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Diurnal depression in leaf hydraulic conductance at ambient and elevated [CO_2] reveals anisohydric water management in field-grown soybean and possible involvement of aquaporins

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ABSTRACT

Diurnal cycles of photosynthesis and water use in field-grown soybean (*Glycine max*) are tied to light intensity and vapor pressure deficit (VPD). At high mid-day VPD, transpiration rates can lead to a decline in leaf water potential (Ψ_{leaf}) if leaf hydraulic conductance (K_{leaf}) is insufficient to supply water to intercellular airspaces in pace with demand. K_{leaf} is determined by leaf xylem conductivity to water, as well as extra-xylem pathways that are likely mediated by aquaporin water transport proteins. When transpiration demand exceeds the maximum capacity of K_{leaf} to supply water, high tension in the water column can cause cavitation in xylem, and these emboli-blocked xylem vessels reduce water transport and thus lower K_{leaf}. Stomatal conductance typically remains high at mid-day for soybean, suggesting either a mid-day increase in K_{leaf} or that photosynthesis may be maintained at the cost of leaf water status, indicative of an anisohydric water management strategy in soybean. This study examined diurnal fluctuations in K_{leaf} and Ψ_{leaf}, showing a mid-day depression in K_{leaf} in a pattern closely reflecting that of Ψ_{leaf}, indicating that K_{leaf} depression is the result of cavitation in leaf xylem. The diurnal depression of K_{leaf} was not prevented by growth at elevated [CO_2], which lowered stomatal conductance. Diurnal transcription patterns of aquaporin genes showed that a total of 34 genes belonging to 4 aquaporin families were expressed in soybean leaves, of which 22 were differentially expressed between at least two time points. These data suggest that mid-day K_{leaf} depression was driven primarily by cavitation at increasing xylem water tensions, but that aquaporins are also likely involved in diurnal regulation of soybean leaf water status. It is further concluded that because soybean photosynthesis is typically sustained at mid-day, K_{leaf} even at the depressed level was in excess of that needed to sustain a stomatal conductance sufficient to prevent depression of photosynthesis in soybean.

1. Introduction

Leaves must contend with dramatic environmental changes over the course of even a single day. Light and air temperature both tend to peak around the middle of the day, and vapor pressure deficit (VPD) typically peaks with leaf temperature, coinciding with maximum light and air temperature. For plants in temperate climates during the peak growing season, this means that transpiration demand is very high while the potential for maximum light-driven carbon acquisition requires fully open stomata. Maintenance of open stomata is only possible if the leaf interior can remain sufficiently hydrated, maintaining leaf water potential (Ψ_{leaf}), even as high VPD drives rapid evaporation of water from the intercellular air spaces. Two water management strategies have been described in response to high mid-day VPD: isohydric, in which stomatal conductance declines to maintain constant Ψ_{leaf}, or anisohydric, in which stomata remain open at the cost of a drop in Ψ_{leaf}. Thus, the anisohydric strategy allows a more variable Ψ_{leaf} in order to maintain open stomata open and higher photosynthetic rates for longer periods, even as leaf water potential declines. This strategy allows anisohydric plants to attain higher carbon gain than isohydric plants when water is

Abbreviations: K_{leaf}, leaf hydraulic conductance; Ψ_{leaf}, leaf water potential; *A*, photosynthesis; PPFD, photosynthetic photon flux density; VPD, vapor pressure deficit.

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abundant and even when moderately limiting (Sade et al., 2012). However, under conditions of intense drought, this risk-taking behavior could lead to a persistent collapse in carbon gain that the more conservative behavior of isohydric plants would avoid.

Leaf hydraulic conductance (K_{leaf}) determines the capacity for water transport through the leaf, and leaves are often the hydraulic bottleneck in the whole-plant transpiration stream (Sack and Holbrook, 2006). K_{leaf} can be dynamic and is determined both by xylem conductivity as well as the resistance to water transport in the leaf mesophyll. If g_s does not decrease, high VPD creates a steep water potential gradient through the leaf when K_{leaf} is insufficient to match evaporative demand. As Ψ_{leaf} decreases, resulting high tensions in the water column can cause cavitation, allowing an air embolism to fill the vessel. Cavitation renders the vessel temporarily unusable for water transport, decreasing K_{leaf} (Tyree and Sperry, 1989). Although it was originally thought that emboli could only be refilled under positive root pressure, after transpiration demand abates, embolism refilling under negative xylem pressure has now been demonstrated in several species (Salleo, 1996; Canny, 1997; Hacke and Sperry, 2003; Zwieniecki and Holbrook, 2009). However, negative-pressure refilling must come at an energetic cost; both the release of osmotically active solutes and production of transport proteins may be involved in the negative-pressure embolism repair mechanism, although the mechanism of negative-pressure refilling is uncertain (Alves et al., 2004; Salleo et al., 2004, 2009; Secchi and Zwieniecki, 2011).

K_{leaf} is known to decline over the course of the day in several species, with peak K_{leaf} ranging from early to late morning then decreasing throughout the afternoon (Brodribb and Holbrook, 2004; Lo Gullo et al., 2005; Yang et al., 2012), and mid-day decreases of K_{leaf} in other species are also expected based on xylem vulnerability curves and *in situ* mid-day Ψ_{leaf} values (Woodruff et al., 2007; Bucci et al., 2012). These diurnal depressions in conductance are interpreted to be the result of cavitation in the xylem at high tensions (McCully et al., 1998; Brodribb and Holbrook, 2004; Woodruff et al., 2007; Bucci et al., 2012). Light environment and circadian rhythms may also play a role in diurnal fluctuations of K_{leaf} (Sack et al., 2002; Tyree et al., 2005), and light-driven diurnal cycles of K_{leaf} have been linked to PIP aquaporin expression and activity (Nardini et al., 2005; Cochard et al., 2007; Hachez et al., 2008). Diurnal aquaporin expression cycles also correlated with cycles of root hydraulic conductance in *Vitis vinifera* and *Lotus japonicus* (Clarkson et al., 2000; Moshelion et al., 2002; Siefritz et al., 2004; Vandeleur et al., 2009). Increased expression or activation of aquaporins likely controls the bundle sheath- or mesophyll-based component of K_{leaf} (Clarkson et al., 2000; Moshelion et al., 2002; Sack et al., 2004; Nardini et al., 2005; Hachez et al., 2008; Chaumont and Tyerman, 2014), and they also may play a role in vessel refilling following cavitation (Secchi and Zwieniecki, 2011). As PIPs primarily localize to the plasma membrane and are known to increase plasma membrane water permeability (Kaldenhoff and Fischer, 2006), this aquaporin subfamily likely has the most direct control on the transpiration stream, but aquaporins from other subfamilies may play a role in regulating cell water status and in embolism refill mechanisms. The contribution of aquaporins to overall K_{leaf} likely varies among species, but chemical inhibition of aquaporin function reduces rosette hydraulic conductance in *Arabidopsis* by 21–23% (Postaire et al., 2010), and in soybean, chemical aquaporin inhibitors reduced the transpiration rate by 42–82% (Sadok and Sinclair, 2010).

Diurnal patterns of K_{leaf} have yet to be examined in any herbaceous crop species such as soybean (*Glycine max*), despite this crop covering over 100 million hectares worldwide. In field-grown soybean, photosynthesis (A) typically peaks at mid-day, closely following the pattern of photosynthetic photon flux density (PPFD) (Rogers et al., 2004; Bernacchi et al., 2005). Despite high leaf temperatures, and thus VPD, throughout most of the growing season in soybean-growing regions, soybean stomata typically remain open during the middle of the day, thereby maximizing carbon gain. This suggests either compensatory diurnal increases in K_{leaf} or that soybean is an anisohydric regulator of leaf water status, thereby leaving K_{leaf} highly vulnerable to cavitation at mid-day and through the afternoon especially on warm, sunny days.

Elevated [CO_2] decreases stomatal conductance and transpiration on a leaf-area basis in virtually all plant species (Ainsworth and Long, 2005), and in field-grown soybean elevated [CO_2] caused seasonal transpiration to decrease between 9% and 16%, depending upon inter-annual variation in weather conditions (Bernacchi et al., 2007). Reduced transpiration demand decreases hydrostatic tension in the water column, reducing the risk of cavitation. Growth at elevated [CO_2] has previously been shown to not affect maximum K_{leaf} in soybean (Locke et al., 2013). Thus, because water supply does not change at elevated [CO_2] while Ψ_{leaf} is less likely to decrease during transpiration due to lower stomatal conductance, we predicted that a mid-day K_{leaf} decrease due to cavitation would be smaller for plants grown at elevated [CO_2]. This study examined the fluctuation of soybean leaf water status and K_{leaf} at ambient and elevated [CO_2] over the course of the day to test the hypothesis that K_{leaf} does not increase with increasing VPD and limits soybean photosynthesis on a daily basis.

2. Materials and methods

2.1. Plant material and growth conditions

Soybean cultivar 93B15 (Pioneer Hi-Bred, Johnston, IA) was planted on 27 May 2010 at the SoyFACE research facility in Champaign, Illinois. This field site is managed according to standard agricultural practices in central Illinois, including yearly rotation with *Z. mays* (corn) and no irrigation. CO_2 was fumigated in open-air, 20 m diameter octagonal plots, with a computer-controlled target elevated [CO_2] of 585 ppm. Elevated [CO_2] was within 10% of the target 75% of the time. A detailed description of the SoyFACE fumigation procedure has been published previously (Rogers et al., 2004). CO_2 fumigation began 13 days after planting and continued throughout the growing season, so soybeans experienced their assigned CO_2 treatment for the almost their entire life cycle.

2.2. Diurnal measurements

Two diurnal sets of K_{leaf} and Ψ_{leaf} measurements were made in 2010, the first between 10 July and 22 July and the second between 14 August and 24 August. Leaves were sampled in the field at four time points: 8:00, 11:00, 14:00, and 17:00. Three leaves (subsamples) were sampled from each plot (ambient or elevated [CO_2]) at each time point. Due to throughput limitations with K_{leaf}, measurements could only be made for one SoyFACE block (one ambient CO_2 plot and one elevated CO_2 plot) per day. Thus, each diurnal data set contains measurements taken on four days. This design allowed environmental variation among sampling days to be accounted for with the block term in the statistical model, distributed equally across treatments and time points.

2.3. Leaf hydraulic conductance

K_{leaf} was measured with the evaporative flux method, in which the flow rate of water through the leaf is measured while the leaves are placed in an environment favorable to transpiration (Sack et al., 2002; Locke et al., 2013). Leaves in the field were cut at

the base of the petiole and immediately placed into a tube of distilled water, and returned to the lab, where the petioles were immediately re-cut another 2–3 cm under water. It was recently reported that leaf excision under negative pressures, even under water, can introduce air bubbles into the xylem, which may bias diurnal measurements of conductivity (Wheeler et al., 2013). In this experiment, leaves were transported from the field to the lab in a closed cooler, which took at least 30 min. The evaporative flux apparatus only allowed measurement of four leaves at a time, and the remaining two leaves were stored in the cooler for up to three hours before the final re-cutting of the petiole under water just prior to K_{leaf} measurement. This time in a dark, closed environment would allow xylem tensions to relax prior to re-cutting the petiole in the lab. There was no correlation between leaf storage time and K_{leaf} (data not shown), providing evidence that reported K_{leaf} values reflected the field condition and were not biased by the sampling artifact revealed by Wheeler et al. (2013). To measure flow rate, petioles were inserted into tubing (Tygon R-3603, Saint-Groban Performance Plastics Corporation, Paris, France) connected to a cylinder of degassed, distilled water on a high-precision balance (XS 250, Mettler Toledo, Columbus, OH). A tight seal with the tubing was ensured by filling crevices in the petiole with petroleum jelly and then wrapping the petiole in Parafilm (Pechiney Plastic Packaging Company, Chicago, IL). Once connected to the balance, the leaf was placed under a 750 watt halogen lamp. A dish of water was placed between the lamp and the leaf to absorb infrared radiation, resulting in approximately 700 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetically active radiation (PAR) at leaf level. To increase throughput, four balances were connected to a single datalogger (CR1000, Campbell Scientific, Inc., Logan, UT) through a serial I/O interface (SDM-SIO4, Campbell Scientific, Inc., Logan, UT) and monitored in real-time on a single computer screen. The mass of water on the balance was electronically recorded every 30 s. Water flow through the leaves was allowed to stabilize for at least 30 min, after which leaf temperature was measured with an infrared thermometer (Fluke 574, Fluke Corporation, Everett, Washington), and final leaf water potential (Ψ_{final}) was measured with thermocouple psychrometers. Four psychrometer chambers were used per leaf, and three 1.2 cm disks were cut from the leaf for each chamber. Leaf area was calculated using leaf photographs and ImageJ (NIH, http://rsbweb.nih.gov/ij/), and K_{leaf} was calculated as:

$$K_{leaf} = \frac{J}{(\Psi_{leaf} \times a_{leaf})}$$

where J is the flow rate and a_{leaf} is leaf area, and K_{leaf} was then normalized for leaf temperature according to Yang and Tyree, 1993.

2.4. Leaf water potential

Leaf water potential was measured using thermocouple psychrometers (Wescor C-30, Wescor, Inc., Logan, UT). Three 1.2 cm leaf disks were cut from a single trifoliate leaf and within 15 s sealed together into a stainless steel chamber housing the thermocouple. The temperature and humidity inside the thermocouple chambers was allowed to equilibrate in a controlled-temperature room for three hours prior to measurement, and then water potential was recorded using a datalogger (Campbell CF-1000, Campbell Scientific, Logan, UT). For determinations of Ψ_{leaf} under field conditions, one thermocouple chamber was used per leaf, and three leaves were measured per plot. For Ψ_{final}, four thermocouple chambers were used for each leaf, and these values were averaged to calculate K_{leaf}.

2.5. Meteorological data

Temperature and humidity data for SoyFACE were collected hourly at the nearby Surface Radiation site, approximately 8 miles away, as described in detail by Vanloocke et al. (2010). Saturation vapor pressure was calculated according to the equation:

$$e_s(t) = a \times e^{\left(\frac{bT}{T+c}\right)}$$

where the constants a,b, and c are 0.611 kPa, 17.502, and 240.97 °C (Campbell and Norman, 1998). Actual vapor pressure was calculated as:

$$e_a = RH \times e_s(t),$$

and vapor pressure deficit is then calculated as:

$$\mathrm{VPD} = (e_s(t) - e_a).$$

2.6. Statistical analysis of physiological data

The field experiment was arranged as a randomized complete block design, with one ambient CO_2 and one elevated CO_2 ring in each of four spatially separated blocks. For both K_{leaf} and Ψ_{leaf} analyses, these blocks were considered replicates ($n = 4$), and individual leaves measured from each block (1 to 3 per treatment per time point) were treated as subsamples. Data were analyzed by ANOVA using SAS software (SAS Institute, Cary, NC). Time of day and CO_2 treatment were the main effects, while block was treated as random and as the subject of repeated measures. Time of day significance was determined using the repeated measures ANOVA p-value. Overall [CO_2] effect was determined using the repeated measures ANOVA p-value, but p-values for pairwise comparisons between [CO_2] treatments for individual time points were considered as well. specify when pairwise comparison p-values are being reported instead of the default, main-effect ANOVA p-values. The data for each month were analyzed separately.

2.7. RNA extraction and sequencing

Three whole leaflets, each from a different plant, were sampled in ambient [CO_2] plots and flash-frozen in liquid nitrogen at each time point during the August diurnal measurements. Thus, mRNA from this tissue represents a snapshot of transcription at the same moment leaves were excised for K_{leaf} measurement. These three leaflets were combined to create one sample per plot. Leaf tissue was stored at −80° C until RNA extraction. Total RNA was extracted from the leaves with a phenol/chloroform method developed specifically for field-grown soybean (Bilgin et al., 2009). Total RNA was treated with the DNA-free kit (Ambion, Inc. Austin, TX). cDNA libraries were constructed with the TruSeq RNA Sample Preparation kit (Illumina, Inc. San Diego, CA). 16 samples (four biological replicates at four time points) were randomly assigned to two flow cells and sequenced on the HiSeq2000 (Illumina, Inc. San Diego, CA).

2.8. Sequence alignment and processing

Reads were filtered and trimmed for quality and mapped to the soybean genome (Schmutz et al., 2010) with the TopHat2 alignment program, version 2.0.7 (Kim et al., 2013). Once mapped, reads per gene were counted with HTSeq (freeware, www-huber.embl.de/users/anders/HTSeq/).

2.9. Aquaporin gene annotation and differential expression analysis

Aquaporin genes were annotated based on Zhang et al. (Zhang et al., 2013). Two genes, Glyma02g42220 and Glyma18g03330, were previously annotated as PseudoPIP#2 and PseudoPIP#4, due to missing NPA amino acid motifs which are characteristic of aquaporin proteins. Gene searches in Phytozome (www.phytozome.org) for these genes revealed alternate gene models for Glyma02g4220 which contained both NPA motifs and similarity to other PIP2 genes, and this gene was renamed GmPIP2;15 in continuity with Zhang et al. (Zhang et al., 2013).

Differential expression analysis for aquaporin genes was performed with SAS PROC MIXED (SAS Institute, Cary, NC). Read counts for sample *x* were normalized to control variance with the equation:

$$\log\left[\frac{\text{read count}}{\text{gene length}} \times \frac{\text{mean total counts across samples}}{\text{total counts for sample}x}\right].$$

Separate repeated measures ANOVAs were calculated for each aquaporin gene, and *p*-values from each pairwise time contrast were corrected for multiple comparisons. Log 2 fold changes were calculated with reads per kilobase gene length per million base pairs (RPKM) for each differentially expressed pairwise comparison, presented as the fold change in expression for the later time relative to the earlier time.

3. Results

3.1. Soybean K_{leaf} varied throughout the day

K_{leaf} changed significantly over the course of the day in both July ($p = 0.0486$) and August ($p = 0.0033$), showing a midday depression in both months (Fig. 1A and B). The greatest change in K_{leaf} occurred between 8:00 and 11:00, when K_{leaf} decreased by an average of 30% across treatments. In July, K_{leaf} began to recover by 17:00 in both ambient and elevated [CO_2], increasing 25% from 14:00 to 17:00 (Fig. 1A). In August, however, K_{leaf} for plants grown at elevated [CO_2] actually decreased by 26% between 14:00 and 17:00 (Fig. 1B). K_{leaf} differences between time points were very similar in magnitude and opposite in direction to the changes in VPD over the course of the day (Fig. 2).

3.2. Daytime Ψ_{leaf} trajectory mirrored K_{leaf}

Ψ_{leaf} was significantly different among time points in July ($p < 0.0001$) and August ($p < 0.0001$). The greatest change in Ψ_{leaf} was also between 8:00 and 11:00, when it decreased by an average of 0.27 MPa, or 75% (Fig. 1C and D). The early evening recovery of Ψ_{leaf} in July was not as large as the recovery in K_{leaf}, with Ψ_{leaf} increasing by only 7% from 14:00 to 17:00 (Fig. 1D) compared to the 25% increase in K_{leaf}.

3.3. [CO_2] effects on K_{leaf} and Ψ_{leaf} were small

K_{leaf} was statistically different between ambient and elevated [CO_2] plants in August ($p = 0.0220$), which was driven primarily by the 8:00 time point (Fig. 1B), where K_{leaf} for elevated [CO_2] plants was 28% lower than ambient ($p = 0.0216$). Although pairwise comparisons between ambient and elevated [CO_2] were not statistically different at other individual time points, the average K_{leaf} values were lower at elevated [CO_2] at every time point.

In July and August, Ψ_{leaf} was nearly equal at the final two time points in ambient [CO_2], but in August the elevated [CO_2] Ψ_{leaf} decreased 25% from 14:00 to 17:00 (Fig. 1D). However, Ψ_{leaf} was

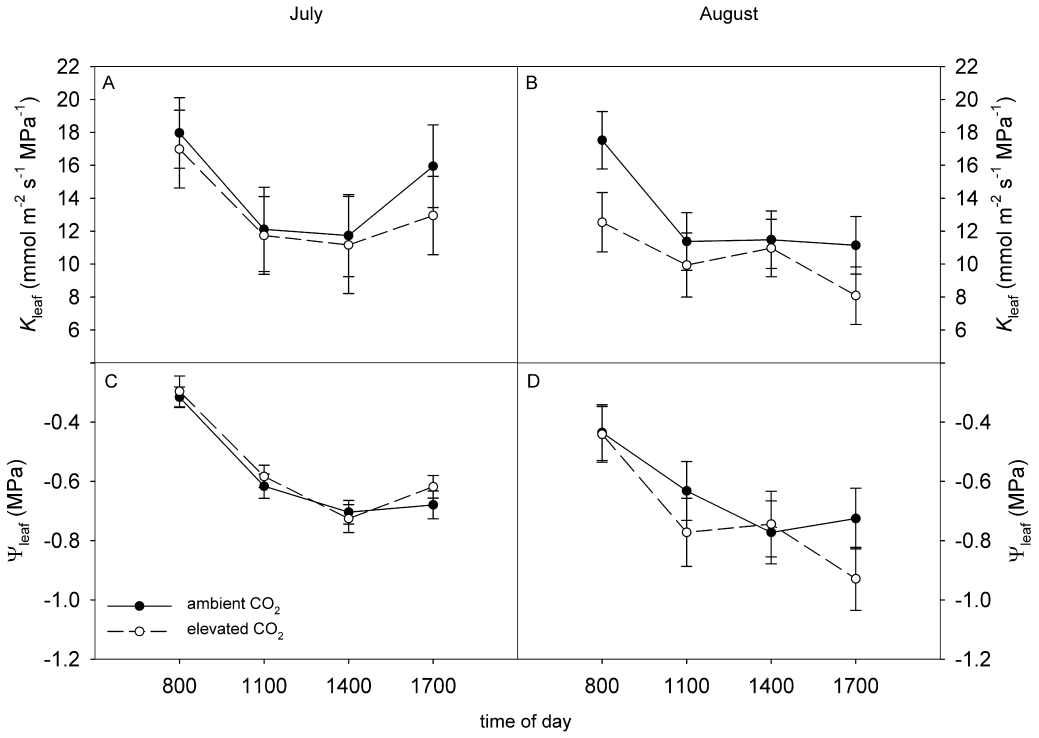

Fig. 1. Diurnal measurements of K_{leaf} (A and B) and Ψ_{leaf} (C and D) for field-grown soybean. Two sets of diurnal measurements were made in 2010; one in July (A and C) and one in August (B and D). Leaves were sampled at four time points: 8:00, 11:00, 14:00 and 17:00, in ambient (closed circles, solid lines) and elevated [CO_2] (open circles, dashed lines). K_{leaf} changed significantly over the course of the day for both July (A) and August (B). Error bars indicate standard error; asterisk denotes a significantly different ($p < 0.05$) pairwise comparison between ambient and elevated [CO_2] for a time point. Each point represents four replicates, and each replicate was comprised of measurements on 1–3 individual leaves (subsamples).

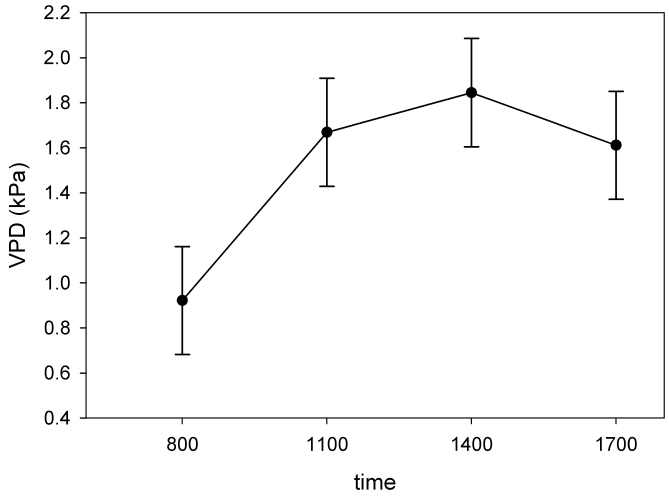

Fig. 2. Atmospheric vapor pressure deficit (VPD) at diurnal sampling time points. Temperature and humidity data were continuously collected at the Surface Radiation site 12.8 km from the SoyFACE field site throughout the growing season. VPD was calculated from the temperature and humidity at each time point. Each VPD value represents the mean ± standard error of the four sampling days during each month.

not significantly different for elevated and ambient [CO_2] plants at any time point in either month.

3.4. Aquaporin gene expression in soybean leaves

In a RNA-seq experiment investigating the transcription of aquaporin genes at four time points over the course of the day, a total of 34 aquaporin genes were expressed in soybean leaves, including 14 PIPs, 10 TIPs, 5 NIPs, and 5 SIPs (Fig. 3). Of these, 22 were differentially expressed ($p < 0.05$) between at least two time points: 9 PIPs, 4 TIPs, 4 NIPs, and 5 SIPs (Fig. 3). There were no consistent patterns of up- or down-regulation over the course of the day across any subfamily, but some trends were apparent for specific individual genes (Fig. 4).

Transcription of *GmPIP1;8* and *GmPIP2;14* progressively decreased over the course of the day. PseudoPIP#4 transcription decreased at every time point in comparison to 8:00, but transcription was stable across the later time points. In contrast, *GmPIP2;7* transcription increased from 8:00 to 11:00 and then was stable for the rest of the day.

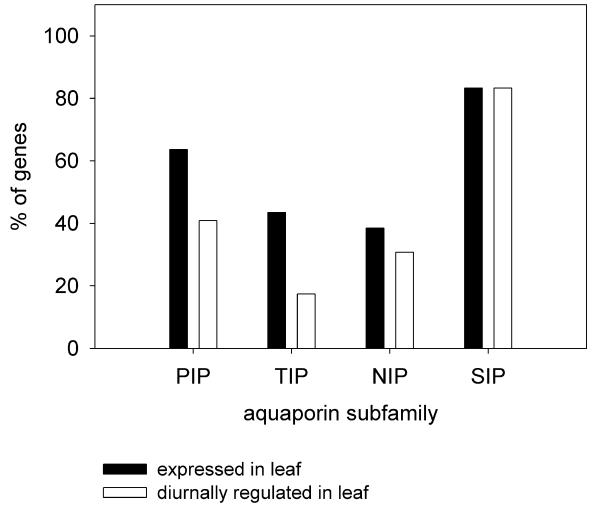

Fig. 3. Percentage of soybean aquaporin genes by subfamily that are expressed in leaves (black bars) and percentage of aquaporin genes by subfamily that are differentially regulated between time points (open bars).

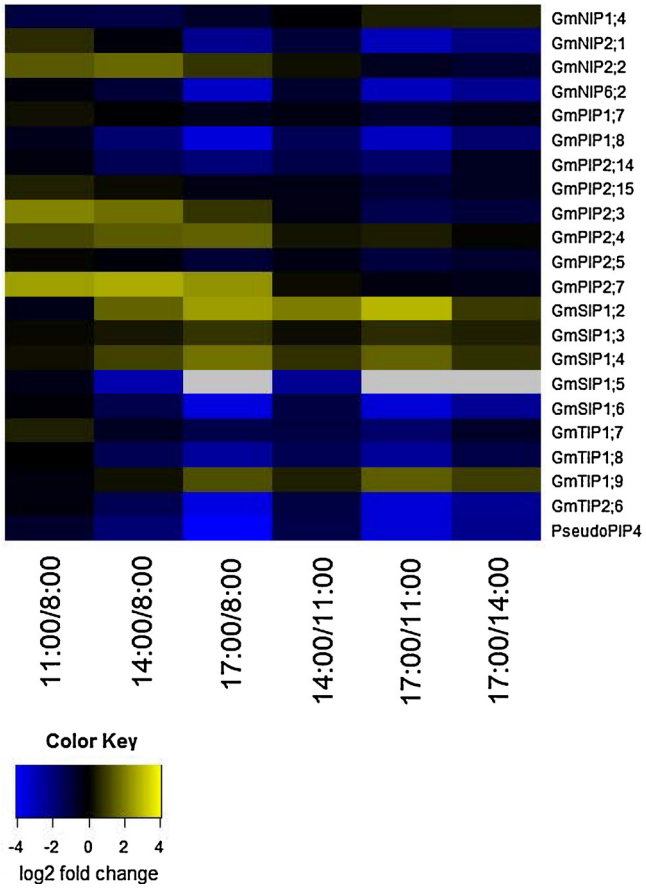

Fig. 4. Gene expression analysis of soybean aquaporin genes for pair-wise comparisons between sampling points. 11:00/8:00 indicates the log fold change in expression for 11:00 compared to 8:00. Leaf tissue was collected from plants grown in ambient [CO_2] during all August measurement points. Only those aquaporin genes with differential diurnal transcription are shown. Color scale indicates log 2 fold change in gene transcription from the later time relative to the earlier time, from −4.0 (brightest blue) to 4.0 (brightest yellow). Non-colored spaces for *Gm*SIP1;*5* indicate that no expression of this gene was detected at 17:00.

Among the TIPs, *GmTIP1;8* transcription progressively decreased over the course of the day. Transcription of *GmPIP1;7* and *GmPIP2;6* only decreased at 17:00 relative to 8:00. Transcription of *GmPIP1;7* actually increased from 11:00 to 14:00, but decreased at 17:00 relative to 8:00 and 11:00. *GmTIP1;9* transcription was stable from 8:00 to 14:00, but it then increased at 17:00. *GmPIP2;3*, *GmPIP2;4*, and *GmPIP2;5* showed mostly stable transcription patterns, each with differential expression at only one time point.

The NIP subfamily shows the most consistent transcriptional changes, with *GmNIP1;4*, *GmNIP2;1*, and *GmNIP6;2* transcription all decreasing over the course of the day. For *GmNIP1;4*, this decrease is slight and only significant at 11:00 and 14:00, while by 17:00, gene expression was similar to its 8:00 level. Both *GmNIP2;1* and *GmNIP6;2* gene expression decreased consistently over the course of the day. *GmNIP2;2* had a transcriptional pattern opposite to *GmNIP1;4*, with increased expression at 11:00 and 14:00 relative to 8:00, while expression at 17:00 was similar to that at 8:00.

Three SIP genes (*GmSIP1;2*, *GmSIP1;3*, and *GmSIP1;4*) were expressed more at 17:00 relative to all other times. For *Gm*SIP1;6,

gene expression progressively decreased over the course of the day, while *GmSIP1;5* expression was only lower at 14:00 relative to 8:00.

Genes for which transcription was detected but that were not differentially expressed between any time points include *GmNIP1;3*, *GmPIP1;4*, *GmPIP1;5*, *GmPIP2;10*, *GmPIP2;13*, *GmPIP2;9*, *GmTIP1;4*, *GmTIP1;6*, *GmTIP2;2*, *GmTIP2;3*, *GmTIP2;5*, and *GmTIP4;1*.

4. Discussion

The decrease in K_{leaf} (Fig. 1A and B) and Ψ_{leaf} (Fig. 1C and D) between early and late morning indicates anisohydric hydraulic regulation in soybean, consistent with a previous report (Allen et al., 1994). The steep decrease in K_{leaf} in conjunction with decreasing Ψ_{leaf} and increasing VPD (Fig. 2) suggests that cavitation, which reduces xylem conductivity, drives the late morning drop in K_{leaf} in soybean. *Simarouba glauca*, a tropical evergreen tree, responded similarly to mid-day evaporative stress, but K_{leaf} recovery began by early afternoon in *S. glauca* (Brodribb and Holbrook, 2004), whereas in the current soybean study, K_{leaf} recovery was not apparent until late afternoon (Fig. 1A) and was minimal (Fig. 1B).

Aquaporin proteins are known to influence K_{leaf} in several species (Cochard et al., 2007; Lovisolo et al., 2007; Hachez et al., 2008; Heinen et al., 2009; Postaire et al., 2010; Sadok and Sinclair, 2010). These proteins mediate membrane permeability to water and certain other small molecules, and thus can be a major component of the bundle sheath and mesophyll hydraulic conductance. Diurnal hydraulic conductivity fluctuations have been studied more in roots than in leaves, and some *Arabidopsis thaliana*, *L. japonicus*, and *V. vinifera* PIP aquaporins have diurnal expression cycles in roots correlating with transpiration (Clarkson et al., 2000; Vandeleur et al., 2009; Takase et al., 2011). Rapid, light-dependent aquaporin transcriptional regulation correlated with K_{leaf} in *Juglans regia* (Cochard et al., 2007 Baaziz et al., 2012). If the transcription of aquaporins were directly involved in the diurnal control of K_{leaf} observed in soybean (Fig. 1A and B) it would be anticipated that transcript levels would decrease over the course of the day as K_{leaf} decreases, and two soybean PIPs in this study, *GmPIP1;8* and *GmPIP2;14*, had this transcription pattern. The highly similar transcription patterns of *GmPIP1;8* and *GmPIP2;14* could be indicative of a positive PIP1/PIP2 heteromeric interaction, as has been observed with *A. thaliana* and *Zea mays* PIPs (Fetter et al., 2004; Vandeleur et al., 2009), although no specific PIP1/PIP2 pairings have been studied in soybean to date. *Nicotiana tabacum* AQP1, which has high sequence similarity to *GmPIP2;15* differentially regulated in this study (Fig. 4), has demonstrated CO_2 transport capabilities, so differential PIP regulation could also influence photosynthesis via mesophyll conductance to CO_2 rather than by modulating hydraulic conductance (Flexas et al., 2006; Uehlein et al., 2008). These aquaporin transcript data, in combination with the physiological data presented above, suggest that aquaporins likely play a role in diurnal regulation of leaf water status, especially since of those aquaporins expressed in the leaf, a large percentage were differentially transcribed over the course of the day (Fig. 3). However, a more thorough functional analysis of aquaporin protein levels and activity would be necessary to draw specific conclusions regarding the contribution of individual aquaporins to the observed diurnal changes in soybean K_{leaf}. Furthermore, the transcript data represents a snapshot of what was happening in the field at the moment of leaf excision. While these are the same conditions that determined K_{leaf} as measured in the laboratory, we cannot be certain that these were the aquaporin transcript levels during K_{leaf} measurement.

TIPs, NIPs, and SIPs in other plant species may have low or no water permeability, but may instead transport nitrogenous compounds, ions, sugars (Wallace et al., 2002; Ishikawa et al., 2005; Kaldenhoff and Fischer, 2006). Thus, while differential regulation of aquaporins in these categories could affect diurnal leaf water status via osmoregulation, they are unlikely to have a direct impact on K_{leaf} though membrane water transport. While differential transcription is certainly a mechanism of aquaporin regulation, sometimes on a time scale of hours (Clarkson et al., 2000; Martre et al., 2002; Moshelion et al., 2002; Siefritz et al., 2004; Cochard et al., 2007), they are also subject to post-translational regulation, including phosphorylation, pH, and Ca^{2+} (Chaumont et al., 2005), so the mechanisms by which aquaporins affect soybean K_{leaf} merit further investigation.

We have previously reported that soybean K_{leaf} does not acclimate to growth at elevated [CO_2], despite decreased stomatal conductance and transpiration (Locke et al., 2013). In the earlier study, leaves were sampled before sunrise, which gives maximum K_{leaf} for fully hydrated, non-transpiring leaves. Although stomatal conductance and therefore transpiration demand are lower at elevated [CO_2], there was no apparent difference in daytime K_{leaf} regulation to balance the different water transport needs for elevated and ambient [CO_2] plants. This interpretation is supported by the large difference between [CO_2] treatments at 8:00, when VPD is still low, and the similarity of Ψ_{leaf} for both [CO_2] treatments at this time point predicts no differential tension between the water columns that could drive greater cavitation in xylem of elevated [CO_2]-grown soybean. This, coupled with the absence of a [CO_2] effect on K_{leaf} for leaves sampled pre-sunrise (Locke et al., 2013), suggests that the slightly lower K_{leaf} for elevated [CO_2] plants in this experiment are more likely related to aquaporin-dependent water transport rather than leaf structural differences. Further investigation of aquaporin expression and function at different [CO_2] would illuminate these differences.

Because the decrease in K_{leaf} observed in this study occurred in conjunction with similar decreases in Ψ_{leaf}, it is likely that K_{leaf} depression was driven primarily by xylem cavitation rather than an aquaporin-mediated decrease in hydraulic permeability. Diurnal fluctuations in K_{leaf} that have been linked to aquaporins suggest increasing hydraulic permeability in response to light (Sack et al., 2002; Nardini et al., 2005; Tyree et al., 2005; Cochard et al., 2007), and a diurnal K_{leaf} response dominated by aquaporins would thus be predicted to peak with high mid-day light. Aquaporins may contribute to evening and/or overnight restoration of K_{leaf} via embolism refilling (Secchi and Zwieniecki, 2011). Soybean K_{leaf} decreased 30% by 11:00 at Ψ_{leaf} of only −0.6 to −0.7 MPa, values typically not low enough to indicate severe leaf water stress. Soybean leaf xylem is thus more vulnerable to cavitation than many other species; most species for which xylem vulnerability has been measured only lose 30% of leaf and stem xylem conductivity at Ψ_{leaf} below −1 MPa, although the majority of these studies have been with trees, and stem xylem of the herbaceous *Chenopodium album* is similarly vulnerable to that of soybean (Cochard et al., 1992; Sperry and Sullivan, 1992; Tyree et al., 1994; Alder et al., 1996; Mencuccini and Comstock, 1997; Sperry and Ikeda, 1997; Kocacinar and Sage, 2003; Hukin et al., 2005; Johnson et al., 2011). However, given that the mid-day K_{leaf} decrease observed over several days in this study, if typical, is not severe enough to hydraulically limit mid-day photosynthesis or g_s (Rogers et al., 2004; Leakey et al., 2006), then the ratio of maximum K_{leaf} to photosynthetic capacity and g_s in must be exceptionally high in soybean. However, variation in K_{leaf} among soybean cultivars suggests that mid-day K_{leaf} depression has the potential to limit photosynthesis in some genotypes (Sinclair et al., 2008). Further investigation of the mechanisms underlying mid-day K_{leaf} decrease, and subsequent refilling, could aid efforts to optimize soybean water use efficiency. Anisohydric regulation of water use confers the advantage of maximizing carbon acquisition for

photosynthesis throughout the day, but carries the risk of excessive water loss and energy spent to refill embolized vessels.

5. Conclusions

Soybean maintains high mid-day photosynthesis by keeping stomata open at the cost of a drop in leaf water potential. This study demonstrated that leaf hydraulic conductance experienced a large mid-day depression in conjunction with Ψ_{leaf} in field-grown soybean, reflective of anisohydric regulation of leaf water status. Over half of the soybean aquaporin genes found to be expressed in leaves were differentially transcribed over the course of the day, suggesting a role for these proteins in maintaining leaf water balance and modulating K_{leaf} on a diurnal basis. While it is unlikely that soybean photosynthesis is regularly limited by K_{leaf}, this diurnal decline in K_{leaf} likely renders the leaf more vulnerable during conditions of high stress resulting in a very large transpiration demand.

6. Authors' contributions

AML participated in the design of the experiment, performed leaf hydraulic conductance and leaf water potential measurements and analyzed these data, analyzed diurnal vapor pressure deficit data, collected soybean leaf tissue, and analyzed RNA-seq data for aquaporin gene expression. DRO participated in the design of the experiment and in the analysis and interpretation of the results, and helped draft the manuscript. Both authors read and approved the final manuscript.

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