Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass

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Abstract

Responses of plant leaf stomatal conductance and photosynthesis to water deficit have been extensively reported; however, little is known concerning the relationships of stomatal density with regard to water status and gas exchange. The responses of stomatal density to leaf water status were determined, and correlation with specific leaf area (SLA) in a photosynthetic study of a perennial grass, Leymus chinensis, subjected to different soil moisture contents. Moderate water deficits had positive effects on stomatal number, but more severe deficits led to a reduction, described in a quadratic parabolic curve. The stomatal size obviously decreased with water deficit, and stomatal density was positively correlated with stomatal conductance ($g_s$), net CO2 assimilation rate ($A_n$), and water use efficiency (WUE). A significantly negative correlation of SLA with stomatal density was also observed, suggesting that the balance between leaf area and its matter may be associated with the guard cell number. The present results indicate that high flexibilities in stomatal density and guard cell size will change in response to water status, and this process may be closely associated with photosynthesis and water use efficiency.

Key words: Gas exchange, guard cell size, photosynthesis, stomatal density, water stress, water use efficiency (WUE).

Introduction

In an agricultural context, water deficit is one of the most important environmental factors constraining plant photosynthesis and productivity in arid and semi-arid areas. Consequently, plant responses to drought have been extensively investigated from molecular, physiological, and individual plant to ecosystem levels (Bray, 1997; Chaves et al., 2003). Water stress may reduce leaf net photosynthetic assimilation ($A_n$) by both stomatal and metabolic limitations (Farquhar and Sharkey, 1982; Chaves et al., 2003; Ghannoum et al., 2003; Ripley et al., 2007). In addition, many studies have reported that stomatal effects are major under moderate stresses, but biochemical limitations are quantitatively important during leaf ageing or during severe drought (Grassi and Magnani, 2005; Gallé et al., 2007). An early response to water deficit is a reduction in leaf area and plant growth, which allows plants to reduce their transpiration, thus increasing water use efficiencies (WUE) (Xu and Zhou, 2005; Monclus et al., 2006; Aguirrezabal et al., 2006), and promoting interspecies competition capacity under drought (Xu et al., 2007).

Plant stomata, the vital gate between plant and atmosphere may play a central role in plant/vegetation responses to environmental conditions, which have been and are being investigated from molecular and whole plant perspectives, as well as at ecosystem and global levels (Nilson and Assmann, 2007, and references therein). Many researchers have reported stomatal density responses to various environmental factors, such as elevated CO2 concentration (Woodward, 1987), heat stress (Beerling and Chaloner, 1993), salt stress (Zhao et al., 2006), drought (Lecoeur et al., 1995; Zhao et al., 2001; Galmés et al., 2007), precipitation change (Yang et al., 2007), and plant density (Zhang et al., 2003). Moreover, many studies have shown that water deficit leads to an increase in stomatal density (McCree and Davis, 1974; Cutler et al., 1977; Yang and Wang, 2001;
Zhang et al., 2006), and a decrease in stomatal size (Cutler et al., 1977; Quarrie and Jones, 1977; Spence et al., 1986), indicating this may enhance the adaptation of plant to drought (Cutler et al., 1977; Spence et al., 1986; Martinez et al., 2007).

Leaf morphological traits, including stomatal density and distribution, and epidermal features may affect gas exchange quite remarkably and their relationships with key environmental factors such as light, water status, and CO₂ levels (Woodward, 1987; Nilson and Assmann, 2007, and references therein). For example, increasing the blue-light response of stomata through red light is closely associated with guard cell chloroplast activity (Roelfsema et al., 2006; Shimazaki et al., 2007), indicating that leaf photosynthetic activity may involve stomatal movement and development. The balance between carbon gain through photosynthesis and water loss through transpiration may also affect instantaneous WUE. Unfortunately, how stomatal density affects gas exchange has received little attention to date.

Grassland dominated by Leymus chinensis (Trin.) Tzvel. represents one of the most widely distributed grassland communities in the Eurasian steppe zone. This native species provides a good livestock forage and has been used widely for natural grazing lands (Wang and Gao, 2003; Bai et al., 2007). However, the grassland has been severely degraded during recent decades due to the adverse effects of climatic change, such as water scarcity, and improper land use practices, such as overgrazing. The responses of plant growth and photosynthesis of grass to water deficit have been recently reported (Chen et al., 2005; Xu and Zhou, 2006a, b; Niu et al., 2008) in both field and greenhouse experiments. Unfortunately, studies describing how stomatal density and guard cell size of the perennial grass respond to different water stresses, and their relationships with gas exchange are few so far. The purposes of this study were to determine the response patterns of stomatal density to different water status, and to develop the relationship of stomatal density with gas exchange, based on a greenhouse experiment with a soil moisture gradient.

Materials and methods

Plant material, growth conditions, and treatments

Experiments were carried out at the experimental farm of the Institute of Botany, CAS (39°48’ N, 116°28’ E, 67 m asl), Beijing, China. The growth conditions and treatments were similar to our previous report (Xu and Zhou, 2006a). Briefly, the seeds of Leymus chinensis (Trin.) Tzvel. were collected from natural grassland in Xilinhot, Inner Mongolia. They were sown in plastic pots with a density of six plants per pot. Soil water-withholding treatments were initiated 30 d after sowing, in a sunlit greenhouse [day/night, air temperature 26/19 ± 2 °C, 13 h photoperiod (05.00–18.00 h)]. The daily maximum photosynthetically active radiation (PAR) was approximately 1000 µmol m⁻² s⁻¹ above the plant canopy. Soil moisture levels were maintained with manual irrigation by weighing individual pot at 17.00 h daily. Each target of the required soil moisture range was achieved by decreasing the water supply progressively about 20 d after the beginning of withholding water. To obtain a relatively stable water moisture gradient, the soil relative water content (SRWC) was roughly divided into five levels: 75–80% of field capacity (FC), 60–65%, 50–55%, 35–40%, and 25–30%, respectively. Each treatment (SRWC level) had 10 pots, i.e. 10 replicates, with a total of 50 pots. The different treatment pots were randomized at each irrigation to avoid effects from other environmental factors, such as light conditions or temperature.

Plant biomass and leaf area

For the biomass measurements, for each treatment, samples from four pots were obtained 40 d after starting the water treatment, dried at 80 °C to a constant weight, and weighed. Leaf area was measured using a Li-3000 leaf area meter (Li-Cor, Inc., Lincoln, Nebraska, USA), and the specific leaf area (SLA) calculated from the measurements of leaf area and dry weight.

Leaf stomatal density and guard cell size

The impression approach was used to determine leaf stomatal density, which was expressed as the number of stomata per unit leaf area (Radoglou and Jarvis, 1990). The leaves selected were those for which gas exchange was measured. The abaxial epidermis of the leaf was cleaned first using a degreased cotton ball, and then carefully smeared with nail varnish in the mid-area between the central vein and the leaf edge, for approximately 20 min. The thin film (approximately 5 mm × 15 mm) was peeled off from the leaf surface, mounted on a glass slide, immediately covered with a cover slip, and then lightly pressured with fine-point tweezers. Numbers of stomata (s) and epidermal cells (c) for each film strip were counted under a photomicroscope system with a computer attachment (MPS 60, Leica, Wetzlar, Germany). Impressions were taken from the six youngest, fully expanded leaves for each treatment. The leaf stomatal index was estimated using the formula [s/(c − s + 1)] × 100. The number of guard cells was estimated by doubling the number of counted stomata in the same leaf area (Radoglou and Jarvis, 1990). Stomatal size was defined as the length in micrometres between the junctions of the guard cells at each end of the stoma, and may indicate the maximum potential opening of the stomatal pore, but not the aperture of opening that actually occurs (Malone et al., 1993; Maherali et al., 2002).

Leaf water potential

Leaf water potential was measured at midday (11.30–12.30 h) using the youngest, fully expanded leaves (those for which gas exchange was measured) to avoid the effects of overnight water recovery. A WP4 Dew-point Potential Meter (Decagon Device, Pullman, Washington, USA) was used for leaf water potential measurement, using six leaves from each treatment.

Leaf gas exchange

Six plants from each treatment were selected from different pots. Gas exchange parameters were measured on an attached youngest fully expanded leaf 37–40 d after withholding water. The order in which the measurements were conducted (six leaves from six plants for each type of treatment) was the same every time.

Net photosynthetic rate per unit leaf area (A), stomatal conductance (gₛ) and transpiration rate (E) were measured using a 0.25 l chamber connected to a portable photosynthesis system (LI-6200, Li-Cor, Inc., Lincoln, NE, USA) under ambient temperature (25–27 °C).
and irradiance (approximately 900 μmol m⁻² s⁻¹). Readings were not terminated until at least 30 s had elapsed. The gas exchange parameters were calculated automatically using the software of the photosynthesis system. WUE was calculated from instantaneous values of A and E.

**Statistical analysis**

The layout of the experiment was a randomized block design. All statistical GLM-ANOVA analyses were performed using SPSS 10.0 (SPSS for Windows, Chicago, Illinois, USA). Linear regression and curve estimation were analysed in the present study using a probability value of 0.05 as the benchmark of significance.

**Results**

**Correlation of stomatal density and size with leaf water potential**

There was a non-linear response of stomatal density to leaf water potential, described by a quadratic parabolic curve with the maximum of 76.5 pores mm⁻² when \( \psi_l \) was –1.78 MPa at the early stage (Fig. 1A). At a later stage, a similar response pattern was also observed, increasing from –2.50 to –1.67 MPa with a stomatal density peak value of 79.7 pores mm⁻² that decreased with increasing \( \psi_l \) (Fig. 1B). For the stomatal index, there was also a similar non-linear response to \( \psi_l \), but the relationship was weaker compared to stomatal density.

![Fig. 1. Responses of stomatal density (closed squares, solid lines) and stomatal index (open squares, dotted lines) to leaf water potential (\( \psi_l \)) at 80 d (A) stomatal density: \( y = -17.07x^2 + 59.89x + 3.36, R^2 = 0.53, P = 0.011, n = 20 \) and at 90 d after sowing (B) stomatal density: \( y = -20.53x^2 + 56.79x + 40.48, R^2 = 0.58, P = 0.048, n = 10 \).](image)

The lengths in micrometres between the junctions of the guard cells at each end of the stoma, which indicate the maximum potential opening of stomatal pore, were also examined. The stomatal size dramatically decreased with increasing water deficit, and approximate linear relationships between stomatal size and water potential were seen (Fig. 2) at both 80 d (\( R^2 = 0.50; P = 0.003 \)) and 90 d after sowing (\( R^2 = 0.72; P = 0.002 \)). These results indicate that severe water stress obviously reduced stomatal size.

**Correlation of gas exchange with stomatal density**

Leaf stomatal density was positively correlated with stomatal conductance to water vapour (\( g_s \), \( R^2 = 0.52, P = 0.003 \)), and leaf net CO₂ assimilation rate (\( A_n \), \( R^2 = 0.35, P = 0.026 \)) (Fig. 3). However, the relationship between stomatal index and gas exchange parameters was remarkably scattered with no significant association found (data not shown). Leaf water transpiration rate (\( E \)) increased with increasing stomatal number, but the linear
relationship was not significant ($R^2=0.33, P>0.05$) (Fig. 4A). Responses of water use efficiency ($WUE, A/E$) to water deficit (Fig. 4B) showed a positive correlation of stomatal density with $WUE$ ($R^2=0.58, P=0.011$), which indicated that an increase in leaf stomatal density was closely associated with higher leaf $WUE$ because $A$ increased more than $E$.

**Correlation of specific leaf area with stomatal density**

Figure 5 illustrates the relationship between leaf characteristics and stomatal density. A significant correlation between green leaf area per individual plant and stomatal density (data not shown) was not found. However, the correlation of $SLA$ with stomatal density was significant and negative ($R^2=0.46, P=0.036$; Fig. 5), suggesting that the balance of leaf area development with its tissue may be closely linked to the occurrence of guard cells under different water status.

**Discussion**

The leaf stoma is a pivotal gate controlling the exchange of CO$_2$ and water vapour, although such processes may be affected by many environmental variables, including light, water status, temperature, and CO$_2$ concentration (Boyer et al., 1997; Buckley, 2005). As we know, CO$_2$ is the photosynthetic substrate in the intercellular space, and its concentration can be calculated from water diffusion through the leaf, showing a coupling interaction between CO$_2$ entry for photosynthesis and water vapour emitted via transpiration (von Caemmerer and Farquhar, 1981; Boyer et al., 1997). Under water stress, photosynthesis limitation can result from both stomatal and non-stomatal...
Stomatal density and water status

Fig. 5. Correlation of stomatal density with specific leaf area (SLA, $y = -0.653x+230.32, R^2=0.46, P=0.036$). Error bars represent ±SE of mean when these exceed the size of the symbol (n=10).

effects, depending on drought intensification and species (Chaves et al., 2003; Grassi and Magnani, 2005; Gallé et al., 2007). Early reports showed an increase in stomatal density and a decrease in cell size under water deficit, indicating that an adaptation to drought could occur (Quarrie and Jones, 1977; Spence et al., 1986; Martinez et al., 2007). Our results show that stomatal density and guard cell size may have a higher plasticity in response to a larger range of water status, and these parameters are clearly associated with photosynthesis and WUE.

In the present study, leaf stomatal size decreased with drought, which is consistent with the results of rice leaves (Quarrie and Jones, 1977; Yang et al., 1995; Meng et al., 1999; Fig. 2). Stomatal density was also negatively correlated with stomatal length under different water conditions in some Jujube leaves (Liu et al., 2006) and Platanus acerifolia leaves (Zhang et al., 2004). However, Zhang et al. (2006) reported that stomatal length increased under limited irrigation conditions, whereas its width decreased. Nevertheless, different effects of abiotic factors on stomatal size may depend on plant species/varieties (Maherali et al., 2002; Liu et al., 2006). In addition, stomatal and cuticle effects on gas exchange depend on the stomatal conductance level ($g_s$), and for leaves with a large $g_s$, all leaf gas exchange has been reported to be stomatal (Boyer et al., 1997). A greater stomatal size can also facilitate CO$_2$ diffusion into the leaf (Parkhurst, 1994), because its conductance is proportional to the square of the effective radius of the stomatal pore, leading to an increased $g_s$ (Maherali et al., 2002). However, Spence et al. (1986) reported that small guard cells may cause stoma to remain open under drought, which demonstrates a balance between carbon gain through photosynthesis and the prevention of excessive water loss through transpiration in an adaptive response to dry conditions. In the present study, however, a decrease in guard cell size did not seem to be directly linked to the change in $g_s$ of the L. chinensis leaf.

Several reports have shown that the stomatal density and its index increase with water stress (Yang and Wang, 2001; Zhang et al., 2006), but the number of stomata per leaf decreases (Quarrie and Jones, 1977). With decreasing precipitation, stomatal density also increases, whereas plant height, density, and leaf area decrease (Wang and Gao, 2003; Yang et al., 2007; Gazanchian et al., 2007). An increase in stomatal density was observed under moderate drought, but a decrease occurred with drought severity (Fig. 1), which is consistent with a study of rice leaves (Meng et al., 1999). Stomatal densities of leaves from several varieties of Jujube also have similar patterns under a drought severity gradient: initially increasing, then declining (Liu et al., 2006), similar to response patterns in rice leaves under salt stress (Zhao et al., 2001), and in wheat leaves in response to plant density (Zhang et al., 2003). However, wheat stomatal density always increases with continually increasing drought severity (Zhang et al., 2006). Stomatal densities of tree leaves rise with increasing urban integrative environmental stresses, indicating this may provoke a regulative capacity to deal with multiple simultaneous stresses including air pollution, high aerosol levels, and drought (Zhang et al., 2004). However, Yin et al. (2006) have suggested that the change in stomatal density might not be associated with drought-resistance in different genetic types of wheat. Thus, how guard cell development responds to environmental stresses and/or leaf development requires further research.

Leaf stomatal density and the stomatal index (the percentage of stomatal number to total cell number on a given leaf area) may be affected by cell expansion, depending on leaf development, ageing, and position (Ceulemans et al., 1995; Loesche et al., 1995). Thus, both guard cell and epidermal cell numbers per unit area of a small leaf at a later plant growth stage would be expected to increase. However, the present study showed that the correlation of stomatal density with water potential better fitted a hump-shaped curve compared to the stomatal index (Fig. 1), which suggests that a trade-off may occur. Nevertheless, further research in detail at the cell development level is still needed to elicit the differing responses between guard cells and epidermal cells to water status.

Drought may initially inhibit leaf growth and development, significantly reducing leaf area (Chaves et al., 2003; Yin et al., 2006; Gazanchian et al., 2007), and although stomatal density is closely associated with leaf development (Yang et al., 1995), response patterns of cell number and size to water stress depend on the actual period of leaf development (Loesche et al., 1995). At the cellular level, moderate water deficits had opposite effects on cell number and cell size, but more severe deficits reduced both variables (Aguirrezabal et al., 2006; Figs 2, 6). For
youngest leaves under moderate drought might favour leaves compared with young leaves under a given stress closely associated with leaf age, decreasing more in older leaf curliness. In addition, leaf stomatal conductance is subjected to more severe drought were not used because gas exchange measurement, and the plants that were the youngest and most fully expanded leaves were used in traits and soil drought severity. In our experiment, only the youngest and most fully expanded leaves were used in (Fig. 3). The disparity may be due to the age-related leaf drought; this is not consistent with the present results (Fig. 3). This implies that a stomatal density increase under long-term moderate drought may help to maintain the value of \( g_s \) to a certain extent, or even produce an acclimated increase in \( g_s \). Yang et al. (2007) reported that the increase in stomatal density is positively correlated

early wheat leaves, lower stomatal density could also arise because of the limitation imposed by guard cell development under stress conditions (Yin et al., 2006). However, our results indicate that although severe drought might lead to a reduction in stomatal density, an increase is possible under moderate drought conditions, since the response is characteristic of a parabola rather than a linear regression. This pattern of response may also explain why a decrease in leaf area results in an increase in stomatal density under moderate drought, but an inhibition of guard cell division in relation to senescence induced by severe drought can lead to a reduction in the total stomatal number on a given leaf, i.e. stomatal density (Fig. 6). Although our results showed that stomatal density was not significantly associated with leaf area per plant (data not shown), it was negatively correlated with specific leaf area (Fig. 5), indicating that enhanced leaf thickness may produce more guard cells for a given leaf area. Enlarged leaf thickness and the associated increased stomatal density may also be useful in enhancing the plasticity to a certain degree under moderate drought (Galmés et al., 2007).

Meng et al. (1999) reported that net photosynthetic rate (A) had a significant negative correlation with stomatal density due to a marked reduction in A induced by severe drought; this is not consistent with the present results (Fig. 3). The disparity may be due to the age-related leaf traits and soil drought severity. In our experiment, only the youngest and most fully expanded leaves were used in gas exchange measurement, and the plants that were subjected to more severe drought were not used because of leaf curliness. In addition, leaf stomatal conductance is closely associated with leaf age, decreasing more in older leaves compared with young leaves under a given stress (Yang et al., 1995). Thus, compared to severe drought, the youngest leaves under moderate drought might favour more gas exchange, demonstrating an adaptation to environmental stress, and leading to high \( g_s \) and \( A \). On the other hand, moderate water stress always limits leaf \( A \) by both stomatal resistance and carboxylation inhibition (Schulze, 1986; Munné-Bosch et al., 2003). Moreover, stomatal conductance does not always parallel changes in the photosynthetic capacity of tobacco plants (von Caemmerer et al., 2004) and \( S. \) \textit{dimidiatum} (Maherali et al., 2002), depending on the cultivars (Lizana et al., 2006), thus highlighting the complexity in the relationship. However, Zhang et al. (2006) reported that the relationship between stomatal density, and \( g_s \) and \( A \) is positive under limited irrigation conditions, while Galmés et al. (2007) indicated that \( g_s \) is related to stomatal density for a wide range of water status, which is consistent with our results (Fig. 3). The present results indicate that both \( A \) and \( g_s \) were closely associated with stomatal density under different water status, suggesting that stomatal density may also play an important role in CO\(_2\) exchange under drought stress. However, Galmés et al. (2007) indicated that high variability and uncertainty are present among Mediterranean plants in response to changing water status. Thus, this hot and unsettled topic is still worthy of more focus in the future.

Assmann and Wang (2001) reported that the responses of guard cell size and stomatal number to environmental variables clearly depend on a time scale from milliseconds to millions of years. Actually, the physiological mechanisms of stomatal response are very complex and not yet fully understood to date (Sousa et al., 2006; Gudesblat et al., 2007). Short-term responses to humidity are fundamentally similar—that is the typical two-phase stomatal response. When humidity around a leaf is reduced, \( g_s \) typically increases for 5–15 min, and then declines for another 20–75 min, ultimately approaching a steady-state \( g_s \) that is lower than the initial value (Cowan and Farquhar, 1977; Oren et al., 1999; Buckley, 2005). When plant roots are subjected to water stress, ABA (abscisic acid) accumulation may be initiated by a drought-sensing mechanism located in the roots, where it can be exported to leaves (Pei and Kuchitsu, 2005), thus reducing water loss by stomatal regulation (Cominelli et al., 2005; Gudesblat et al., 2007). On the other hand, long-term soil drought can also lead to up-regulation of leaf osmotic pressure and lower water potential around the stomata while osmoregulation promotes greater \( g_s \) under moderate soil drought (Buckley, 2005). In the present study, although a response of \( g_s \) to short-term low humidity was not observed, the relationship between \( g_s \) and stomatal density was positive under long-term drought (Fig. 3). This implies that a stomatal density increase under long-term moderate drought may help to maintain the value of \( g_s \) to a certain extent, or even produce an acclimated increase in \( g_s \). Yang et al. (2007) reported that the increase in stomatal density is positively correlated
with WUE, which is confirmed by our results (Fig. 4). Furthermore, plants with elevated WUE also have a higher $g_s$, implying a positive balance between carbon and water exchange (Figs 3, 4). An increase in WUE with high stomatal density might also indicate a high acclimation capacity to a gradually increasing water deficit, and suggest an evolutionary adaptation to environmental stresses.

It is noted that stomatal density increased with increasing water stress (Fig. 1), and $g_s$ was positively correlated with stomatal density (Fig. 3), but stomatal size decreased with increasing water stress (Fig. 2). This suggests that a greater $g_s$ may appear under water stress concurrent with high stomatal density and small guard cell size. Moreover, small guard cells may cause stomata to remain open under drought to some extent (Spence et al., 1986) or when the effects of abscisic acid are felt (Quarrie and Jones, 1977), indicating that there is greater $g_s$ with a small guard cell size, which seems to be confirmed by our results. However, a parallel increase in $g_s$ and A with stomatal density might not imply greater $g_s$ and A under water stress, because severe drought might cause simultaneous declines in $g_s$, A, as well as stomatal density. Just as $g_s$ is not always closely associated with A (Maherali et al., 2002; von Caemmerer et al., 2004), the relationships of stomatal density and size with gas exchange may be complex, suggesting that some compromises can occur during plant adaptation to varying degrees of water status.

Conclusions

Our study suggests that stomatal density increases with decreasing water potential under moderate water deficit, but declines under severe drought, demonstrating that this grass exhibits a degree of leaf trait plasticity in response to environmental changes. An increase in stomatal density and a decrease in guard cell size may also be closely associated with leaf area development, and cell division under certain degrees of water deficit stress (Fig. 6). That balance may, in addition, determine the direct effect of stomatal on gas exchange, photosynthesis, and water use efficiency. The present findings of high regulation via changing stomatal number and guard cell size induced by water deficit are of great ecophysiological significance, because L. chinensis grows in regions with severe water scarcity during its growing season, and are also of high importance to gain an insight into how plants acclimate to long-term climate change. Improving regulation capacity in stomatal traits by breeding selection and/or genetic methods would enable plants to acclimate to environmental stresses, such as drought.

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