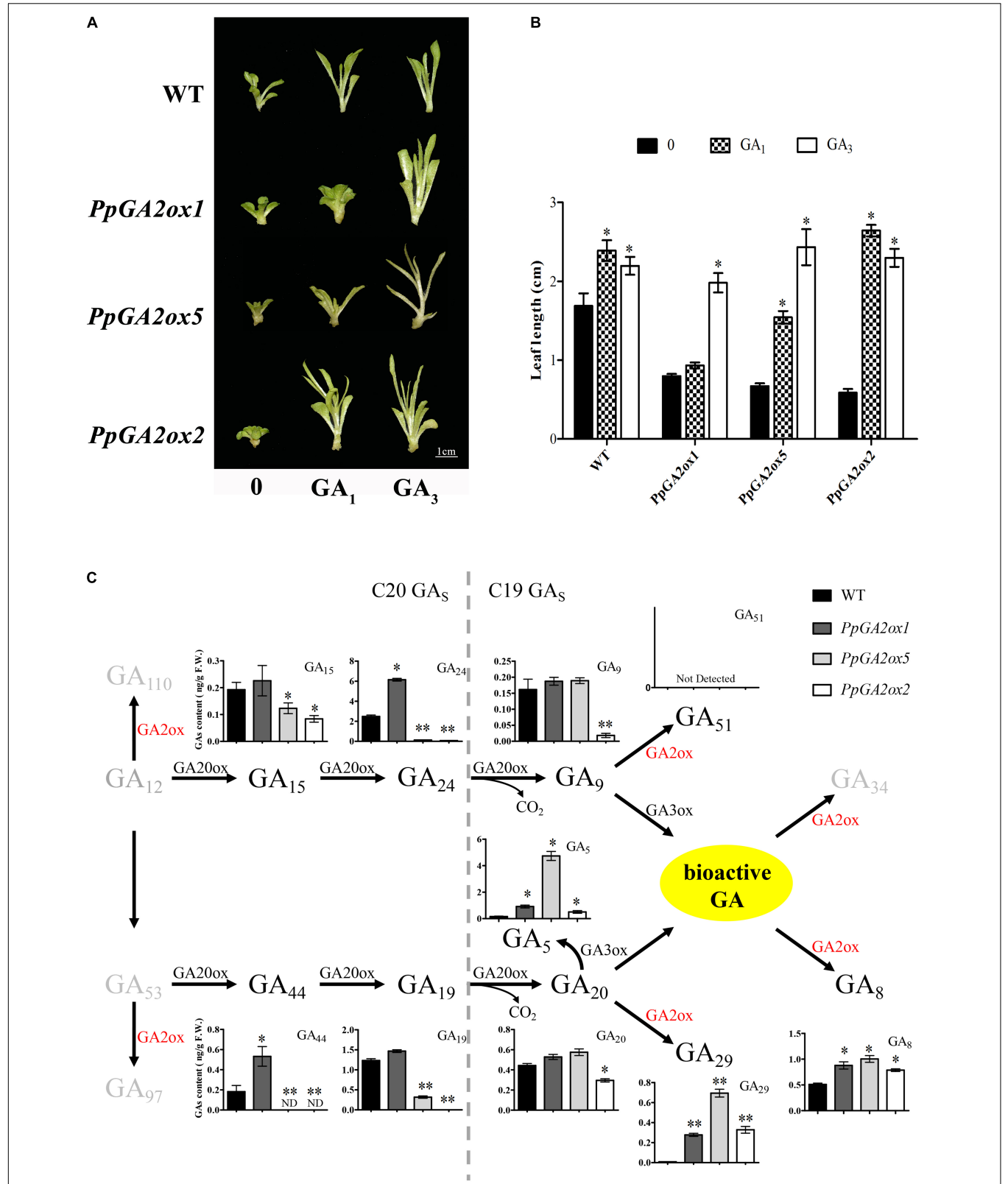

FIGURE 6 | Analysis the function of PpGA2ox1, PpGA2ox5, and PpGA2ox2. **(A,B)** Effects of GA_1 and GA_3 on phenotypes of transgenic plants. Shoots cultivated in MS medium containing 0.4 or 0.0 mg/L GA_1 or GA_3. WT represents the non-transgenic plant. Bar represents 1 cm. **(C)** Effects of PpGA2ox1, PpGA2ox5, and PpGA2ox2 on the biosynthesis pathway of GA in tobacco measured using HPLC-MS/MS. The values are presented as the means ± standard error. *Significantly different from the wild type or untreated plant ($P < 0.05$). **Significantly different from the wild type ($P < 0.01$).

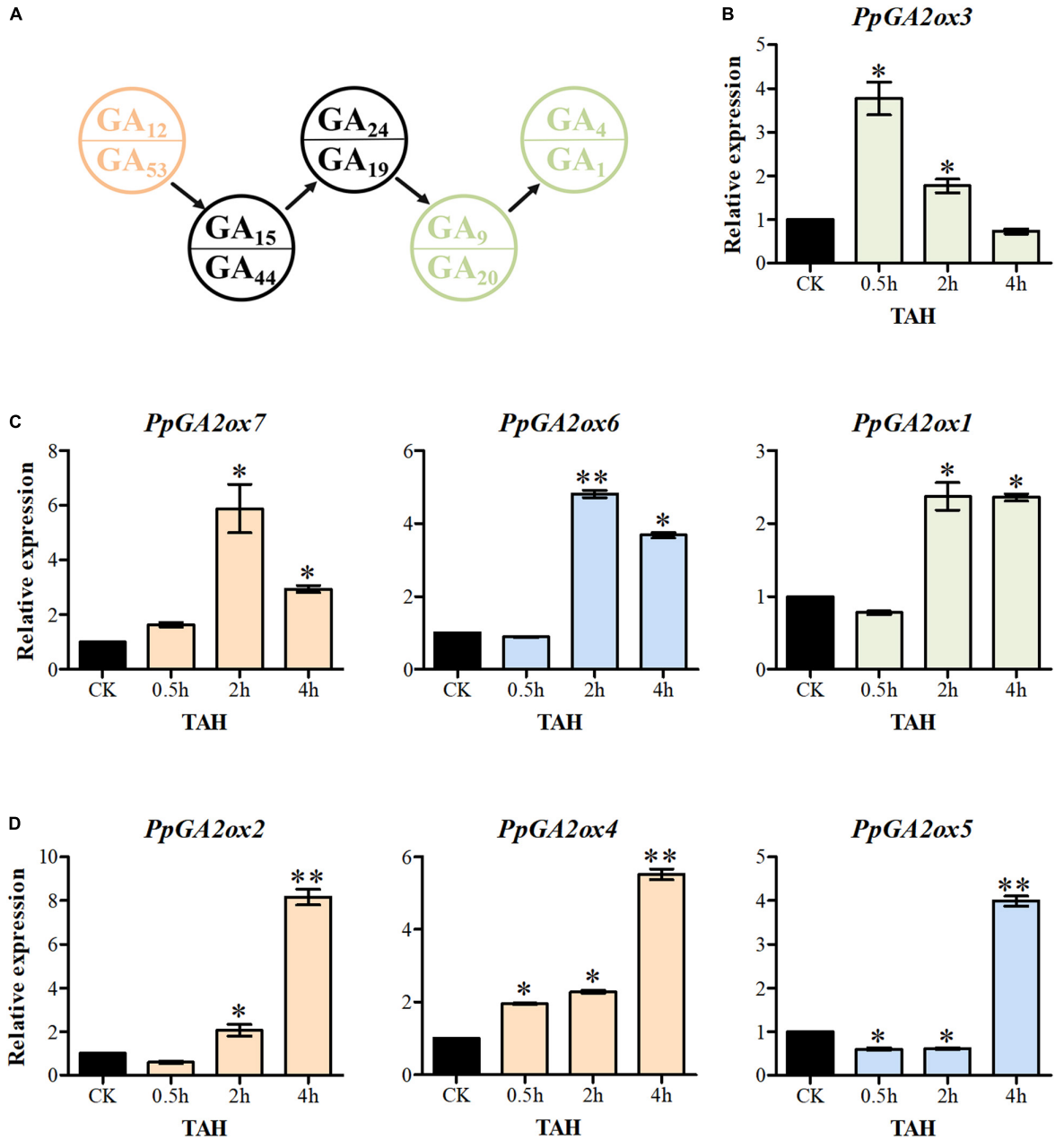

FIGURE 7 | The transcription of *PpGA2ox* genes after GA_3 treatment. **(A)** Part of GA biosynthesis way. Pink and green font represent the substrates for C_{20}-GA2ox and C_{19}-GA2ox, respectively. Seven *PpGA2ox* genes were divided into three type based on the response time to GA_3 treatment. **(B)** The type 1 showed the most rapid response to GA_3 treatment. **(C)** The type 2 reached to the peak transcript level at 2 h. **(D)** The type 3 reached to the peak transcript level at 4 h. Green, blue and pink columns represent the genes from the C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I subfamilies, respectively. TAH, treatment after hours. CK was the control treatment sprayed with distilled water. The values are presented as the means ± standard deviation. *Significantly different from the control ($P < 0.05$). **Significantly different from the control ($P < 0.01$).

seven *PpGA2ox* genes were divided into three types based on their transcriptional responses. The type 1 response, which was observed only for *PpGA2ox3*, was the most rapid response to GA_3 treatment, with peak expression at 0.5 h followed by a reduction at 2 and 4 h after GA_3 treatment. The type 2 response, which was observed for *PpGA2ox1*, *PpGA2ox6*, and *PpGA2ox7*, was characterized by peak transcript levels at 2 h after GA_3 treatment that remained high at 4 h after treatment. The type 3 response to GA_3, as observed for *PpGA2ox2*, *PpGA2ox4*, and *PpGA2ox5*, was slower, with gradually increasing transcript levels at the first two time points and a peak at 4 h. These results suggest that the transcription of the C_{19}-GA2ox genes is activated more rapidly than the transcription of C_{20}-GA2ox genes after GA_3 treatment.

DISCUSSION

GAs are important phytohormones that control diverse aspects of plant growth and development, including seed germination, stem elongation, leaf expansion, and flower and seed development (Yamaguchi, 2008). The homeostasis of bioactive GA levels is crucial for plant growth and development. GA2ox enzymes catalyze the deactivation of GA, thereby helping to maintain GA homeostasis. In the current study, we identified seven *PpGA2ox* genes in the peach genome. Our findings suggest that C_{19}-GA2ox-II enzymes might be evolutionary intermediates between C_{20}-GA2oxs and C_{19}-GA2oxs and that the PpGA2ox family is responsible for maintaining the delicate balance of GA homeostasis.

C_{19}-GA2ox-II Enzymes Might Be Intermediates Between C_{20}-GA2oxs and C_{19}-GA2oxs

The GA2 oxidase family is divided into three groups: C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I. These groups are highly conserved in monocots and dicots (**Table 1**), suggesting there are likely functional differences among the groups. Here, we demonstrated that PpGA2ox5 belongs to subfamily C19-GA2ox-II and is simultaneously active against both C_{20}-GAs and C_{19}-GAs. Tobacco plants overexpressing PpGA2ox5 contained lower levels of four C_{20}-GAs (GA_{15}, GA_{24}, GA_{44}, and GA_{19}) than the wild type, which was similar to the results for PpGA2ox2-OE (a predicted C_{20}-GA2ox) but opposite to those for of PpGA2ox1-OE (a predicted C_{19}-GA2ox). These changes in C_{20}-GA levels in the PpGA2ox5-OE lines were consistent with those in Arabidopsis *c_{19}-ga2ox* and *c_{20}-ga2ox* mutants (Magome et al., 2008; Rieu et al., 2008). In addition, PpGA2ox5-OE contained significantly higher levels of GA_5 and GA_{29} than the wild type. These results suggest that PpGA2ox5-OE is active against C_{19}-GAs.

PpGA2ox1-OE showed higher levels of GA_{15}, GA_{24}, GA_{44}, and GA_{19} than the WT. Perhaps these plants exhibited increased GA 20-oxidase activity resulting from feedback regulation due to reduced levels of bioactive GAs. PpGA2ox2-OE also contained significantly higher levels of GA_5 and GA_{29} than the wild type, suggesting that PpGA2ox2 also has activity against C_{19}-GAs. While PpGA2ox2-OE plants had a dwarf phenotype, they grew rapidly after GA_1 treatment. These results suggest that PpGA2ox2-OE might also have two activities against C_{20}-GAs and C_{19}-GAs, although its major activity is against C_{20}-GAs. VvGA2ox7, a member of C_{20}-GA2ox-I in grapevine, is also active against C_{19}-GAs (GA_9, GA_{20}, GA_4, and GA_1) (Giacomelli et al., 2013).

Understanding the evolutionary relationships among gene subfamilies can uncover information about the course of functional divergence of genes. C_{19}-GA2oxs, C_{20}-GA2oxs, and GA biosynthetic oxidases (GA20ox and GA3ox) are all members of the 2OG-Fe (II) oxygenase superfamily (Han and Zhu, 2011). Although the evolutionary relationships among the C_{19}-GA2ox, C_{20}-GA2ox, GA20ox, and GA3ox subfamilies is unclear, the four subfamilies likely share a common ancestor (Giacomelli et al., 2013; Huang et al., 2015). Our results suggest that C_{20}-GA2ox-I is an older clade of the GA2ox family compared to C_{19}-GA2ox. Indeed, several studies have suggested that C_{19}-GA2ox genes were derived from C_{20}-GA2ox genes (Han and Zhu, 2011; Giacomelli et al., 2013; Huang et al., 2015). In an addition, only *ent*-kaurenoic acid, a gibberellin precursor, was detected in *Physcomitrella patens*, and *ent*-kaurenoic acid oxidase (KAO), GA20ox, and GA3ox were absent in this moss (Hirano et al., 2007; Miyazaki et al., 2018). *ent*-kaurenoic acid contains 20 carbons and can be converted into the bioactive GAs by KAO, GA20ox, and GA3ox. This observation suggested that C_{20}-GA arose before C_{19}-GA during evolution, implying that enzymes active against C_{20}-GAs evolved earlier than enzymes active against C_{19}-GAs.

Since PpGA2ox2 showed activity against C_{19}-GAs, perhaps the ancient GA2ox was able to catalyze the 2β-hydroxylation of both C_{19}-GAs and C_{20}-GAs but was mainly active against C_{20}-GAs and was only weakly active against C_{19}-GAs. Perhaps once the ancient GA2ox evolved into two clades, one of the clades kept the original activity and formed the C_{20}-GA2ox-I subfamily, while the other clade gradually gained increased activity against C_{19}-GAs, resulting in the formation of the C_{19}-GA2ox clade. The C_{19}-GA2ox-II clade, containing PpGA2ox5, might have retained the ancient function of catalyzing the 2β-hydroxylation of C_{20}-GAs (**Figure 8**).

Our analysis showed that the conserved tryptophan (W) in the signature motif of GA2ox is highly conserved in both C_{19}-GA2ox-II and C_{20}-GA2ox proteins, while a glutamic acid (E) is highly conserved in C_{19}-GA2ox-I proteins. This tryptophan is also highly conserved in four clades of the 2OG-Fe (II) oxygenase superfamily (GA20ox, GA3ox, GAox-A, and GAox-C) (Huang et al., 2015). We speculate that this tryptophan is important for the ancient function of catalyzing the 2β-hydroxylation of the C_{20}-GAs. More work is needed to verify this hypothesis.

TABLE 1 | The number of *GA2ox* genes from six species distributed into the three subgroups.

	C19-GA2ox-I	C19-GA2ox-II	C20-GA2ox-I	References
Arabidopsis	2	3	2	Han and Zhu, 2011
Rice	2	4	4	
Cucumber	1	3	1	Pimenta Lange et al., 2013
Grapevine	2	3	4	Giacomelli et al., 2013
Tomato	2	5	4	Chen et al., 2016
Peach	2	2	3	

PpGA2ox Family Members Help Maintain the Delicate Balance of Endogenous GA Levels

Plants must cope with variable environmental conditions and are subjected to daily variations in light and temperature

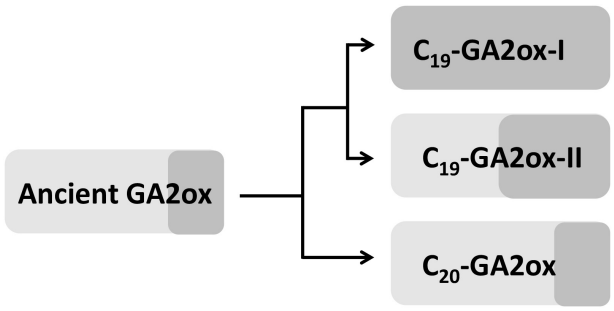

FIGURE 8 | Hypothesis for the evolution of GA2 oxidases in higher plants. Light and dark gray box represents the activity of GA2ox against C_{20}-GAs and C_{19}-GAs, respectively. The area of box is proportional to the degree of enzyme activity.

along with various biotic and abiotic stresses. Endogenous phytohormones help regulate plant growth and development and function in plant responses to the variable environment. GAs generally stimulate organ growth by enhancing cell elongation and cell division (Hedden and Thomas, 2012). The regulation of endogenous GA levels is important for regulating plant growth and development. The deactivation of GAs by GA2 oxidases is an important mechanism that influences GA responses. For example, bioactive GAs accumulate in the hypocotyls of dark-grown Arabidopsis seedlings and are deactivated in the hypocotyls of light-grown seedlings via the activated transcription of *GA2ox1* (Achard et al., 2007). Low temperature (15°C) promotes GA deactivation by activating the transcription of *GA2ox* genes in rice (Wang et al., 2018). High salinity upregulates *GA2ox7* in leaves, hypocotyls, and roots, resulting in reduced levels of active GAs (Magome et al., 2008). *GA2ox7* is also upregulated by cold stress (Zhou et al., 2017).

In the current study, we treated tobacco shoot tips with a high concentration of GA_3, which appeared to be responsible for increasing the expression of *GA2ox* genes over time. The different response times of different *PpGA2ox* genes coupled with the different GA targets of the subgroups within the GA2ox family (C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I) illustrate how these enzymes fine-tune GA levels. Three *PpGA2ox* genes, one each from the C_{19}-GA2ox-I, C_{19}-GA2ox-II, and C_{20}-GA2ox-I subgroups, were significantly upregulated after GA_3 treatment at similar time points, whereas one gene from C_{19}-GA2ox-II and one from C_{20}-GA2ox-I were significantly upregulated more slowly after GA_3 treatment. After GA_3 treatment, the C_{19}-GA2oxs were activated first, followed by the C_{20}-GA2oxs. Six C_{20}-GAs and four C_{19}-GAs are intermediates in the GA biosynthesis pathway. The C_{20}-GA2oxs significantly depleted the C_{20}-GAs and some of the C_{19}-GAs, while the C_{19}-GA2oxs significantly depleted the C_{19}-GAs but increased the levels of some of the C_{20}-GAs (**Figure 6**). Ultimately, the C_{20}-GA2oxs were activated, thereby blocking GA biosynthesis. These findings indicate that the plants employed an active response to cope with high GA_3 treatment. Together, these results suggest that the *PpGA2ox* family contributes to the fine-tuning of endogenous GA levels via the different response times and different enzymatic activities of the individual PpGA2 oxidases.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The peach cultivar used in this study, "QiuMiHong" (QMH), was provided by the Fruit Tree Germplasm Repository of Henan Agricultural University (Henan Province, China). For RNA extraction, young fruits, flower buds, flowers, sepals, petals, young leaves, mature leaves, and shoot tips were collected from 3 year old "QMH" trees in the spring, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent experiments. Tobacco variety "K326" seedlings were grown *in vitro* in a temperature-controlled growth chamber at 24 ± 2°C under a 16 h light/8 h dark cycle. To calculate the leaf aspect ratio, a digital Vernier caliper was used to measure the distance from the bottom to the top of the leaf (length) and the maximum width of the leaf (width).

Sequence Analysis and Cloning

To identify *GA2ox* genes in the peach genome, a BlastP with default parameters of the Phytozome database[1] was performed based on the conserved domain of genes in the 2-oxoglutarate-dependent oxygenase family using the amino acid sequences of seven Arabidopsis GA2oxs as queries. The search results were aligned by ClustalW algorithm with default parameters to reduce duplicate and redundant sequences (Larkin et al., 2007). Gene information from the JGI database was then used to predict the genomic positions of the *PpGA2ox* genes. The *PpGA2ox* genes were renamed, plotted on the peach chromosomes, and visualized with MapGene2 Chrom[2] (Jiangtao et al., 2015).

The amino acid sequences of all GA oxidases used in this study were downloaded from the Phytozome and NCBI databases[3]. Sequence logos were generated using the online WebLogo platform[4] (Crooks et al., 2004). A phylogenetic tree was constructed using the neighbor-joining method (NJ) method in MEGA (version 5.1) (Tamura et al., 2011). The main parameters were set as follows: the model was set to "p-distance," the GAPs was set to "pairwise deletion," and bootstrapping was performed with 1000 replicates. Default parameters were used for all bioinformatics analyses, except for those specifically mentioned.

The full-length sequences of the *PpGA2ox* genes were cloned from RNA obtained from peach shoot tips using specific primers and ligated into the pEASY®-Blunt Vector (TransGen Biotech). PCR products that produced a single band on the gel were purified and sent to Sangon Biotech (Shanghai[5]) for sequencing. All primers used in this study are listed in **Supplementary Table S3**.

[1]https://phytozome.jgi.doe.gov/pz/portal.html
[2]http://mg2c.iask.in/mg2c_v2.0/
[3]https://www.ncbi.nlm.nih.gov/
[4]http://weblogo.berkeley.edu/logo.cgi
[5]https://www.sangon.com/

Construction of the Overexpression Vector and Transformation of Tobacco

The full-length sequences of the *PpGA2oxs* were amplified from the pEASY® -Blunt Vector (TransGen Biotech) using specific primers containing restriction sites and inserted into the pSAK277 vector containing the *CaMV35S* promoter. The plant overexpression vectors *35S:PpGA2ox1, 35S:PpGA2ox5*, and *35S:PpGA2ox2* were introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform tobacco leaf discs as described previously (Horsch et al., 1985; Lee and Zeevaart, 2005). Briefly, the leaves of aseptic tobacco seedlings were cut into small pieces (∼1 cm × 1 cm), pre-cultured in Murashige and Skoog (MS) medium for 2 days, incubated with *A. tumefaciens* (OD_{600} = 0.6) harboring the chosen construct for 5 min, and transferred to MS medium. After 2 days of co-culture in the dark, the leaf discs were transferred to selection medium (MS, 1 mg/L 6-benzylaminopurine, 0.1 mg/L NAA, 50 mg/L kanamycin, and 400 mg/L cefotaxime). When shoots appeared, individual shoots were cut and transferred to root-inducing medium (MS, 0.1 mg/L NAA, 50 mg/L kanamycin, and 400 mg/L cefotaxime). After rooting, the plantlets were grown in pots in a greenhouse and used for phenotypic analysis and further experiments. A pSAK277-GFP vector was transformed into tobacco in our lab and the transgenic lines showed no dwarf phenotype and smaller leaves.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from the samples using a Column-type Plant Total RNA Extraction and Purification Kit (Sangon Biotech. Shanghai, China). Purified total RNA (0.5–2 μg) was reverse transcribed into first-strand cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Beijing, China). The cDNA product was used for semiquantitative RT-PCR and qRT-PCR analysis. qRT-PCR analysis was conducted using SYBR Select Master Mix (Applied Biosystems, United States) according to the manufacturer's protocol on an ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems, Madrid, CA, United States). All experiments were conducted in triplicate. To quantify relative transcript level, *TEF2* (Peach EST database accession number: TC3544) and β-*tubulin* (accession number: U91564) were used for data normalization (Tong et al., 2009; Schmidt and Delaney, 2010).

Exogenous GA_3 Treatment

One year old branches of "QMH" peach trees were sprayed with GA_3 (100 mg/L) or with distilled water (as a control). Three biological replicates were performed for all treatments. Shoot tips were collected at 0.5, 2, and 4 h after treatment for RNA extraction to analyze *GA2ox* transcript levels.

Quantification of Endogenous GAs

To measure endogenous phytohormone contents, approximately 1.0 g of young leaf tissue was collected from transgenic and wild-type tobacco plants and frozen in liquid nitrogen. The concentrations of different GAs were measured by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) as described previously (Cheng et al., 2019). Three biological replicates were used to measure the level of each hormone.

Treating Tobacco With GA_1 and GA_3

The shoots of transgenic tobacco were cultivated in MS medium containing 0.4 mg/L GA_1 or 0.4 mg/L GA_3. Changes in growth were observed 10 days after treatment. The lengths of 8–10 leaves were measured and averaged. The experiments were performed three times.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JF, XZ, and JC conceived and designed the experiments. JM, HL, and MZ performed the experiments. JF and JC wrote the manuscript. BT, XY, and WW performed the GA metabolic analysis. LZ, ZL, and JL revised the manuscript. All authors contributed to the article 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.2021.619158/full#supplementary-material

Supplementary Figure 1 | Amino acid residues presumed to bind Fe at the active site (*). Putative 2-oxoglutarate binding sites (▲) and the signature motif of GA2ox (Black overline). Two conserved domains DIOX_N (red overline) and 2OG-FeII_Oxy (green overline) were detected in all GA2ox proteins. Black shading indicates identity, magenta and cyan shading indicate that similar residues are greater than 75 and 50%, respectively.

Supplementary Figure 2 | Analysis the evolutionary relationship of C_{19}-GA2ox-I, C_{19}-GA2ox-II and C_{20}-GA2ox-I subgroups. A phylogenetic tree of GA2ox and GAox. All GA2oxs were searched through the Phytozome database (https://phytozome.jgi.doe.gov). Gene accession numbers of the sequences used in this tree are listed in **Supplementary Table S2**. ▲ represents the GA oxidases from liverwort (*Marchantia polymorpha*), *Sphagnum fallax*, *Selaginella moellendorffii*; ● represents the GA2 oxidases from rice, peach and *Arabidopsis thaliana*.

Supplementary Figure 3 | qRT-PCR analysis of PpGA2ox-overexpressing transgenic and non-transgenic (WT) lines using RNA isolated from the tobacco young leaves. The expression levels of each gene were relative to Nt β-Tubulin. Bars represent mean values of three biological repeats indicated standard deviation. ***Significantly different from the wild type ($P < 0.001$).

Supplementary Table 1 | Comparison of the deduced amino acid sequences between PpGA2ox proteins.

Supplementary Table 2 | The accession numbers of GAoxs used in this study.

Supplementary Table 3 | Primer used in this study.

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