



Role of Hydraulic Signal and ABA in Decrease of Leaf Stomatal and Mesophyll Conductance in Soil Drought-Stressed Tomato

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Drought reduces leaf stomatal conductance (g_s) and mesophyll conductance (g_m). Both hydraulic signals and chemical signals (mainly abscisic acid, ABA) are involved in regulating g_s . However, it remains unclear what role the endogenous ABA plays in g_m under decreasing soil moisture. In this study, the responses of g_s and g_m to ABA were investigated under progressive soil drying conditions and their impacts on net photosynthesis (A_n) and intrinsic water use efficiency (WUE_i) were also analyzed. Experimental tomato plants were cultivated in pots in an environment-controlled greenhouse. Reductions of g_s and g_m induced a 68–78% decline of A_n under drought conditions. While soil water potential (Ψ_{soil}) was over -1.01 MPa, g_s reduced as leaf water potential (Ψ_{leaf}) decreased, but ABA and g_m kept unchanged, which indicating g_s was more sensitive to drought than g_m . During Ψ_{soil} reduction from -1.01 to -1.44 MPa, Ψ_{leaf} still kept decreasing, and both g_s and g_m decreased concurrently following to the sustained increases of ABA content in shoot sap. The g_m was positively correlated to g_s during a drying process. Compared to g_s or g_m , WUE_i was strongly correlated with g_m/g_s . WUE_i improved within Ψ_{soil} range between -0.83 and -1.15 MPa. In summary, g_s showed a higher sensitivity to drought than g_m . Under moderate and severe drought at $\Psi_{soil} \leq -1.01$ MPa, furthermore from hydraulic signals, ABA was also involved in this co-ordination reductions of g_s and g_m and thereby regulated A_n and WUE_i .

Keywords: drought, leaf water potential, abscisic acid, stomatal conductance, mesophyll conductance, intrinsic water use efficiency

INTRODUCTION

Soil water scarcity is one of the major environmental constraints to the plant physiological processes and yield (Easlon and Richards, 2009; Olsovska et al., 2016). To achieve high plant water-use efficiency under a drier environment in the future, it is essential to improve crop photosynthesis and productivity with a given unit of water (Flexas et al., 2013). For C_3 plants, leaf photosynthesis is strongly limited by three factors,

i.e., stomatal conductance (g_s), mesophyll diffusion conductance to CO_2 (g_m), and biochemical photosynthetic capacity (Grassi and Magnani, 2005; Cano et al., 2013). g_s and g_m determine the diffusion of CO_2 from ambient air of leaf to sub-stomatal cavities and from the sub-stomatal cavities to chloroplast stroma, respectively (Flexas et al., 2002; Niinemets et al., 2009). Recent studies have shown that both g_s and g_m were the main limitations for maximum photosynthesis under drought conditions (Tosens et al., 2016; Wang et al., 2018). Therefore, revealing the mechanisms underlying the decreases of g_s and g_m in response to drought is necessary for enhancing our understanding of plant adaptation to water limitation.

Different regulatory mechanisms such as chemical messengers like abscisic acid (ABA), electrical signals, and hydraulic signals have been identified in the control of stomatal movement (Dodd, 2005; Ache et al., 2010; Tombesi et al., 2015; Huber et al., 2019). Despite the large list of candidates in regulating guard cells, ABA and hydraulic signals have gained most of the attention in regulating stomatal aperture. ABA is a phytohormone that has been involved in different strategies of plants to avoid excessive water loss, and many reports demonstrated its important role in stomatal control (Wilkinson and Davies, 2002; Assmann and Jegla, 2016). The decrease of g_s in response to drought has been generally modulated by the accumulation of leaf ABA in a wide number of plant species including soybean, grapevine and tomato (Liu et al., 2005; Tombesi et al., 2015; Yan et al., 2017). However, stomata closed with a wide range of variations of leaf hydraulic signals, such as leaf water potential (Ψ_{leaf}), possibly due to differences of experimental plant materials and the intensity of applied drought under investigation. For example, g_s decreased with decreasing Ψ_{leaf} during leaf dehydration (Kim et al., 2012; Wang et al., 2018). On the contrary, other studies showed that stomata closed with little change in Ψ_{leaf} under moderate soil drying, but both parameters decreased under severe drought (Tardieu, 1998; Yan et al., 2017), or g_s decreased as Ψ_{leaf} increased under mild soil drying but then no significant relationship existed between both variables with continued soil drying (Kudoyarova et al., 2007). It is difficult to explore the response of Ψ_{leaf} and g_s under a single soil water condition. Progressive soil drying, representing a natural process of soil water loss, could help us explore the dynamic responses of g_s to Ψ_{leaf} during drying process.

Leaf mesophyll conductance to CO_2 (g_m) has been recognized to be finite, variable, and rapid acclimation to varying environmental conditions. Although a reduction in g_m response to soil drought has been reported in many studies, the mechanisms underlying this reduction have not been elucidated substantially (Flexas et al., 2002; Th eroux-Rancourt et al., 2014; Sorrentino et al., 2016). Recent studies on hydraulic signals suggested that the parallel decreases in g_s and g_m were caused by leaf hydraulic vulnerability as a result of decrease in Ψ_{leaf} (Wang et al., 2018). Similarly, g_m was strongly correlated with leaf hydraulic conductance (K_{leaf}), as the ratio of transpiration rate to the water potential driving force across the leaf ($K_{\text{leaf}} = \text{transpiration}/\Delta\Psi_{\text{leaf}}$), across species under light-saturated conditions (Xiong et al., 2018). This correlation between g_m and leaf hydraulic signals might be due to CO_2 partially shared

common diffusion pathways with H_2O through mesophyll tissues (Ferrio et al., 2012). These studies confirmed that leaf hydraulic signals played an essential role in controlling g_m in response to drought. However, the effects of chemical ABA signal on g_m are not consistent. Vrabl et al. (2009) did not observe any reduction in g_m when applied exogenous ABA in *Helianthus annuus* plants. In line with this, Flexas et al. (2013) found that g_m was highly insensitive to endogenous ABA among ABA-insensitive and ABA-hypersensitive genotypes or to exogenous ABA application in *Arabidopsis thaliana*. However, several studies yielded contrasting results. For instance, Mizokami et al. (2015) compared the responses of g_m to leaf ABA in wild type and ABA-deficient mutant of *Nicotiana plumbaginifolia* and confirmed that the increase in leaf ABA concentration was crucial for the decrease in g_m under drought conditions. Still, g_m reduced effectively in response to ABA in a short term in three of the four species in Sorrentino et al. (2016). Recently, Mizokami et al. (2018) examined the responses of g_m to high CO_2 and ABA application and revealed that g_m was able to respond to high ABA levels, which was intrinsically different from the response to the elevated CO_2 . These contrasting results possibly due to species differences or the experimental approaches utilized to modify ABA, e.g., the exogenous ABA concentration or the applying period. In brief, it has been largely demonstrated that hydraulic signals play an important role in regulating g_m , while the role of ABA on g_m is still not unequivocal. Therefore, a deep understanding about the mechanisms of g_m response to endogenous ABA under progressive soil drying conditions awaits further investigation.

Leaf intrinsic water use efficiency (WUE_i), expressed as the ratio of net photosynthetic rate (A_n) to g_s at leaf level, can explain instantaneous responses to environmental factors (Flexas et al., 2016; Qiu et al., 2019). Improving WUE_i need increase A_n and decrease g_s simultaneously. Using A_n/g_s to explain the changes of WUE_i would be too coarse due to the decrease in g_s inevitably affect CO_2 uptake and thereby limit A_n . g_m determines the CO_2 concentration at the carboxylation site in the chloroplast, increasing g_m would increase A_n without increasing water loss. Therefore, g_m might play a role in improving WUE_i . Despite all of the negative impacts of drought stress on leaf gas exchange, many studies reported that drought was beneficial to improve WUE_i (Liu et al., 2005; Xue et al., 2016). However, the reasons of this improvement of WUE_i have not been elucidated clearly. Evidences have suggested that g_m/g_s played a key role on increasing WUE_i in response to water limitation (Flexas et al., 2016; Han et al., 2016). Revealing the exact responses of g_m/g_s or WUE_i to stressed signals especially ABA under progressive soil drought would be of great interest in the selection of varieties with high yield in breeding and strong adaptability under varied environmental conditions.

In this study, relationships between g_s , g_m , and Ψ_{leaf} or ABA were examined in tomato seedlings under progressive soil drying conditions. The objectives of this study were (i) to evaluate the effects of limiting factors of g_s and g_m on A_n in tomato plants during progressive soil drying, (ii) to investigate the responses of g_s and g_m to drought signals (Ψ_{leaf} and ABA) under increasing drought stress, and (iii) to reveal the effects

of g_s/g_m on WUE_i in tomato seedlings during the progressive soil drying.

MATERIALS AND METHODS

Plant Material and Soil Water Treatments

Seeds of tomato (*Solanum lycopersicum* L., cv. Helan108) were sown in nursery seedling plate with substrate (sphagnum peat, Pindstrup Mosebrug A/S, Ryomgaard, Denmark). When the second true leaf emerged, tomato seedlings were transplanted into 5.3 L pots (height 30 cm, diameter 15 cm). Each pot was filled with 6.5 kg air-dried sandy loam soil. The gravimetric field water capacity (θ_{FC}) and wilting point were 22% ($g\ g^{-1}$) and 6.8% ($g\ g^{-1}$), respectively. After transplanting, all pots were irrigated to 85% θ_{FC} with Hoagland solution [5 mM KNO_3 , 5 mM $Ca\ (NO_3)_2\ 4H_2O$, 1 mM KH_2PO_4 , and 1 mM $MgSO_4\ 7H_2O$, 1 ml l^{-1} micronutrients, pH = 6.0]. Seedlings were cultivated in an environment-controlled chamber [day/night air temperature 25/18°C, 50–60% relative humidity, 12 h photoperiod at 600 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD) supplied by LED lamps from 7:00 to 19:00]. All pots were weighted daily at 8:00 a.m. to calculate daily irrigation amount. During the experiment, same volume of Hoagland solution was applied to all pots to avoid nutrient differences. Soil water content was expressed as relative soil water content (RSWC), i.e., the ratio between the current soil moisture (θ_C) and θ_{FC} .

Water treatments (including well-watered and progressive drought-stressed treatments) were conducted at the 27 day after transplanting (DAT). For the well-watered treatment, RSWC was maintained within the range of 70–82% θ_{FC} throughout the experiment. Plants remained well-watered acted as a control group (CK). For the drought-stressed treatment (withholding water), RSWC decreased from 82.90% θ_{FC} to 37.27% θ_{FC} from 27 to 33 DAT. On each day of the drying period (28–33 DAT), the relevant experimental indexes were measured and collected for the two treatments.

Leaf Gas Exchange and Chlorophyll Fluorescence Measurements

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously using an open gas exchange system Li-Cor 6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) equipped with an integrated leaf fluorometer chamber (Li-Cor 6400-40) from 9:00 to 14:00 h. All measurements were recorded on the same fully expanded leaves (the 6th or 7th leaves from the base of the plant) during 28–33 DAT, using two or six replicate plants for CK and water stressed treatment, respectively. During the measurements, the PPFD was kept at 1500 $\mu mol\ m^{-2}\ s^{-1}$, the sample CO_2 concentration was maintained at 400 $\mu mol\ mol^{-1}$ with a CO_2 cylinder. Relative humidity was kept at 55%. Leaf gas exchange, chlorophyll fluorescence and leaf temperature were recorded when A_n was stabilized on these conditions (usually 20 min after clamping the leaf). After that, A- C_i response curves were conducted. During the measurements, the PPFD was kept as constant of 1500 $\mu mol\ m^{-2}\ s^{-1}$, sample CO_2 concentration was adjusted in a series

of: 400, 300, 200, 150, 100, 50, 400, 400, 600, 800, 1000, 1200, 1400, 1600 $\mu mol\ mol^{-1}$.

The intrinsic water use efficiency (WUE_i , $\mu mol\ CO_2\ mol^{-1}\ H_2O$) was calculated as the ratio of net photosynthetic rate divided by stomatal conductance:

$$WUE_i = A_n/g_s \quad (1)$$

where A_n is net photosynthesis rate ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$), g_s is stomatal conductance ($mol\ H_2O\ m^{-2}\ s^{-1}$).

The actual photochemical efficiency of photosystem II (Φ_{PSII}) was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence (F_m') during a light-saturating pulse of ca. 8000 $mmol\ m^{-2}\ s^{-1}$:

$$\Phi_{PSII} = (F_m' - F_s)/F_m' \quad (2)$$

The electron transport rate (J_f) was then calculated as:

$$J_f = \Phi_{PSII} \times PPFD \times \alpha \times \beta \quad (3)$$

where PPFD was maintained at 1500 $\mu mol\ m^{-2}\ s^{-1}$ on both the well-watered and water-stressed leaves. α represents the leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. α and β were assumed to be 0.84 and 0.5 in the study, respectively (Laisk and Loreto, 1996; Flexas et al., 2002).

Estimation of g_m by Gas Exchange and Chlorophyll Fluorescence Method

g_m was calculated by the variable J method of Harley et al. (1992), as follows:

$$g_m = \frac{A_n}{C_i - \frac{\Gamma^*(J_f + 8(A_n + R_d))}{J_f - 4(A_n + R_d)}} \quad (4)$$

where C_i represents intercellular CO_2 concentration ($\mu mol\ CO_2\ mol^{-1}$), R_d represents the light mitochondrial respiration ($\mu mol\ CO_2\ mol^{-1}$), which was calculated as 1/2 of the dark respiration Xiong et al. (2018), Γ^* is the chloroplast CO_2 compensation point ($\mu mol\ CO_2\ mol^{-1}$), a leaf temperature-dependent parameter, and estimated as:

$$\text{Parameter} = \exp\left(c - \frac{\Delta H_a}{R \cdot T_K}\right) \quad (5)$$

where c is the scaling constant (dimensionless), H_a is the energies of activation ($KJ\ mol^{-1}$), and R is the molar gas constant ($8.314\ J\ K^{-1}\ mol^{-1}$). At the leaf temperature of 25°C, c and H_a in *S. lycopersicum* were equal to 12.7 and 23.2 ($KJ\ mol^{-1}$), respectively (Hermida-Carrera et al., 2016). T_k is the leaf absolute assay temperature (K), which was recorded by the LI-6400 system and corrected to Kelvin temperature.

Given the potential errors in estimation made by the variable J method, sensitivity analyses were conducted to determine the effect of $\pm 20\%$ error of R_d , Γ^* , J_f , and C_i on calculation of g_m .

Photosynthetic Limitation Analysis

The relative photosynthesis limitations of A_n resulting from g_s (l_s), g_m (l_m), and biochemical photosynthetic capacity (l_b) ($l_s + l_m + l_b = 1$) was determined using the method of Grassi and Magnani (2005), as follows:

$$l_s = \frac{g_t/g_{sc} \cdot \partial A/\partial C_c}{g_t + \partial A/\partial C_c} \quad (6)$$

$$l_m = \frac{g_t/g_m \cdot \partial A/\partial C_c}{g_t + \partial A/\partial C_c} \quad (7)$$

$$l_b = \frac{g_t}{g_t + \partial A/\partial C_c} \quad (8)$$

where g_{sc} is the stomatal conductance to CO_2 ($mol\ CO_2\ m^{-2}\ s^{-1}$), $g_{sc} = g_s/1.6$, g_t is the total conductance to CO_2 from the leaf surface CO_2 to chloroplast ($1/g_t = 1/g_{sc} + 1/g_m$). According to the Farquhar model (Farquhar, 1980), $\partial A/\partial C_c$ can be calculated as follows:

$$\partial A/\partial C_c = \frac{V_{cmax} \cdot (\Gamma^* + K_c(1 + O/K_o))}{(C_c + K_c \cdot (1 + O/K_o))^2} \quad (9)$$

where K_c and K_o are the Rubisco Michaelis–Menten constants for CO_2 and O_2 , both of them were temperature-dependent and calculated as Equation (5). Specific values of these parameters in Equation (5) were obtained from Sharkey et al. (2007). O is the atmospheric O_2 concentration ($210\ mmol\ mol^{-1}$). V_{cmax} is the maximum carboxylation capacity ($\mu mol\ m^{-2}\ s^{-1}$). V_{cmax} was calculated from the A/C_i curve fitting method (Long and Bernacchi, 2003).

Soil and Leaf Water Potential Measurement and Shoot Sap Collection

Leaf water potential (Ψ_{leaf}) was measured on the same leaves as the measurement of gas exchange. Soil samples at the 10–12 cm under soil surface were collected to measure soil water potential (Ψ_{soil}). Both Ψ_{leaf} and Ψ_{soil} were measured by the WP4C Dewpoint Potentiometer (Meter Group Inc., Pullman, WA, USA) with two or six repetitions for CK and water stressed treatment. Meanwhile, the shoot part (including stem and leaf) was put into the Model 3115 pressure chamber (Plant Moisture Equipment, Santa Barbara, CA, USA). Pressure was increased gradually until sap outflowed at the cut surface. After discarding the first 1–2 drops, nearly 2 ml of sap was collected into centrifuge tube frozen in liquid nitrogen and then stored at $-80^\circ C$ for ABA analysis.

ABA Determination

The concentration of ABA was determined as previously described by Li et al. (2020). Briefly, sap ABA concentration was measured with a high-performance liquid chromatography-tandem mass spectrometry (Agilent Technologies Inc., Santa

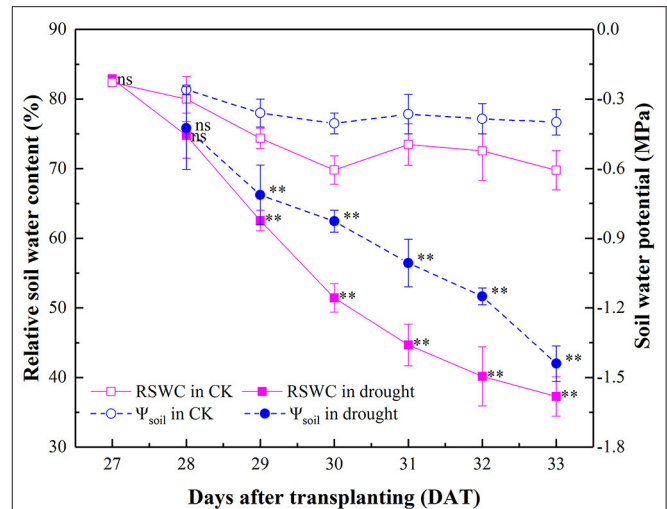


FIGURE 1 | Dynamics of RSWC and Ψ_{soil} in the well-watered (CK) and drought-stressed tomato seedlings during 27–33 DAT. Mean values and SD were presented ($n = 6$). ns indicated no significant difference and ** indicated significant difference at $P < 0.01$ level between drought and well-watered treatment.

Clara, CA, USA), quantitated as the methods of isotope internal standard.

Statistical Analysis

All statistical analyses were performed using SPSS 16.0 (IBM Corp., Armonk, NY, United States). The significance of differences between mean values was assessed by One-way analysis of variance (ANOVA) according to Dennett's test at $P < 0.05$ level. Regressions were fitted by linear or non-linear models, and the model with higher regression coefficient (r^2) was selected. Regression lines was shown when $P < 0.05$. All graphics and regressions were performed in Origin-Pro 2017 (Origin Lab, Northampton, MA, USA).

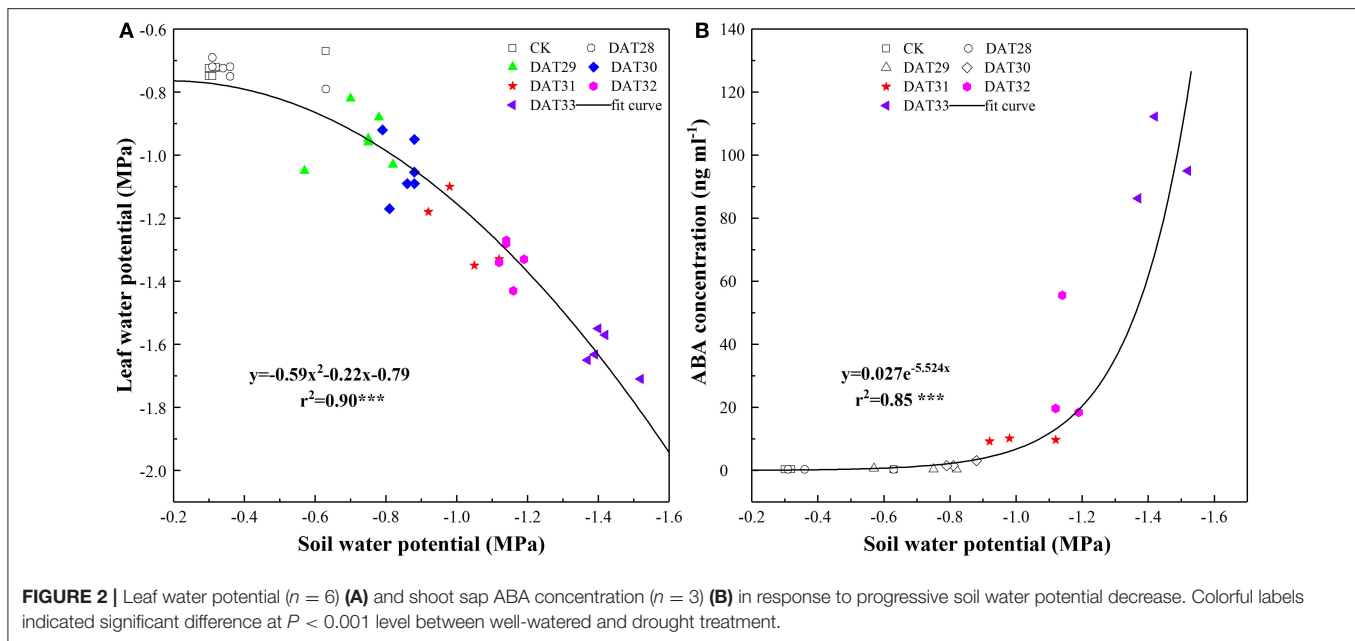
RESULTS

Dynamic of Soil Water Status

Relative soil water content (RSWC) and Ψ_{soil} of the well-watered pots were maintained at an average of 75.13% and -0.43 MPa, indicating no water stress occurred during the experiment. By withholding irrigation from 27 to 33 DAT during the progressive drying process, RSWC in the drought treatment decreased gradually from 82.90 to 37.27% and Ψ_{soil} decreased by 1.04 MPa correspondingly. Interestingly, significant reduction of both RSWC and Ψ_{soil} occurred simultaneously at 29 DAT (Figure 1).

Effects of Drought on Ψ_{leaf} and ABA

In the well-watered treatment, Ψ_{leaf} maintained at an average of -0.72 MPa from 27 to 33 DAT. Along with decreasing Ψ_{soil} in the pots, Ψ_{leaf} of the drought-stressed tomato seedlings kept almost constant until Ψ_{soil} reached to -0.71 MPa (Figure 2A). However, ABA did not statistically increase within the range of Ψ_{soil} from -0.42 to -0.83 MPa, indicating that compared to Ψ_{leaf} , chemical



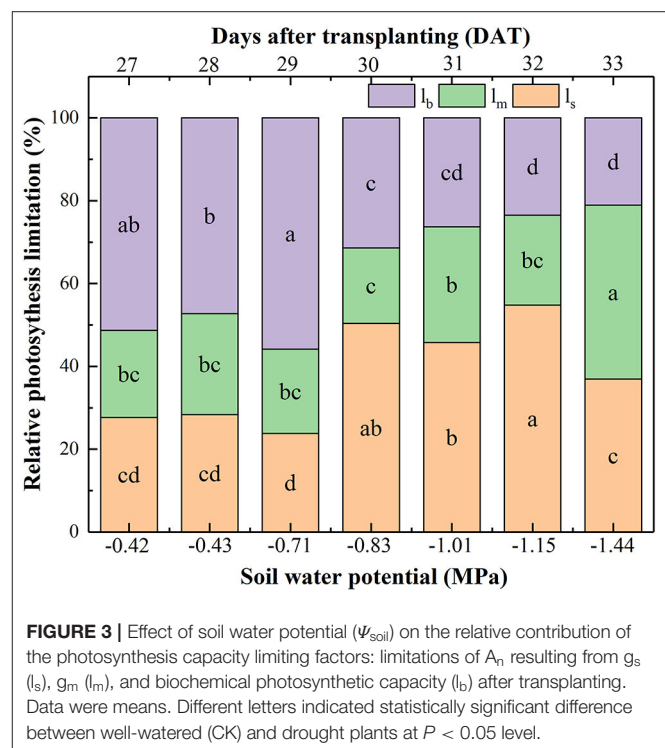
signal, ABA showed a delayed response in face to mild soil drying. As soil further drying, ABA increased exponentially with Ψ_{soil} decreasing from -1.01 to -1.44 MPa (Figure 2B). It should be noteworthy that ABA in the drought-stressed plants increased up to an average of 97.86 ng ml^{-1} at the end of experiment, resulting in an around 300 times higher than the well-watered treatment.

Quantitative Analysis of Photosynthetic Limitation in Response to Soil Drying

The relative contributions of all limiting factors (l_s , l_m , l_b) to photosynthetic capacity can be divided into three stages (Figure 3). Firstly, l_b contributed to around an average of 51.46% limitation when Ψ_{soil} was > -0.71 MPa, suggesting that photosynthetic biochemistry was the main factor under no water stressed condition. Secondly, with Ψ_{soil} decreasing from -0.83 to -1.15 MPa, l_b declined, whereas both l_s and l_m increased, but l_s was higher than l_m , which contributed solely to an almost 50.30% reduction in A_n , indicating that g_s was the main limiting factor to photosynthetic capacity under mild and moderate drought. Thirdly, with Ψ_{soil} decreasing to -1.44 MPa, l_m contributed to 41.99% reduction in photosynthesis, followed by l_s (36.93%) and l_b (21.08%), showing that g_m was the most important limiting factor to photosynthetic capacity under the severe drought condition.

Ψ_{leaf} and ABA in the Regulation of g_s , g_m , g_t , and A_n

As compared to g_s in CK, g_s in the water-stressed tomato seedlings increased firstly with Ψ_{leaf} decreasing from -0.72 to -0.95 MPa and then decreased with Ψ_{leaf} decreasing from -1.05 to -1.63 MPa (Figure 4A). However, g_m kept unchanged within the range of Ψ_{leaf} from -0.72 to -1.05 MPa and decreased significantly when Ψ_{leaf} was < -1.28 MPa (Figure 4C). The



output of ANOVA showed that drought had significant effect on the slopes of the regression lines between g_s and g_m to Ψ_{leaf} (Supplementary Figure 1). In addition, under mild and moderate drought, the ratio of g_s reduction was higher than g_m during 30–32 DAT (Supplementary Figure 2). These results indicated that g_s was more sensitive to mild and moderate drought stress than g_m . In summary, there was a significant

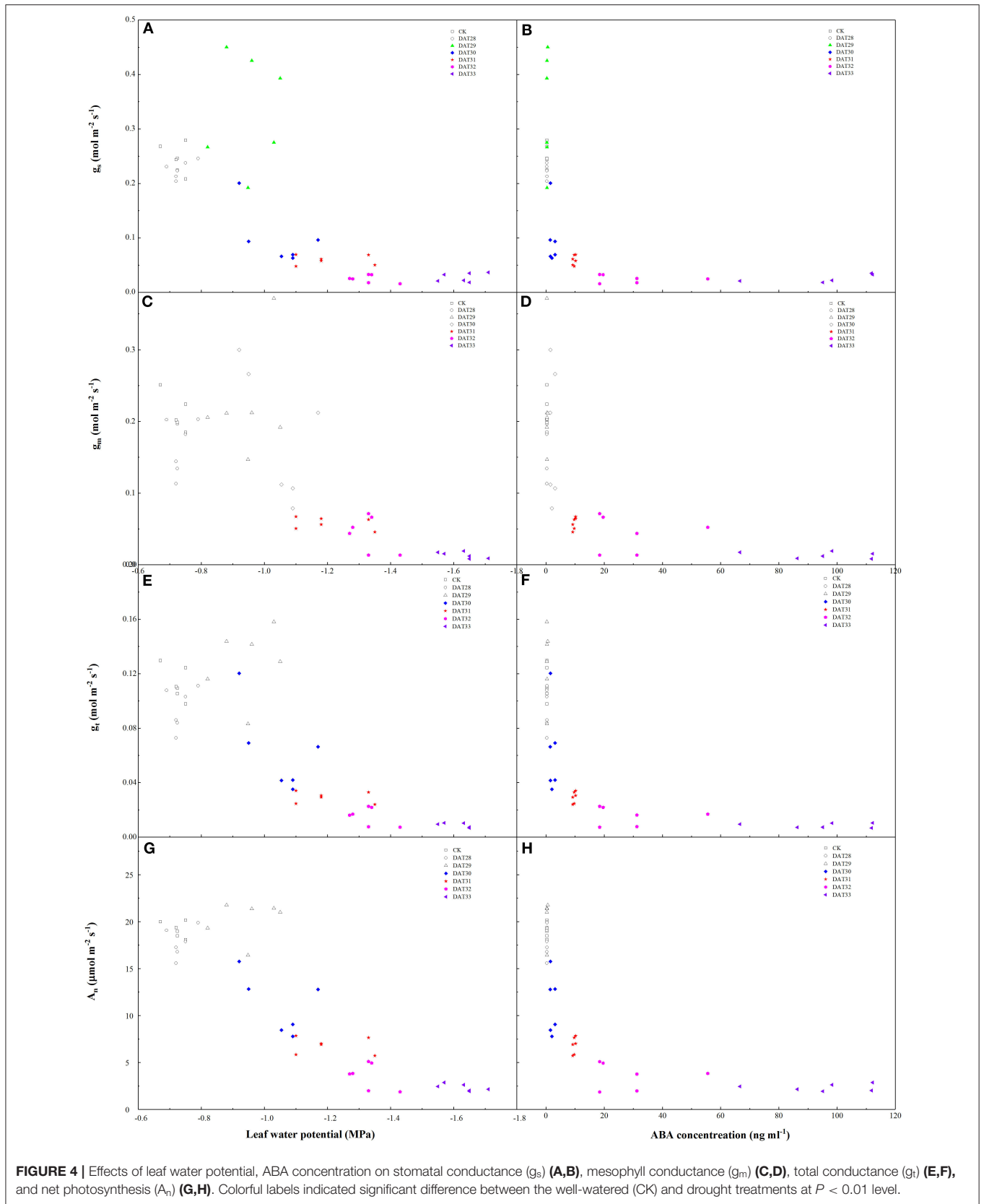
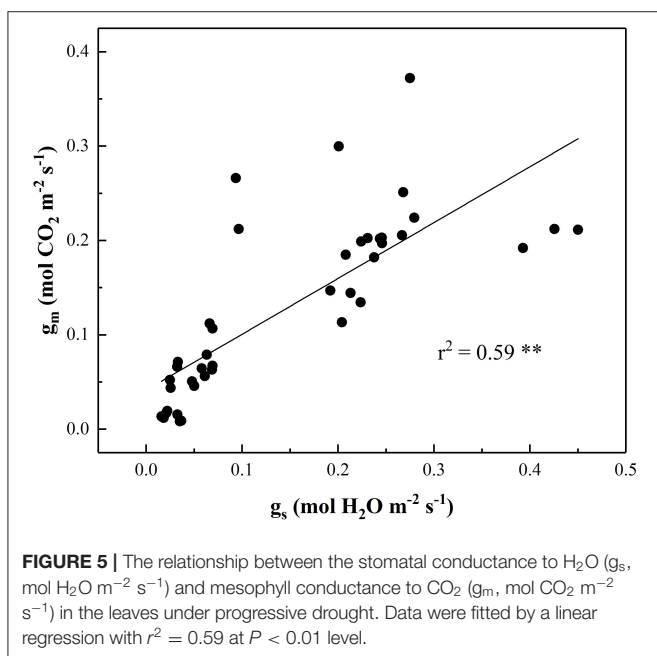


TABLE 1 | Correlation matrix between studied parameters including intrinsic water use efficiency (WUE_i), net photosynthesis (A_n), mesophyll conductance (g_m), stomatal conductance (g_s) and the ratio (g_m/g_s), abscisic acid (ABA), and leaf water potential (Ψ_{leaf}).

	A _n	g _s	g _m	g _m /g _s	WUE _i	Ψ _{leaf}	ABA
A _n	1	0.938**	0.892**	-0.164	0.639**	0.885**	-0.695**
g _s		1	0.777**	0.339*	0.759**	0.740**	-0.548**
g _m			1	0.160	0.439**	0.760**	-0.643**
g _m /g _s				1	0.771**	-0.109	-0.229
WUE _i					1	-0.395**	0.072
Ψ _{leaf}						1	-0.816**
ABA							1

* and **mean statistically significant relationship according to the Pearson correlation analysis at $P < 0.05$ and $P < 0.01$.



positive relationship between Ψ_{leaf} and g_s ($r = 0.74$, $P < 0.01$) and g_m ($r = 0.76$, $P < 0.01$) during progressive soil drying (Table 1). We also investigated the relationship between ABA and g_s or g_m (Figures 4B,D). g_s changed with no significant increasing ABA during 28–29 DAT. As soil further dried, g_s continued decreasing and g_m started to decrease with significant increase in ABA (Figures 4B,D). g_m was closely related to g_s during drying ($r^2 = 0.59$, $P < 0.01$) (Figure 5). Notably, g_m and ABA changed concurrently at the threshold of Ψ_{soil} = -1.01 MPa (Figures 2B, 4D). In summary, ABA was negatively related to g_m ($r = -0.64$, $P < 0.01$) and g_s ($r = -0.55$, $P < 0.01$) (Table 1). These results indicated that the decline of g_s was regulated by Ψ_{leaf} in the early stage of drought, whereas under moderate or severe drought, g_s and g_m were controlled by both Ψ_{leaf} and ABA.

Drought significantly affected A_n and g_t during 30–33 DAT. When Ψ_{leaf} decreased to -1.05 MPa or ABA increased to 2.04 ng ml⁻¹, A_n and g_t declined by 40.18 and 45.13%, respectively

(Figures 4E–H). As soil further dried, i.e., Ψ_{leaf} decreasing from -1.28 to -1.63 MPa, A_n and g_t reduced by 62.84–88.94% and 74.33–92.92% in the drought-stressed plants as compared with the well-watered plants, respectively (Figures 4E,G).

g_m/g_s and WUE_i in Response to Ψ_{leaf} and ABA Under Progressive Soil Drying

The dynamics of g_m/g_s in response to Ψ_{leaf} and ABA during progressive soil drying were presented in Figure 6. Higher g_m/g_s was observed as Ψ_{leaf} decreased from -1.05 to -1.33 MPa or as ABA increased from 2.04 to 31.23 ng ml⁻¹ (Figures 6A,B), indicating that g_s declined more than g_m under mild or moderate drought. However, no significant difference of g_m/g_s between CK and the intense water stress with Ψ_{leaf} = -1.63 MPa was found. WUE_i in response to these signals changed in the same way as g_m/g_s (Figures 6C,D), it increased firstly and then decreased. In addition, WUE_i was positively related to g_m/g_s with a logarithmic relationship ($r^2 = 0.62$, $P < 0.001$) during the progressive soil drying (Figure 6E), indicating that WUE_i was strongly correlated to g_m/g_s.

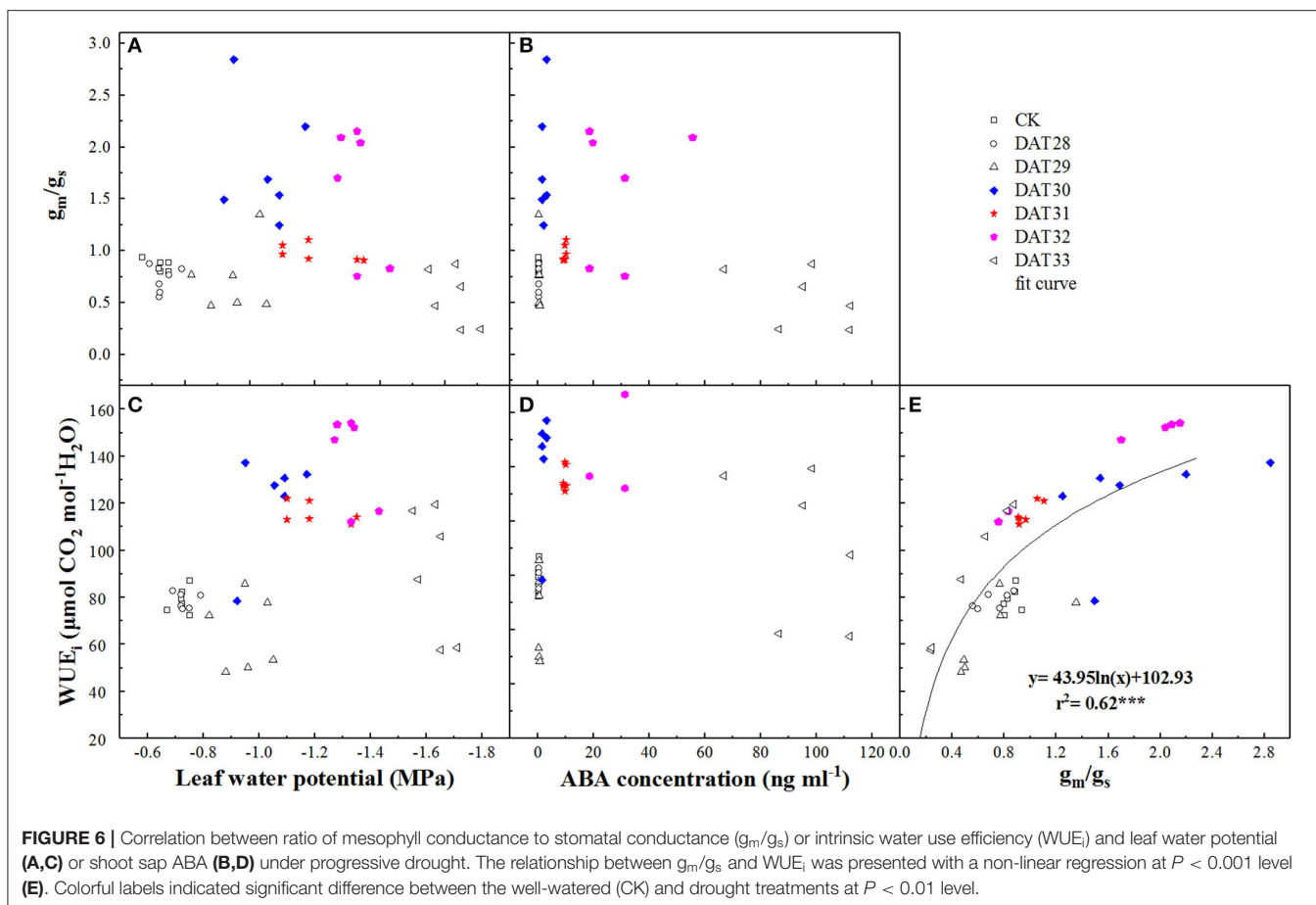
Sensitivity Analyses of Parameters in the Estimation g_m

10% variation of R_d and J_f did not affect g_m significantly, whereas Γ* has a significantly effect on g_m in well-watered plants (Table 2). As compared to g_m in the well-watered plants, g_m in the drought treatment was unaffected by the 20% underestimation of J_f, showing that g_m in the drought treatment was less sensitive to J_f than in the well-watered plants. Variation of C_i resulted in an overestimation of g_m in well-watered plants, whereas g_m in drought treatment was unaffected by overestimation of C_i. These results indicated that overestimation of C_i had a slighter effect on calculation of g_m than underestimation in the current study.

DISCUSSION

Effects of g_s and g_m on A_n Under Soil Drought

Efficient CO₂ fixation is important for plant acclimation to environmental factors. In the present study, the total diffusion conductance of CO₂ (g_t) and A_n declined synchronously under drought (Figures 4E–H). The total diffusion conductance of CO₂ mainly includes g_s and g_m (Grassi and Magnani, 2005). Many authors have reported that CO₂ diffusion from sub-stomatal cavities to chloroplasts is a significant factor determining photosynthetic capacity in C₃ plants such as tomato (Han et al., 2016; Du et al., 2019; Xu et al., 2019). Our analysis showed that l_s and l_m increased as soil drying proceeded and contributed to an almost 68–78% reduction in A_n when Ψ_{soil} was < -0.83 MPa (Figure 3). Our results, as well as those of previous studies (Niinemets et al., 2009; Wang et al., 2018), confirmed the significance of g_s and g_m on assimilation rate under various drought conditions. It should be acknowledged that, many authors have highlighted the effects of leaf anatomical traits on g_m, such as cell thickness, cell packing and area of chloroplasts



exposed to the intercellular air spaces (S_c/S) across many species including tomato (Tomas et al., 2013; Muir et al., 2014). This effect was a result of plants acclimation to the long-term stressed environmental factors lasting for weeks. However, rapid response of g_m to stress could occur within minutes response to elevating CO_2 (Mizokami et al., 2018) or hours response to application of ABA (Sorrentino et al., 2016). Perhaps this meant that different mechanisms of g_m determination existed under short and long term drought conditions. Therefore, to minimize the effects of leaf anatomy on g_m , we focused on the responses of g_m to drought stress and the involvement of ABA in a short water stress cycle.

Response of g_s to ψ_{leaf} and ABA Under Soil Drought

We found that g_s generally decreased as ψ_{leaf} decreased (Figures 2A, 4A), suggesting that ψ_{leaf} might induce stomatal closure at the early stage of drought. The mechanisms of this hydraulic regulation remain unclear, but the reduction in ψ_{leaf} has been tightly associated with decreasing leaf hydraulic signals (leaf turgor or K_{leaf}) in understanding the closure of stomata (Ripullone et al., 2007; Wang et al., 2018). On the one hand, evidences have suggested that decline of leaf turgor could explain the decrease in g_s within no change of ABA

(Rodriguez-Dominguez et al., 2016; Huber et al., 2019), possibly due to the decrease of elastic modulus and the activity of anion channel in guard cell during leaf dehydration (Ache et al., 2010; Saito and Terashima, 2010). On the other hand, progressive drop of plant water potential might decrease xylem pressure and increase the likelihood of embolism and hydraulic failure (Martorell et al., 2014; Tombesi et al., 2015). Responding to the future unpredictable soil water availability, stomata closed to prevent water loss and avoid xylem cavitation. Here, the increase of shoot sap ABA concentration was statistically insignificant, which implied that stomatal closure was not initiated by ABA with ψ_{soil} not approaching to -1.01 MPa (Figures 2B, 4B). Indeed, the delayed increase in leaf ABA in the present study was consistent with the recent findings that leaf ABA did not increase until after stomata closed, which was different from the actions of leaf turgor subjected to drought stress (Huber et al., 2019). However, as soil drought proceeded, g_s continued decreasing with significant changes in both ABA and ψ_{leaf} , suggesting that ψ_{leaf} was not solely controlling g_s , but chemical ABA was also involved in the reduction of g_s . A similar variation between ABA and g_s was also reported by Tombesi et al. (2015), who indicated that ABA played a crucial role in maintaining stomatal closure under long and severe drought. However, it should be noteworthy that our data need to be further interpreted, as shoot sap ABA

TABLE 2 | Sensitivity analyses of the effects of $\pm 20\%$ error of light mitochondrial respiration (R_d), chloroplast CO_2 compensation point (Γ^*), electron transport rate (J_f), and intercellular CO_2 concentration (C_i) on calculation of g_m in well-watered and severe drought tomato at $\Psi_{\text{soil}} = -1.44$ MPa as compared with the original value of g_m .

Factors	g_m in CK	g_m in drought	Factors	g_m in CK	g_m in drought
$R_d-20\%$	0.182 ± 0.006 ns	0.013 ± 0.002 ns	$J_f-20\%$	1.208 ± 0.74 **	0.014 ± 0.002 ns
$R_d-10\%$	0.189 ± 0.005 ns	0.013 ± 0.002 ns	$J_f-10\%$	0.309 ± 0.020 ns	0.014 ± 0.002 ns
$R_d+10\%$	0.206 ± 0.07 ns	0.014 ± 0.002 ns	$J_f+10\%$	0.160 ± 0.005 ns	0.013 ± 0.002 ns
$R_d+20\%$	0.216 ± 0.08 ns	0.014 ± 0.002 ns	$J_f+20\%$	0.141 ± 0.004 ns	0.013 ± 0.002 ns
$\Gamma^*-20\%$	0.146 ± 0.005 **	0.013 ± 0.002 ns	$C_i-20\%$	0.433 ± 0.025 **	0.020 ± 0.003 *
$\Gamma^*-10\%$	0.168 ± 0.009 **	0.013 ± 0.002 ns	$C_i-10\%$	0.270 ± 0.011 **	0.017 ± 0.003 ns
$\Gamma^*+10\%$	0.238 ± 0.015 **	0.014 ± 0.002 ns	$C_i+10\%$	0.155 ± 0.005 **	0.013 ± 0.002 ns
$\Gamma^*+20\%$	0.301 ± 0.011 **	0.014 ± 0.002 ns	$C_i+20\%$	0.127 ± 0.004 **	0.011 ± 0.002 ns

Data were mean \pm SD ($n = 6$). ns indicated no significant difference and ** indicated significant difference at $P < 0.01$ level between drought and well-watered treatment.

was collected in the pressurized stem and leaf tissues instead of in localized guard cells.

Response of g_m to Ψ_{leaf} and ABA Under Soil Drought

The variable J method (Harley et al., 1992), as the most commonly and easily accessible approach, was used to determine g_m during the dry-down stage. To obtain precise calculation of g_m , the highest possible accuracy of gas exchange and chlorophyll fluorescence were required during the process of measurement. As reported previously, the decrease in g_m under drought was likely to associate with an overestimation of C_i due to stomatal closure (Pons et al., 2009). However, the sensitivity analyses showed that an overestimation of C_i did not induce g_m decline in drought-stressed plants (Table 2). Thus, overestimation of C_i was unlikely to have a significant effect on g_m in this study, might due to the influence of other environmental variations was ruled out under controlled environment. Therefore, it is reasonable to conclude that the reduction in g_m during drought was mostly attributed to the decline of g_m *per se* rather than the overestimation of C_i .

Compared to the response of g_s , g_m in the drought-stressed seedlings remained almost constant with Ψ_{leaf} not decrease to -1.28 MPa (Figures 4A,C), indicating that g_m was less sensitive to the decrease in Ψ_{leaf} than g_s at the beginning of soil drought. This result was in agreement with an earlier study conducted by Th  roux-Rancourt et al. (2014), who found that g_m only responded to more negative Ψ_{leaf} or more severe soil drought, e.g., $\Psi_{\text{soil}} < -1.01$ MPa in the present study. Hydraulic compartmentalization of the mesophyll cell from the transpiration stream may account for this delayed response of g_m to Ψ_{leaf} (Zwieniecki et al., 2007; Th  roux-Rancourt et al., 2014). This delayed response of g_m under the mild soil drought might be beneficial for mesophyll cells to be buffered against little variation in leaf water status and allow plants to maintain a greater A_n (Figure 4G).

However, as soil drought proceeded, g_m declined as Ψ_{leaf} continued decreasing (Figures 4C,D). Based on literature surveys, the causes of this decrease in g_m may be influenced by three main factors: mesophyll structure, membrane permeability, and biochemical enzymes activity (Flexas et al., 2008; Evans et al., 2009; Sorrentino et al., 2016). Mesophyll structural properties

may not be involved in this rapid reduction of g_m under the short-term drought. Instead, it is well-established that the K_{leaf} -induced reduction in g_m was associated with the decrease in mesophyll density or membrane permeability under drought conditions (Aasamaa et al., 2005; Xiong et al., 2018). Water moves through leaf mesophyll tissues via apoplastic, symplastic and vapor phase pathways, which shared a part of pathways of CO_2 diffusion (Xiong and Nadal, 2020). The decline in hydraulic conductance under drought usually leads to reductions in water supply to the leaves, therefore affecting mesophyll cells water relations and functions. Although the effect of K_{leaf} on g_m was not investigated in this study, we observed a strong and positive relationship between Ψ_{leaf} and g_m ($r^2 = 0.77$, $P < 0.01$) (Figure 4C), because K_{leaf} was strongly influenced by Ψ_{leaf} under drought stress (Wang et al., 2018). Therefore, the decline in Ψ_{leaf} might contribute to this decrease in g_m , as CO_2 diffusion and liquid water shared partly common pathways within leaves (Xiong et al., 2018).

Most notably, rapid reduction of g_m occurred following with increase of ABA when Ψ_{soil} was below -1.01 MPa in the current study. Fast fluctuations in g_m have also been recorded in response to ABA application (Sorrentino et al., 2016; Mizokami et al., 2018). The concurrent responses between g_m and ABA with Ψ_{soil} decreasing from -1.01 to -1.44 MPa was not a mere coincidence. This might suggest that Ψ_{leaf} was not the only factor influencing g_s and g_m under drought, other signals (ABA) could be involved in this reduction. Though mechanisms for the effect of ABA on g_m remain unclear, the results from both Sorrentino et al. (2016) and the current studies indicated that the reduction in g_m was most likely regulated by biochemical components due to the rapid reduction of g_m to ABA (Flexas et al., 2008; Kaldenhoff et al., 2008; Xiong et al., 2018). Evidences have indicated two candidates are likely to play this biochemical role: carbonic anhydrase and aquaporins. CO_2 molecules passing from sub-stomatal cavities to chloroplasts diffuse through the gas phase among intercellular air spaces and the liquid phase from the cell wall to stroma. Carbonic anhydrase (CA) plays a key role on the conversion of gaseous CO_2 to aqueous carbonic acid (H_2CO_3) (Flexas et al., 2008). Higher ABA accumulation was likely to change the extracellular pH and decrease the activity of H^+ -ATP-ase, an important ion transporter in plant cell plasma membrane, thus affect the CA activity (Hayat et al., 2001; Sukhov

et al., 2017). Aquaporins (AQPs) are pore-forming integral membrane proteins that transport of water, CO₂ and other small neutral molecules across the plasma membrane (Flexas et al., 2006; Kaldenhoff, 2012). A higher abundance of AQPs increased the cellular CO₂ uptake rates several folds. Expressions of plant AQPs could be influenced by drought stress and ABA (Kapilan et al., 2018). Additionally, an indirect role of ABA on decreasing K_{leaf} might also be involved in regulating g_m , due to the ability of ABA on inactivation bundle sheath aquaporins such as the plasma membrane intrinsic proteins (PIPs) (Shatil-Cohen et al., 2011; Pantin et al., 2013). Based on these, we considered that the reduction in g_m was not attributed solely to hydraulic regulation, ABA seemed to maintain the decrease in g_m under moderate or severe soil drought, e.g., $\Psi_{soil} < -1.01$ MPa in the present study. The regulation of g_m is complex, and regulated by many factors, including hydraulic or chemical signaling and mesophyll structure. It is still unclear the mechanism of g_m response to ABA under stress, further analysis of the expressions of carbonic anhydrase and cooporin protein in membrane may elucidate the biochemical mechanisms underlying this response. Notably, g_s and g_m decreased as ABA significantly increased (Figures 4B,D). Pooling all the data, a strong and positive relationship between both variables was observed in Table 1. In addition, 59% of the variation in g_m can be explained by g_s (Figure 5). Coupled changes between g_s and g_m was also found in response to drought (Perez-Martin et al., 2009; Han et al., 2016; Olsovska et al., 2016) or ABA application (Mizokami et al., 2018). Therefore, it seems that drought regulated g_m in order to match the variation of g_s , thereby optimization balance between CO₂ uptake and water loss. However, the role of g_s on regulating g_m response to ABA is still debated by many scientists (Sorrentino et al., 2016; Mizokami et al., 2018), further detail investigations are needed to address this issue.

Variability of WUE_i Under Drought Depends on g_m/g_s

In this study, g_m/g_s and WUE_i increased concurrently with Ψ_{soil} in the range of -0.83 to -1.15 MPa with a strong correlation (Figure 6E). Our results showed that WUE_i was closely related to g_m/g_s compared to the correlation between WUE_i and g_m or g_s (Table 1). This result was consistent with Han et al. (2016) who also found WUE_i and g_m/g_s were closely correlated compared to the correlation between WUE_i and g_s or g_m . These suggested that variations in WUE_i were much more sensitive to changes of g_m/g_s . Stomata controls the water loss and mesophyll determines the photosynthesis, thus it would be better that using g_m/g_s instead of A_n/g_s explained the variations of WUE_i. Interestingly, this improvement of WUE_i were coupled with increase in ABA. This might due to g_s reduced more in response to ABA than g_m under moderate drought. Though the mechanisms of ABA improving WUE_i remain largely unknown, it is likely to be one of the most promising strategies to improve WUE_i by means of decoding of the ABA signaling pathway or manipulating the expression of ABA-related genes on stomatal conductance or CA activity (Flexas et al., 2016; Cardoso et al., 2020). Nonetheless, such improvement of WUE_i controlled by ABA could only be

beneficial for maintaining water status under short-term drought during Ψ_{soil} reduction from -0.83 to -1.15 MPa, not for long and serious drought (Figure 6D). This was because the increase in WUE_i at leaf scale may not always mean an improvement of WUE at the whole plant scale under serious soil drought, as the closure of stomata restricts CO₂ uptake and hence diminish plant productivity (Xue et al., 2016).

CONCLUSION

The limitation of g_s and g_m increased along with progressive soil drying and diffusive conductance to CO₂ from ambient air to chloroplasts was the crucial constraints to photosynthesis under drought conditions. The decrease in Ψ_{leaf} triggered stomata closure at the onset of drought. As soil drying proceeded, g_s and g_m declined synchronously. Both hydraulic and ABA signals were involved in this consistent decrease under moderate and severe drought. WUE_i improved as g_m/g_s increased under mild and moderated drought due to a larger reduction of g_s to ABA than g_m . Manipulation of ABA levels might be a promising approach to improve plant water use efficiency for breeding project. For future research, examining the influence of stomatal closure on g_m response to ABA will give further detailed insight on working of g_m to ABA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

AD and YG planned and designed the experiments. SL and JL performed the experiments and analyzed the data. SL wrote the draft manuscript. AD, YG, HL, and RQ revised the manuscript. 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.2021.653186/full#supplementary-material>

Supplementary Figure 1 | Relationships between stomatal conductance (g_s) or mesophyll conductance (g_m) under drought during 30-33 DAT. Closed circles indicated g_s , open circles indicated g_m . Slope with P value indicates significant difference between the slopes of the regression lines for g_s and g_m to Ψ_{leaf} .

Supplementary Figure 2 | The decreasing ratio of stomatal conductance (g_s) or mesophyll conductance (g_m) under drought compared CK during 30 to 33 DAT.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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