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 \( (\psi_{soil}) \) was over \(-1.01 \) MPa, \( g_s \) reduced as leaf water potential \( (\psi_{leaf}) \) decreased, but ABA and \( g_m \) kept unchanged, which indicating \( g_s \) was more sensitive to drought than \( g_m \). During \( \psi_{soil} \) reduction from \(-1.01 \) to \(-1.44 \) MPa, \( \psi_{leaf} \) 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 \( \psi_{soil} \) 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; Olsovská 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 C3 plants, leaf photosynthesis is strongly limited by three factors,
i.e., stomatal conductance (gs), mesophyll diffusion conductance to CO2 (g*m), and biochemical photosynthetic capacity (Grassi and Magnani, 2005; Cano et al., 2013). gs and g*m determine the diffusion of CO2 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 gs 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 gs 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 Jegl, 2016). The decrease of gs 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 (Ψleaf), possibly due to differences of experimental plant materials and the intensity of applied drought under investigation. For example, gs decreased with decreasing Ψleaf during leaf dehydration (Kim et al., 2012; Wang et al., 2018). On the contrary, other studies showed that stomata closed with little change in Ψleaf under moderate soil drying, but both parameters decreased under severe drought (Tardieu, 1998; Yan et al., 2017), or gs decreased as Ψleaf 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 Ψleaf and gs 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 gs to Ψleaf during drying process.

Leaf mesophyll conductance to CO2 (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; Théroux-Rancourt et al., 2014; Sorrentino et al., 2016). Recent studies on hydraulic signals suggested that the parallel decreases in gs and g*m were caused by leaf hydraulic vulnerability as a result of decrease in Ψleaf (Wang et al., 2018). Similarly, g*m was strongly correlated with leaf hydraulic conductance (Kleaf), as the ratio of transpiration rate to the water potential driving force across the leaf (Kleaf = transpiration/ΔΨleaf), across species under light-saturated conditions (Xiong et al., 2018). This correlation between g*m and leaf hydraulic signals might be due to CO2 partially shared common diffusion pathways with H2O 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 CO2 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 CO2. 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 (WUEi), expressed as the ratio of net photosynthetic rate (An) to gs at leaf level, can explain instantaneous responses to environmental factors (Flexas et al., 2016; Qiu et al., 2019). Improving WUEi need increase An and decrease gs simultaneously. Using An/gs to explain the changes of WUEi would be too coarse due to the decrease in gs inevitably affect CO2 uptake and thereby limit An. g*m determines the CO2 concentration at the carboxylation site in the chloroplast, increasing g*m would increase An without increasing water loss. Therefore, g*m might play a role in improving WUEi. Despite all of the negative impacts of drought stress on leaf gas exchange, many studies reported that drought was beneficial to improve WUEi (Liu et al., 2005; Xue et al., 2016). However, the reasons of this improvement of WUEi have not been elucidated clearly. Evidences have suggested that g*m/gs played a key role on increasing WUEi in response to water limitation (Flexas et al., 2016; Han et al., 2016). Revealing the exact responses of g*m/gs or WUEi 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 gs, g*m, and Ψleaf 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 gs and g*m on An in tomato plants during progressive soil drying, (ii) to investigate the responses of gs and g*m to drought signals (Ψleaf 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 capacity was maintained within the range of 70–82% of g$_s$, respectively. After transplanting, all pots were irrigated to 85% $\theta_{FC}$; with Hoagland solution [5 mM KNO$_3$, 5 mM Ca (NO$_3$)$_2$ 4H$_2$O, 1 mM KH$_2$PO$_4$, and 1 mM MgSO$_4$ 7H$_2$O, 1 ml $^{-1}$ 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 µmol 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 ($\theta_C$) and $\theta_{FC}$.

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% $\theta_{FC}$ 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% $\theta_{FC}$ to 37.27% $\theta_{FC}$ 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 µmol m$^{-2}$ s$^{-1}$, the sample CO$_2$ concentration was maintained at 400 µmol 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-Ci response curves were conducted. During the measurements, the PPFD was kept as constant of 1500 µmol 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 µmol mol$^{-1}$.

The intrinsic water use efficiency (WUE$_i$, µmol CO$_2$ mol$^{-1}$ H$_2$O) was calculated as the ratio of net photosynthetic rate divided by stomatal conductance:

$$WUE_i = A_n/g_s$$  \hspace{1cm} (1)

where A$_n$ is net photosynthesis rate (µmol CO$_2$ m$^{-2}$ s$^{-1}$), g$_s$ is stomatal conductance (mol H$_2$O 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$^{-1}$:

$$\Phi_{PSII} = (F_m' - F_i)/F_m'$$  \hspace{1cm} (2)

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

$$J_f = \Phi_{PSII} \times PPFD \times \alpha \times \beta$$  \hspace{1cm} (3)

where PPFD was maintained at 1500 µmol m$^{-2}$ s$^{-1}$ on both the well-watered and water-stressed leaves. $\alpha$ represents the leaf absorptance and $\beta$ reflects the partitioning of absorbed quanta between photosystems II and I. $\alpha$ and $\beta$ 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:

$$g_m = \frac{A_n}{C_i - \frac{\Gamma^* (J_f + 8(A_{n} + R_{d}))}{J_f - 4(A_{n} + R_{d})}}$$  \hspace{1cm} (4)

where $C_i$ represents intercellular CO$_2$ concentration (µmol CO$_2$ mol$^{-1}$), $R_{d}$ represents the light mitochondrial respiration (µmol CO$_2$ mol$^{-1}$), which was calculated as 1/2 of the dark respiration Xiong et al. (2018), $\Gamma^*$ is the chloroplast CO$_2$ compensation point (µmol CO$_2$ mol$^{-1}$), a leaf temperature-dependent parameter, and estimated as:

$$\text{Parameter} = \exp(c - \frac{\Delta H_a}{R \cdot T_K})$$  \hspace{1cm} (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 ±20% error of R_{j}, Γ*, I_{j}, and C_{i} on calculation of g_{m}.

**Photosynthetic Limitation Analysis**

The relative photosynthesis limitations of A_{net} 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:

\[ l_{s} = \frac{g_{s}/g_{sc} \cdot \partial A/\partial C_{c}}{g_{s} + \partial A/\partial C_{c}} \]  
\[ l_{m} = \frac{g_{m}/g_{m} \cdot \partial A/\partial C_{c}}{g_{s} + \partial A/\partial C_{c}} \]  
\[ l_{b} = \frac{g_{b}}{g_{s} + \partial A/\partial C_{c}} \]

where g_{sc} is the stomatal conductance to CO_{2} (mol CO_{2} m^{-2} s^{-1}), g_{sc} = g_{s}/1.6, g_{s} is the total conductance to CO_{2} from the leaf surface CO_{2} to chloroplast (1/g_{sc} = 1/g_{s} + 1/g_{m}). According to the Farquhar model (Farquhar, 1980), ∂A/∂C_{c} can be calculated as follows:

\[ \partial A/\partial C_{c} = \frac{V_{c,max} \cdot (\Gamma^{*}+K_{c}(1+O/K_{o}))}{(C_{i} + K_{c} \cdot (1 + O/K_{o}))^{2}} \]  

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_{c,max} is the maximum carboxylation capacity (µmol m^{-2} s^{-1}). V_{c,max} 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°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 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 $\Psi_{\text{soil}}$ 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, $l_s$ contributed to around an average of 51.46% limitation when $\Psi_{\text{soil}}$ was $>-0.71$ MPa, suggesting that photosynthetic biochemistry was the main factor under no water stressed condition. Secondly, with $\Psi_{\text{soil}}$ decreasing from $-0.83$ to $-1.15$ MPa, $l_s$ declined, whereas both $l_m$ and $l_b$ increased, but $l_m$ was higher than $l_b$, 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 $\Psi_{\text{soil}}$ decreasing to $-1.44$ MPa, $l_m$ contributed to 41.99% reduction in photosynthesis, followed by $l_g$ (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.

### $\Psi_{\text{leaf}}$ 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 $\Psi_{\text{leaf}}$ decreasing from $-0.72$ to $-0.95$ MPa and then decreased with $\Psi_{\text{leaf}}$ decreasing from $-1.05$ to $-1.63$ MPa (Figure 4A). However, $g_m$ kept unchanged within the range of $\Psi_{\text{leaf}}$ from $-0.72$ to $-1.05$ MPa and decreased significantly when $\Psi_{\text{leaf}}$ 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 $\Psi_{\text{leaf}}$ (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
FIGURE 4 | Effects of leaf water potential, ABA concentration on stomatal conductance ($g_s$) (A,B), mesophyll conductance ($g_m$) (C,D), total conductance ($g_t$) (E,F), and net photosynthesis ($A_n$) (G,H). Colorful labels indicated significant difference between the well-watered (CK) and drought treatments at $P < 0.01$ level.
Many studies have shown that abscisic acid (ABA) is a key hormone involved in the regulation of stomatal closure during drought stress (Niinemets et al., 2009; Wang et al., 2018). As soil further dried, gs decreased and ABA increased (Grassi and Magnani, 2005). We also investigated the relationship between ABA and gs during progressive soil drying. The dynamics of gs/γs and ABA during 28–29 DAT. As soil further dried, g_s decreased and ABA increased from 2.04 to 31.23 ng ml⁻¹ (Figures 4E, G), 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 γ_leaf = −1.63 MPa was found.

**Sensitivity Analyses of Parameters in the Estimation g_m**

10% variation of R_d and J_l did not affect g_m significantly, whereas J_× 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_×, showing that g_m in the drought treatment was less sensitive to J_× 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_s) 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 C_3 plants such as tomato (Han et al., 2016; Du et al., 2019; Xu et al., 2019). Our analysis showed that l_4 and l_6 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.

### TABLE 1 | Correlation matrix between studied parameters including intrinsic water use efficiency (WUE), 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).

| Parameters | A_n | g_s | g_m | g_m/g_s | WUE | 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        | 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.

**FIGURE 5 |** The relationship between the stomatal conductance to H₂O (g_s, mol H₂O m⁻² s⁻¹) and mesophyll conductance to CO₂ (g_m, mol CO₂ m⁻² s⁻¹) in the leaves under progressive drought. Data were fitted by a linear regression with r² = 0.59 at P < 0.01 level.
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 \(\Psi_{\text{leaf}}\) and ABA Under Soil Drought

We found that g_s generally decreased as \(\Psi_{\text{leaf}}\) decreased (Figures 2A, 4A), suggesting that \(\Psi_{\text{leaf}}\) might induce stomatal closure at the early stage of drought. The mechanisms of this hydraulic regulation remain unclear, but the reduction in \(\Psi_{\text{leaf}}\) 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 \(\Psi_{\text{soil}}\) 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 \(\Psi_{\text{leaf}}\), suggesting that \(\Psi_{\text{leaf}}\) 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 ±20% error of light mitochondrial respiration ($R_{d}$), chloroplastic CO$_2$ compensation point ($r^*$), electron transport rate ($\Gamma$), and intercellular CO$_2$ concentration ($C_i$) on calculation of $g_m$ in well-watered and severe drought tomato at $\psi_{leaf} = −1.44$ 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 | $J$-20% | 1.208 ± 0.74 ** | 0.014 ± 0.002 ns |
| $R_{d}$-10% | 0.189 ± 0.005 ns | 0.014 ± 0.002 ns | $J$-10% | 0.309 ± 0.020 ns | 0.014 ± 0.002 ns |
| $R_{d}$+10% | 0.206 ± 0.07 ns | 0.014 ± 0.002 ns | $J$+10% | 0.160 ± 0.005 ns | 0.013 ± 0.002 ns |
| $R_{d}$+20% | 0.216 ± 0.08 ns | 0.014 ± 0.002 ns | $J$+20% | 0.141 ± 0.004 ns | 0.013 ± 0.002 ns |
| $r^*$-20% | 0.146 ± 0.005 ** | 0.013 ± 0.002 ns | $C_i$-20% | 0.433 ± 0.025 ** | 0.020 ± 0.003 * |
| $r^*$-10% | 0.168 ± 0.009 ** | 0.013 ± 0.002 ns | $C_i$-10% | 0.270 ± 0.011 ** | 0.017 ± 0.003 ns |
| $r^*$+10% | 0.238 ± 0.015 ** | 0.014 ± 0.002 ns | $C_i$+10% | 0.155 ± 0.005 ** | 0.013 ± 0.002 ns |
| $r^*$+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 ± 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 $\psi_{leaf}$ 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 $\psi_{leaf}$ not decrease to −1.28 MPa (Figures 4A,C), indicating that $g_m$ was less sensitive to the decrease in $\psi_{leaf}$ than $g_s$ at the beginning of soil drought. This result was in agreement with an earlier study conducted by Théroux-Rancourt et al. (2014), who found that $g_m$ only responded to more positive $\psi_{leaf}$ or more severe soil drought, e.g., $\psi_{soil} < −1.01$ 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 $\psi_{leaf}$ (Zwieniecki et al., 2007; Théroux-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 $A_o$ (Figure 4G).

However, as soil drought proceeded, $g_m$ declined as $\psi_{leaf}$ 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 $\psi_{leaf}$ and $g_m$ ($r^2 = 0.77$, $P < 0.01$) (Figure 4C), because $K_{leaf}$ was strongly influenced by $\psi_{leaf}$ under drought stress (Wang et al., 2018). Therefore, the decline in $\psi_{leaf}$ 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 $\psi_{soil}$ 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 $\psi_{soil}$ decreasing from −1.01 to −1.44 MPa was not a mere coincidence. This might suggest that $\psi_{leaf}$ 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-stomal 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$_2$CO$_3$) (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
Aquaporins (AQPs) are pore-forming integral membrane proteins that transport of water, CO$_2$ and other small neutral molecules across the plasma membrane (Flexas et al., 2006; Kaldenhoff, 2012). A higher abundance of AQPs increased the cellular CO$_2$ 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 $K_{\text{leaf}}$ 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., $\Psi_{\text{soil}} < -1.01$ 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; Olsovksa 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 CO$_2$ 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 $\Psi_{\text{soil}}$ 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 $A_i/g_s$ 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 $\Psi_{\text{soil}}$ 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 CO$_2$ 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 CO$_2$ from ambient air to chloroplasts was the crucial constraints to photosynthesis under drought conditions. The decrease in $\Psi_{\text{leaf}}$ 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 moderate 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. 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.

**FUNDING**

This work was supported by the National Natural Science Foundation of China (51790534, 51879267, 51779259), and the Agricultural Science and Technology Innovation Program (ASTIP), Chinese Academy of Agricultural Sciences.

**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 $\Psi_{\text{soil}}$.

**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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