Coordinated variation between veins and stomata in cotton and its relationship with water-use efficiency under drought stress

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Abstract

Drought stress causes changes in vein and stomatal density. The objectives of this study were to determine (1) if the changes in vein and stomatal density are coordinated in cotton (*Gossypium hirsutum* L.) and (2) how these changes affect water-use efficiency (WUE). The results showed significant positive correlations between vein density and stomatal density when cotton was grown under different degrees of drought stress. WUE was significantly positively correlated with the densities of both veins and stomata. Stomatal pore area and stomatal density on the abaxial leaf side, but not the adaxial side, were significantly correlated with WUE, stomatal conductance, leaf net photosynthetic rate, and transpiration rate. In conclusion, coordinated changes in vein and stomatal density improve the WUE of cotton under drought stress. The abaxial leaf side plays a more important role than the adaxial side in WUE and gas exchange.

Additional key words: major leaf veins; maximum stomatal conductance; minor leaf veins; photosynthesis; stomata size.

Introduction

Water transport is a continuous process in plants (Philip 2003). In terrestrial plants, water is taken from soil by roots and then transported to the petiole through the stem. The water then moves from veins into mesophyll cells and evaporation sites near the stomatal cavity. Water near the stomatal pores is rapidly lost to the atmosphere. Thus, at the leaf level, veins and stomata are important structures determining plant water use (Sack and Frole 2006).

Veins are a conduit system for water. The density of veins determines their capacity and efficiency to supply water to leaf cells (Brodribb *et al.* 2007, Boyce *et al.* 2009, McKown *et al.* 2010, Brodribb and Jordan 2011). Vein characteristics directly affect plant water supply, which in turn is closely related to physiological activity. Maximum hydraulic conductance and maximum photosynthetic rate are both closely related to leaf vein density (Sack and Frole 2006, Brodribb *et al.* 2007).

Water transport and CO₂ diffusion inside leaves are two important functional traits determining CO₂ assimilation efficiency (Sack and Holbrook 2006). As channels

for gas exchange between plants and the atmosphere, stomata are another important structure affecting the leaf water balance (Küppers 1984, Meinzer *et al.* 1991, Brodribb and Feild 2000, Brodribb and Jordan 2011). Leaf transpiration is directly related to stomatal density and stomatal size (Franks *et al.* 2009a, Zhang *et al.* 2012).

Studies have shown coordinated development between vein density and stomatal density in the evergreen *Nothofagus cunninghamii* (Hook.) Oerst (Brodribb and Jordan 2011) and in 17 species of *Paphiopedilum* (Zhang *et al.* 2012). In leaves of the woody angiosperm *Toona ciliata* M. Roem, vein density and stomatal density are regulated by leaf expansion so that leaf hydraulic conductance and stomatal conductance (g_s) remain proportional (Carins *et al.* 2012). Several recent studies have examined the effects of environment on vein and stomatal density. One of those studies reported that tobacco (*Nicotiana tabacum* L.) responds to differences in day/night temperature by adjusting the density of minor veins and stomata density in a correlated manner (Hu *et al.*

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Abbreviations: C_i – intercellular CO₂ concentration; E – transpiration rate; g_s – stomatal conductance; MS – mild drought stress; MDS – moderate drought stress; LMA – leaf dry mass per area; P_N – net photosynthetic rate; WUE – water-use efficiency; WUE_i – intrinsic water-use efficiency; WW – well-watered treatment.

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2014). A similar correlation between minor vein and stomatal density was observed when peanut (*Arachis hypogaea* L.) was grown under varying light conditions (Zhao *et al.* 2017). These results suggest coordination between leaf water supply and demand. Relatively little is known about coordination between stomatal density and vein density under drought stress.

Drought is a primary factor limiting crop productivity. High WUE is a major prerequisite for plants to survive under hot and dry conditions (Costa *et al.* 2007, Morison *et al.* 2008). Plants improve WUE by (1) concentrating CO₂, (2) increasing the conductance of CO₂ through the mesophyll, and (3) increasing Rubisco activity (Parry *et al.* 2005, Flexas *et al.* 2010, 2013; Gago *et al.* 2014). A previous study reported that some soybean (*Glycine max* L. Merrill) cultivars exposed to supplemental UV-B radiation increased WUE by altering stomatal development and function (Gitz *et al.* 2005). Franks *et al.* (2015) studied *Arabidopsis* mutants which exhibited differences in stomatal density due to overexpression of epidermal patterning factor. The authors reported that reduced

stomatal density increased both instantaneous and long-term WUE. Yoo *et al.* (2010) reported that the WUE of *Arabidopsis* was regulated by transcription factor *GT-2 Like 1*, which modulated abaxial stomatal density via transgression of stomatal density and distribution. There are some differences between adaxial and abaxial g_s . Under most conditions, including drought, stomatal resistance is considerably greater on the abaxial leaf side than that on the adaxial side (Sharpe 1973).

Cotton (*Gossypium hirsutum* L.) is a common cash crop in arid and semiarid regions of Central Asia. Drought stress can limit cotton yield in those regions. Yields could be increased and irrigation water saved if the WUE of cotton was improved. The objective of this field experiment was to test the following hypotheses: (1) water stress induces coordinated changes in the density of veins and stomata in cotton; (2) the coordinated changes in vein and stomatal density improve WUE; (3) the abaxial leaf side plays a more important role than the adaxial side on WUE and gas exchange.

Materials and methods

Experimental design: The field experiment was carried out at an agricultural experimental field near Shihezi University, Xinjiang, China (45°19'N, 86°03'E). The soil texture at the site is clay loam.

The experiment consisted of a completely randomized block design with three treatments: (1) well-watered (WW, the total irrigation amount between 8 June and 20 August was 4500 m³ ha⁻¹); (2) mild drought stress (MS, 60% of WW, 2700 m³ ha⁻¹); and (3) moderate drought stress (MDS, 20% of WW, 900 m³ ha⁻¹). The total irrigation amount in WW was the same as that used by local farmers. Each treatment was replicated three times.

Upland cotton (*Gossypium hirsutum* L., cv. Xinluzao 45) was sown in the plots on 18 April 2016. The plant density was 1.8×10^5 plants ha⁻¹. All of the plots were irrigated with the same amount of water two days after sowing in order to promote germination. The treatments were imposed 50 d after sowing (8 June). The plots were watered ten times (at 6–10-d intervals) between 8 June and 20 August. The irrigation amounts per application were 450, 270, and 90 m³ ha⁻¹ in the WW, MS, and MDS treatments, respectively. The measurements described in the sections below were performed between 15 July and 30 July (37–52 d after the treatments were imposed).

The plots (31.5 m²) were fertilized before planting with 156 kg(N) ha⁻¹ (urea) and 225 kg(P) ha⁻¹ (KH₂PO₄). Additional fertilizer [520 kg(N) ha⁻¹ and 158 kg(P) ha⁻¹] was applied to the plots during the growing season via drip irrigation.

Water potential: A pressure chamber (SKPM 1400; Skye Instruments, Llandrindod Wells, UK) was used to measure pre-dawn leaf water potential. After excising the leaves

from the plants, the petioles were immediately sealed in a compression gasket with the cut surface exposed and the leaf blade inside the pressure chamber. The pressure inside the chamber was slowly increased with N₂ gas until sap appeared at the cut surface of the petiole.

Gas exchange and WUE: Leaf gas exchange was measured using an open infrared gas-exchange analyzer system (LI-6400; LI-COR Inc., Lincoln, USA) that was equipped with a leaf LED chamber (6400-02B LED). The measurements were made between 11:00 and 14:00 h under light-saturating conditions (PPFD; 1,800 μmol m⁻² s⁻¹). The temperature was 28°C. The measurements were made on one young, fully expanded leaf that was randomly selected in each plot. The leaf chamber was attached to the same portion of each leaf, and the leaf position was not modified. The WUE was calculated by dividing leaf net photosynthetic rate (P_N) by transpiration rate (E). Intrinsic WUE (WUE_i) was calculated as P_N/g_s .

Leaf dimensions: One leaf was collected from each plot. The area of the leaves was measured with an LI-3000 leaf area meter (LI-COR Inc., Lincoln, USA). The leaf samples were then fixed by vacuum infiltration in 4% glutaraldehyde and 3% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.2). The leaf samples were then fixed again in 1% osmium tetroxide overnight, dehydrated in a graded series of acetone solutions, and then embedded in Spurr's resin (or LR White). An ultramicrotome (Leica Ultracut, Wetzlar, Germany) was used to make cut thin (40-nm) sections. The sections were stained with toluidine blue and observed under an electron microscope (JEM1230, JEOL, Tokyo, Japan). Electron micrographs were taken with a

digital camera (*BH-2, Olympus*, Tokyo, Japan) at magnifications of 10 × and 40 ×. Leaf thickness and the distance from the stomata to the nearest vein were determined using *ImageJ* software.

Ten discs (1.56 cm²) were excised from each leaf, dried at 60°C for at least 48 h, and then weighed to determine leaf dry mass per unit leaf area (LMA). Leaf density was calculated by dividing the leaf mass by its volume.

Vein density: In this paper, the term ‘major veins’ refers to veins which extend from the petiole. The term ‘minor veins’ refers to all but the major veins. The length of the major veins was measured with a ruler. Major vein density was calculated by dividing the total length of the major veins by the leaf area.

To measure minor veins, leaf sections (5 × 5 mm) were excised from the middle of the leaves. The sections were soaked in 80% alcohol solutions at 65°C for about 1.5 h. The alcohol solution was then replaced with 10% sodium hydroxide solution at 65°C for 3 h and then bleached. The samples were washed with water and then dyed with safranin. The samples were decolorized with a gradient of alcohol. The leaf samples were photographed with a light microscope (*BH-2, Olympus*, Tokyo, Japan). The lengths of the minor veins and the area of each leaf section were measured using *ImageJ* software. Minor vein density was calculated by dividing minor vein length by the area of the leaf section.

Stomata measurements: Leaf sections (5 × 8 mm) were excised from the leaves and then fixed in FAA (50% alcohol solution, 5% formaldehyde, 5% glacial acetic acid). The samples were sent to the Xinjiang Institute of Ecology and Geography Chinese Academy of Science where photographs were made using an electron scanning

microscope (*SUPRA 55VP, Carl Zeiss AG*, Oberkochen, Germany). The lengths and widths of the stomata and stomatal pores were measured with *ImageJ* software. Stomatal density was calculated as the number of stomata per unit leaf area. Stomatal pore area and stomatal pore perimeter were calculated based on the methods of Zhou and Xie (2016).

Theoretical g_s and theoretical maximum g_s were calculated for both the abaxial and adaxial sides based on the following equation (Franks and Farquhar 2001):

$$g_s = \frac{(\text{diffusion coefficient}) \times (\text{stomata density}) \times (\text{pore area})}{(\text{molar volume of air}) \times [(\text{pore depth}) + \frac{\pi}{2} \sqrt{\frac{\text{pore area}}{\pi}}]}$$

when calculating theoretical g_s , pore depth was estimated based on pore width using the following equation (Franks and Beerling 2009):

$$\text{Pore depth} = [(\text{stomata width} - \text{pore width})/2]$$

For maximum theoretical g_s , pore depth was estimated based on stomatal width using the following equation (Franks and Farquhar 2007):

$$\text{Pore depth} = (\text{stomata width}/2).$$

In both calculations, the molar volume of air and the effective diffusion coefficient for water vapor in air were those for 21°C.

Statistical analysis: One-way analysis of variance was conducted using *SPSS 19.0 for Windows* (SPSS, Chicago, USA). The significance of the differences between treatment means was separated using the *Student-Newman-Keul's* test at the $P \leq 0.05$ level. The data are presented as the means ± SD. Linear regressions were performed using *SigmaPlot version 10*.

Results

Leaf water potential decreased significantly as drought stress increased (Fig. 1). Leaf water potentials were 30% lower under MS and 58% lower under MDS compared with WW.

Leaf dimensions: Drought stress significantly reduced leaf area and leaf thickness but increased LMA and leaf density (Table 1S, *supplement available online*). Leaf surface area was 37% lesser under MS and 53% lesser under MDS compared with WW. Leaf thickness was reduced by 9.7% in the MS treatment and by 24.2% in the MDS treatment. The LMA was 8.6% greater in the MS treatment and 33.3% greater in the MDS treatment compared with WW. Similarly, the MS and MDS treatments increased leaf density by 19.7 and 74.9%, respectively.

Fig. 1. Effect of drought stress on cotton leaf water potential. Error bars represent SD. Values with *different letters* are significantly different at $P < 0.05$. WW – well-watered treatment.

Vein density: Drought stress significantly increased vein density per unit leaf area (Table 1; Fig. 1S, *supplement available online*). Major vein density was 26.2% greater under MS and 40.5% greater under MDS compared with WW. Similarly, the MS and MDS treatments increased minor vein density per unit leaf area by 9.6 and 36.8%, respectively. Minor vein density per unit leaf volume was also significantly greater in the MS and MDS treatments than that in WW. Drought stress influenced the distance from the stomata to the veins. On the adaxial leaf side, the average distance from the stomata to the vein decreased significantly in the order WW > MS > MDS. On the abaxial leaf side, the order was MS > WW, MDS.

Stomata characteristics: Drought stress significantly reduced stomata length on the abaxial leaf side but not on the adaxial leaf side (Table 2). Stomata width was not significantly affected by drought stress; however, stomata width was significantly greater (18.3–41.7%) on the abaxial rather than that on adaxial leaf side. MDS significantly reduced stomata pore perimeter on the adaxial leaf side by 14% compared with WW, whereas MS had no significant effect. On the abaxial leaf side, the MS and MDS treatments reduced stomata pore perimeter by 28.2 and 33.4%, respectively, compared with WW.

The stomata pore area per unit leaf area was 132–235% greater on the abaxial side than that on the adaxial side (Table 2). Drought stress had no significant effect on stomata pore area on the adaxial leaf side. However, on the abaxial side, the MS and MDS treatments reduced stomata

pore area by 13.5% and 15.4%, respectively, compared with WW.

Stomata density was 22, 100, and 144% greater on the adaxial side than that on the adaxial side in WW, MS, and MDS treatments, respectively (Table 2). The MDS treatment increased stomata density on the adaxial side by 22% compared with WW, whereas MS treatment had no significant effect. On the abaxial side, the MS and MDS treatments increased stomata density by 40 and 134%, respectively, compared with WW.

Total stomata density and pore area: The ratio of adaxial:abaxial stomata density decreased significantly as drought stress increased (Table 2S, *supplement available online*). In contrast, ratio of sum of the total adaxial and abaxial stomata density per unit vein density increased significantly as drought stress increased. The MS and MDS treatments both reduced total adaxial and abaxial stomatal pore area per unit leaf area. MDS significantly reduced the total adaxial and abaxial stomatal pore area per unit leaf volume, whereas MS had no significant effect.

Gas exchange: Leaf P_N , g_s , E , and C_i decreased significantly in the order WW > MS > MDS (Table 3). The WUE (*i.e.*, P_N/E) in the MDS treatment was 9.4% greater than that in the MS treatment and 3.1% greater than that in the WW treatment. There was no significant difference in WUE between the MS treatment and WW. The intrinsic WUE (*i.e.*, P_N/g_s) increased significantly as drought stress increased.

Table 1. Effect of drought stress on the leaf vein characteristics of cotton. Values are means $\pm$ SD. Values within a column followed by different letters are significantly different at $P < 0.05$. Major veins are those which extend from the petiole. Minor veins are all but the major veins. MS – mild stress; MDS – moderate stress; WW – well-watered.

Treatment	Major vein density [cm cm ⁻²]	Minor vein density [cm cm ⁻²]	Minor vein density [cm cm ⁻³]	Distance from stomata to vein [μm]	
				Adaxial	Abaxial
WW	0.42 $\pm$ 0.03 ^c	12.52 $\pm$ 0.40 ^c	31.25 $\pm$ 0.25 ^c	283.7 $\pm$ 15.1 ^a	143.8 $\pm$ 5.6 ^b
MS	0.53 $\pm$ 0.02 ^b	13.68 $\pm$ 0.36 ^b	37.75 $\pm$ 0.01	226.6 $\pm$ 13.8 ^b	162.0 $\pm$ 6.9 ^a
MDS	0.59 $\pm$ 0.02 ^a	17.07 $\pm$ 0.30 ^a	56.24 $\pm$ 1.35 ^a	191.4 $\pm$ 7.4 ^c	137.9 $\pm$ 1.7 ^b

Table 2. Effects of drought stress on the stomata characteristics of cotton leaves. Values are means $\pm$ SD. Values within a column followed by different letters are significantly different at $P < 0.05$. MS – mild stress; MDS – moderate stress; WW – well-watered.

Leaf side	Treatment	Stomata length [μm]	Stomata width [μm]	Stomatal pore perimeter [μm]	Stomatal pore area [$\times 10^{-3}$ μm ² μm ⁻²]	Stomata density [stomata mm ⁻²]
Adaxial	WW	27.3 $\pm$ 0.2 ^{bc}	15.3 $\pm$ 0.9 ^b	30.7 $\pm$ 2.8 ^b	3.8 $\pm$ 0.61 ^c	167.7 $\pm$ 20.2 ^d
	MS	27.6 $\pm$ 0.5 ^b	16.9 $\pm$ 0.4 ^b	30.1 $\pm$ 1.5 ^b	3.3 $\pm$ 0.72 ^c	150.0 $\pm$ 20.2 ^d
	MDS	27.0 $\pm$ 0.8 ^{bcd}	16.9 $\pm$ 0.5 ^b	26.4 $\pm$ 1.5 ^c	3.1 $\pm$ 0.29 ^c	205.2 $\pm$ 1.5 ^c
Abaxial	WW	30.0 $\pm$ 0.6 ^a	21.6 $\pm$ 0.7 ^a	34.4 $\pm$ 1.7 ^a	10.4 $\pm$ 0.63 ^a	214.0 $\pm$ 3.8 ^c
	MS	26.4 $\pm$ 0.6 ^{cd}	20.2 $\pm$ 0.5 ^a	24.7 $\pm$ 1.0 ^{cd}	9.0 $\pm$ 0.25 ^b	300.0 $\pm$ 13.8 ^b
	MDS	26.3 $\pm$ 0.38 ^d	20.0 $\pm$ 30.4 ^a	22.9 $\pm$ 0.4 ^d	8.8 $\pm$ 0.80 ^b	500.8 $\pm$ 10.1 ^a

Theoretical stomatal conductance: Theoretical g_s was significantly greater on the abaxial side than that on the adaxial side (Fig. 2A). On the adaxial side, theoretical g_s was not significantly affected by drought stress. However, on the abaxial side, theoretical g_s decreased in the order WW > MDS > MS. The same trend was observed for maximum theoretical g_s , except that on the abaxial side, maximum theoretical g_s was significantly greater under moderate stress than under either mild stress or WW (Fig. 2B).

Relationship between stomata density and vein density: Vein density was positively and significantly correlated with stomata density on both the adaxial and abaxial sides (Fig. 3A,B). Similarly, total stomata density was significantly positively correlated with vein density per unit leaf area and with vein density per unit leaf volume (Fig. 3C,D).

Table 3. Effects of drought stress on the stomatal conductance (g_s), leaf photosynthetic rate (leaf P_N), transpiration rate (E), intercellular CO_2 concentration (C_i), water-use efficiency ($WUE = P_N/E$), and intrinsic water-use efficiency ($WUE_i = P_N/g_s$) of cotton leaves. Values are means $\pm$ SD. Values within a column followed by different letters are significantly different at $P < 0.05$. MS – mild stress; MDS – moderate stress; WW – well-watered.

Treatment	g_s [$\mu\text{mol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{ s}^{-1}$]	P_N [$\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$]	E [$\text{mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{ s}^{-1}$]	C_i [$\mu\text{mol}(\text{CO}_2) \text{ mol}^{-1}$]	WUE [$\mu\text{mol}(\text{CO}_2) \text{ mol}(\text{H}_2\text{O})^{-1}$]	WUE_i [$\mu\text{mol}(\text{CO}_2) \text{ mmol}(\text{H}_2\text{O})^{-1}$]
WW	1.2 ± 0.1^a	35 ± 0.9^a	11.0 ± 0.3^a	251 ± 5.3^a	3.2 ± 0.1^b	30.2 ± 3.2^c
MS	0.5 ± 0.1^b	28 ± 2.8^b	8.4 ± 0.7^b	219 ± 4.5^b	3.3 ± 0.1^b	61.3 ± 6.0^b
MDS	0.2 ± 0.1^c	18 ± 4.8^c	5.2 ± 1.2^c	174 ± 20.1^c	3.5 ± 0.2^a	106.7 ± 17.2^a

Fig. 2. Effects of drought stress on the theoretical stomatal conductance (A) and the maximum theoretical stomatal conductance (B) of cotton leaves. Error bars represent SD. Values with different letters are significantly different at $P < 0.05$. WW – well-watered.

Relationships between vein and stomatal density and WUE_i : Vein density was significantly positively correlated on both a leaf area and a leaf volume basis with WUE_i (Fig. 4A,B). Stomatal density was also positively correlated WUE_i (Fig. 4C,D). Stomatal pore area (on both a leaf

on the adaxial leaf showed no significant correlation with g_s , P_N , E , and WUE_i (Fig. 5). On the abaxial side, stomatal pore area per unit leaf area was significantly negatively correlated with WUE_i and significantly positively correlated with g_s , P_N , and E .

Discussion

Coordinated relationship between stomata density and vein density: As the exit point for water and the entrance point for CO_2 , stomata are at the core of leaf structural diversity. The vein network is important because it distributes water and nutrients throughout the plant. A cost-optimal use of water resources requires the homogenous distribution of veins and stomata; that is, the density of these two structures should remain coordinated (Fiorin *et al.* 2015).

Many studies have shown coordinated development between vein density and stomata density. For example, Brodribb and Jordan (2011) observed that the development of veins and stomata was coordinated in evergreen trees (*Nothofagus cunninghamii*). Correlated evolution between

stomatal traits and vein density also exists in *Paphiopedilum* (Zhang *et al.* 2012). In the leaves of a woody angiosperm (*Toona ciliata* M. Roem.), the differential between vein density and stomata density was regulated by leaf expansion so that leaf hydraulic conductance and g_s remained proportional (Carins *et al.* 2012). In this study, cotton was grown under increasing drought stress. The results showed that vein density per unit leaf area was positively correlated with stomata density per unit leaf area on both the abaxial and adaxial leaf sides (Fig. 3). Furthermore, total adaxial and abaxial stomata density per unit leaf area was significantly positively correlated with vein density per unit leaf area. These results indicate a coordinated relationship between

vein density and stomata density. We propose that this coordinated relationship is essential for maintaining the dynamic balance of water loss and supply in plant leaves. Leaf water transport is affected by several leaf morphological traits including leaf thickness, the depth of the veins inside the leaves, and leaf vein density (Noblin *et al.* 2008, Buckley *et al.* 2015). Previous studies only considered the relationship between stomata and veins on a leaf area basis. In this study, drought reduced leaf thickness (Table 1S). Our analysis also indicated that a coordinated relationship existed between stomata density and vein density on a leaf volume basis (Fig. 3). Drought stress significantly reduced the distance from the stomata to the vein on the adaxial leaf side (Table 1). In contrast, the distance from the stomata to the vein was relatively stable on the abaxial side, regardless of the drought stress treatment. These results suggest that the stability of the distance from the stomata to vein on the abaxial side is important for cotton under drought stress.

During photosynthesis, CO₂ moves through stomata

into mesophyll cells. When the stomata are open, the supply of CO₂ increases, thus benefiting photosynthesis. However, water transport capacity is required to keep the stomata open. High stomata density and high vein density must appear simultaneously. If not, stomata must be partially closed (Dow and Bergmann 2014). Furthermore, we found that drought stress significantly increased the ratio of stomatal density to vein density (Table 2S). This may mean that veins improve the ability of the plant to indirectly support stomatal opening. We speculate that photosynthesis would benefit from open stomata as a result of increasing vein density when plants are recovering from drought stress (Lauriano *et al.* 2004, Chaves and Oliveira 2004).

Vein and stomata density in ferns and angiosperms is determined by epidermal cell size (Zhao *et al.* 2017). In this study, drought stress reduced the size of both mesophyll and epidermal cells, resulting in smaller leaves. As mesophyll cells become smaller, the veins are nearer each other; thus vein density increases (Table 1). Higher

Fig. 3. Correlation between vein density and stomata density on the adaxial (A) and abaxial (B) leaf sides of cotton as affected by drought stress. Correlation between stomata density and vein density per unit leaf area (C) and per unit leaf volume (D). WW – well-watered.

Fig. 4. Correlation between intrinsic water-use efficiency (WUE_i) and vein density per unit area (A) and per unit leaf volume (B) in cotton as affected by drought stress. Correlation between WUE_i and stomata density per unit leaf area (C) and per unit leaf volume (D). WW – well-watered.

stomata density in the drought stress treatments may be because the smaller epidermal cells are closely packed and epidermal cell expansion is reduced (Hsie *et al.* 2015). Under drought conditions, a reduction in leaf size allows plants to reduce transpiration and increase WUE (Table 3).

Coordination between stomata density and vein density may improve WUE under drought: As expected, the WUE and WUE_i of cotton increased under drought stress (Table 3). Water transport efficiency is closely linked with leaf veins. A high density of first order veins equalizes the water potential across the leaf (Zwieniecki *et al.* 2004, Ocheltree *et al.* 2008). In this study, major vein density increased under water stress (Table 1). Acting as water transport superhighways, the major veins can efficiently distribute limited water resources across the leaf, thus increasing WUE. In addition, a high density of first-order (*i.e.*, major) veins improves drought resistance (Sack and Scoffoni 2013).

Generally speaking, high vein density represents a larger number of parallel exit pathways of water. Brodribb *et al.* (2007) examined 43 plant species and observed that permeable xylem surface area increased as vein density

increased. The increase of vein density per unit leaf area or leaf volume means that the indirect or direct area between vein and mesophyll cells changes incrementally, so that under drought stress, the veins can efficiently transport urgently needed water to mesophyll cells for photosynthesis. A previous report indicated that high vein density in *Populus deltoides* leaves may contribute to greater phloem transport (Russin and Evert 1984) and higher rates of gas exchange per unit leaf area (Sack and Frole 2006, Brodribb *et al.* 2007, Boyce *et al.* 2009, Brodribb and Jordan 2010, McKown *et al.* 2010, Feild *et al.* 2011, Walls 2011). Higher vein density can also increase the efficient transport of materials to where they are needed in the plant, so that plant growth increases after rewatering (Lauriano *et al.* 2004, Chaves and Oliveira 2004).

In this study, stomata density was well correlated with WUE_i (Fig. 4). Stomata density and stomata size showed negative correlation in *Eucalyptus globulus* (Franks *et al.* 2009a,b). Previous researchers proposed that small stomata can rapidly respond to changes in the external environment (Xu and Zhou 2008, Zhang *et al.* 2012, Drake *et al.* 2013). In this study, drought stress reduced stomata size on the abaxial leaf side (Table 2). The decrease in

Fig. 5. Correlation between stomatal conductance (g_s) and stomata pore area (A–B) and stomata density (C–D) in cotton as affected by drought stress (E–H). Correlation between leaf net photosynthesis (P_N) and stomatal pore area (E–F) and stomata density (G–H). (I–L) correlation between transpiration (E) and stomatal pore area (I–J) and stomata density (K–L). (M–P) correlation between intrinsic water-use efficiency (WUE_i) and stomatal pore area (M–N) and stomatal pore density (O–P). WW – well-watered treatment.

stomata size allows cotton to quickly and sensitively regulate leaf gas exchange and increase WUE. This suggested that drought-induced increases in stomata density may improve WUE_i . Taken together, the coordinated relationship between stomata density and vein density may contribute to the improvement of WUE.

The abaxial leaf side is more important than the adaxial side in gas exchange and water-use efficiency

under drought: Previous studies indicated that under normal conditions, adaxial g_s is lesser than abaxial g_s (Lu *et al.* 1993, Wang *et al.* 2008). Operating g_s was also lesser on the adaxial side than that on the abaxial side (Fanourakis *et al.* 2015). This suggests that the abaxial side plays a more important role than the adaxial side in gas exchange under normal conditions. In our study, the adaxial:abaxial stomata density decreased as drought stress increased (Table 2S). In addition, theoretical g_s and

maximum theoretical g_s were lesser on the adaxial side than that on the abaxial side (Fig. 2). These results show that under water stress, the abaxial side contributes more to gas exchange than the adaxial side. We also found that gas exchange was significantly correlated with stomatal density and stomatal pore area on the abaxial side but not on the adaxial side (Fig. 5).

From our perspective, preventing water loss and improving WUE are prerequisites for the survival of plants under drought conditions. Theoretic g_s and theoretic maximum g_s are both low on the adaxial side (Fig. 2). This not only prevents water loss but also limits the CO_2 supply to chloroplasts. This suggests that the adaxial side of cotton leaves may contribute less than the abaxial side to photosynthesis. Although it is the dominant side for transpiration under normal conditions, low g_s limits water loss from the adaxial leaf side during drought stress.

Rockwell *et al.* (2014) studied mature red oak (*Quercus rubra* L.) trees and found that increases in the air fraction can increase the competitiveness of vapor transport at the transition to the spongy mesophyll. Recent modeling approaches have discovered that water inside the leaves is transported mainly through the liquid; however,

in leaves with low tissue density, the vapor phase may also have a significant role in water transport under high irradiance and high temperatures (Rockwell *et al.* 2014, Buckley 2015). A 10% increase in leaf mass per unit area reduced the airspace fraction by about 2% in 11 simple-leaved woody angiosperm species (John *et al.* 2017). The airspace fraction of spongy tissue is high and nearly twice that of palisade tissue. As a result, cell-to-cell connectivity in spongy tissue is low, which effectively decreases the area available for liquid-phase flow. Therefore, spongy mesophyll is more resistive compared with other tissues (Buckley *et al.* 2015). Leaf thickness decreases and leaf density increases as water stress increases (Table 1S). The explanation is that water stress may increase the number of mesophyll cells (especially in spongy tissue), thus reducing leaf airspace. The reduction in airspace may limit transpiration from the abaxial side. Our results show that theoretic conductance is greater on the abaxial side than on the adaxial side (Fig. 2); therefore, CO_2 entering the abaxial side may prevent water loss. Overall, under drought conditions, the abaxial side may play a more important role in gas exchange and water use efficiency compared with the adaxial side.

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