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RESEARCH PAPER

# Transpiration response of 'slow-wilting' and commercial soybean (*Glycine max* (L.) Merr.) genotypes to three aquaporin inhibitors

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## Abstract

**The slow-wilting soybean [*Glycine max* (L.) Merr.] genotype, PI 416937, exhibits a limiting leaf hydraulic conductance for transpiration rate (TR) under high vapour pressure deficit (*VPD*). This genotype has a constant TR at *VPD* greater than 2 kPa, which may be responsible for its drought tolerance as a result of soil water conservation. However, the exact source of the hydraulic limitation between symplastic and apoplastic water flow in the leaf under high *VPD* conditions are not known for PI 416937. A comparison was made in the TR response to aquaporin (AQP) inhibitors between PI 416937 and N01-11136, a commercial genotype that has a linear TR response to *VPD* in the 1–3.5 kPa range. Three AQP inhibitors were tested: cycloheximide (CHX, a *de novo* synthesis inhibitor), $HgCl_2$, and $AgNO_3$. Dose–response curves for the decrease in TR following exposure to each inhibitor were developed. Decreases in TR of N01-11136 following treatment with inhibitors were up to 60% for CHX, 82% for $HgCl_2$, and 42% for $AgNO_3$. These results indicate that the symplastic pathway terminating in the guard cells of these soybean leaves may be at least as important as the apoplastic pathway for water flow in the leaf under high *VPD*. While the decrease in TR for PI 416937 was similar to that of N01-11136 following exposure to CHX and $HgCl_2$, TR of PI 416937 was insensitive to $AgNO_3$ exposure. These results indicate the possibility of a lack of a Ag-sensitive leaf AQP population in the slow-wilting line, PI 416937, and the presence of such a population in the commercial line, N01-11136.**

**Key words:** Aquaporin, hydraulic flow, soybean, transpiration.

## Introduction

Leaves are the major site of plant water loss via transpiration. To protect plants from excessive water loss, natural selection has resulted in limitations in liquid water flow within leaves as part of the whole-plant hydraulic system (Sack *et al.*, 2004). Across species, the leaf hydraulic limitation is thought to represent about 30% of the whole-plant resistance to water flow, and, in some cases, can be as high as 80–98% (Sack *et al.*, 2003; Sack and Holbrook, 2006). However, many aspects of the leaf hydraulic system and its connection with plant transpiration rate (TR) remain poorly understood and are currently a matter of extensive research (Sack *et al.*, 2003, 2004; Cochard *et al.*, 2004, 2007; Tyree *et al.*, 2005; Hachez *et al.*, 2008; Ye *et al.*, 2008). Hydraulic resistances to water flow inside the leaf have been linked to environmental variables such as temperature and light (Sack *et al.*, 2004; Cochard *et al.*, 2007). Recently, Levin *et al.* (2007) showed that leaf hydraulic conductance of intact plants of *Arabidopsis thaliana* was higher under high relative humidity (77%) when compared to those measured under low relative humidity (17%), but this response was not isolated from the possibility of hydraulic or chemical signals from the roots.

The soybean genotype PI 416937 expresses a slow-wilting phenotype under water-deficit conditions in the field (Sloane *et al.*, 1990), which may be a result of restricted water use resulting in soil water conservation. This slow-wilting genotype was reported by Fletcher *et al.* (2007) as having no further increase in TR once a *VPD* threshold of about 2 kPa was exceeded. In addition, phenotyping of commercial and recombinant inbred line populations that had PI

416937 in their pedigree resulted in a large genetic variability in TR response to *VPD* (Sadok and Sinclair, 2009a, b). Such variability indicated a complex inheritance for the trait and it was concluded that there may be more than one mechanism controlling the TR limitation trait associated with *VPD*.

The results of Sinclair *et al.* (2008) indicated that the source of the maximum TR response in PI 416937 was associated with a limited hydraulic conductance for water flow from the leaf xylem into the guard cells, which was not observed in two other genotypes studied. One possibility to explain these observations is a lower symplastic conductance (i.e. possibly aquaporin [AQP]-mediated water transport) in the leaf hydraulic pathway of PI 416937 as compared to the other genotypes. Although it is still unclear whether water moves principally apoplastically or symplastically in the leaf (Sack and Holbrook, 2006; Heinen *et al.*, 2009), increasing evidence indicates that the symplastic, AQP-mediated path leading into the guard cells is important, on the basis of biophysical (Sack *et al.*, 2004; Ye *et al.*, 2008) and chemical (e.g. AQP gating by inhibitors; Nardini *et al.*, 2005; Cochard *et al.*, 2007) experiments.

In this study, based on a chemical approach, it is investigated whether symplastic differences in water flow from the xylem leading into the guard cells of two soybean genotypes was associated with differences in TR response to high *VPD* conditions. The slow-wilting genotype (PI 416937) was compared with genotype (N01-11136) with a linear increase in TR over the entire *VPD* range from 1–3.5 kPa. The effect on TR in response to AQP inhibitors under high *VPD* was measured on de-rooted plants. The approach using de-rooted plants differs from previous investigations using leaf protoplasts (Morillon and Chrispeels, 2001; Volkov *et al.*, 2007), leaf tissues/discs (Terashima and Ono, 2002), or single leaves (Cochard *et al.*, 2007). This use of whole, de-rooted plants may be important because leaf AQP activity may differ *in planta* from conditions prevailing in protoplasts, or vary for leaves depending on the location of the sampled tissue (Volkov *et al.*, 2007; Hachez *et al.*, 2008). In addition, the removal of roots nullifies the possible confounding influence of root characteristics, which may occur in whole-plant studies (Voicu and Zwiazek, 2004; Levin *et al.*, 2007).

Dose–response curves were constructed for the TR response to exposure to four concentrations of three protein inhibitors that possibly have differing influences on AQP families and regulation processes. This approach contrasts with a common approach based just on the use of mercurials that are known to have a large array of toxic side-effects in addition to being ineffective against certain AQPs. Recent reviews highlighted the need for using an array of AQP inhibitors besides mercurials (Kaldenhoff *et al.*, 2008; Maurel *et al.*, 2008).

Results are presented from experiments using cycloheximide (CHX), which specifically targets the inhibition of the *de novo* synthesis process and two metallic ions, mercury ($HgCl_2$) and silver ($AgNO_3$). Cycloheximide is known to inhibit peptide initiation and extension (O'Brig *et al.*, 1971) and has been previously used on plants for water transport studies (Moshelion *et al.*, 2002; Voicu and Zwiazek, 2004; Cochard *et al.*, 2007). For instance, on walnut leaves, a 100 μM CHX treatment reduced leaf hydraulic conductance by about 65% and this decrease was attributed to an inhibited AQP (Cochard *et al.*, 2007). Both silver (Ag) and mercury (Hg) react with the sulphydryl group of a cysteine. However, the specific interaction between $Ag^+$ and a histidine (in addition to cysteine) and the non-reversibility of the $Ag^+$ effect by mercaptoethanol strongly indicate different inhibition modes between Ag and Hg (Niemietz and Tyerman, 2002). In contrast to CHX and Hg, there have only been a few studies on the effects of Ag on water transport in plants.

## Materials and methods

### *Plant material*

Soybean line PI 416937 (maturity group VI) is a plant introduction from Japan with unknown parentage (Pantalone *et al.*, 1999; Carter *et al.*, 2003). PI 416937 was identified as a slow-wilting genotype in the field (Sloane *et al.*, 1990; Hudak and Patterson, 1995; King *et al.*, 2009). The second line, N01-11136 (maturity group VII) is a new cultivar (developed by T Carter, ARS-USDA, Raleigh, NC) that has PI 416937 in its pedigree. These genotypes were selected based on differences in their TR response to *VPD* under well-watered greenhouse conditions. In the 0.8–3.2 kPa *VPD* range, TR of PI 416937 reaches a maximum value at a *VPD* of about 2 kPa, and maintains a constant TR as *VPD* is increased further (Fletcher *et al.*, 2007; Sinclair *et al.*, 2008). By contrast, the TR response to *VPD* of genotype N01-11136 showed a continuous linear increase in TR over the same *VPD* range (Sadok and Sinclair, 2009a).

Seeds were sown in pots filled with 1.5–3 kg of composted garden soil (Miracle-Gro lawn products, Inc., Marysville, OH) containing slow-release fertilizer (1.5 g N $kg^{-1}$, 0.2 g P $kg^{-1}$, 0.8 g K $kg^{-1}$). Three to four seeds inoculated with *Bradyrhyzobium japonicum* (Nitragin, Inc., Brookfield, WI) were sown in each pot. The plants were grown in a greenhouse with the temperature regulated for a minimum temperature of 20 °C and maximum temperature of 33 °C. Pots were watered every 1–2 d. Seven to 15 d after sowing, each pot was thinned to one plant.

Plants were grown for approximately 4 weeks to vegetative stages ranging from V2 to V3 (2–3 unfolded trifoliolate leaves, respectively). At that time, pots were over-irrigated daily for 2–3 d. On the afternoon of the day prior to the experiment, two or three replicate plants per genotype (i.e. 4–6 plants) were gently removed from the soil and de-rooted. Although it was found that de-rooting the plants underwater was not necessary to avoid an impact on TR (data not shown), in nearly all cases de-rooting was done by cutting the base of the plant stem underwater. Immediately after cutting, the cut stems were placed in 125 ml beakers containing de-ionized water and placed in a dark room overnight (approximately 14 h) under a temperature maintained at 20.3 °C (±0.18 SE). The following morning, the plants were moved from the dark room and transferred to a new set of 125 ml beakers containing fresh de-ionized water. Laboratory film (Parafilm 'M'®, Pechiney Plastic Packaging, Chicago, IL) was used to seal the stems in the beakers to avoid direct water evapouration. A small hole was made in the film to avoid negative pressure inside the sealed beaker due to water loss.

### *Experiments*

The impact of each AQP inhibitor was measured simultaneously on 4–6 plants placed in a test chamber with a stable atmosphere of

approximately 3.8 kPa. A stable *VPD* was achieved by continuously flowing about 40 l $min^{-1}$ of air into the chamber. The air was dried by first pumping air through two *PVC* cylindrical cartridges (l=0.68 m, d=0.03 m) filled with a silica gel desiccant ($SiO_2$, 10–18 Mesh, S161–500, Fisher Scientific, Fair Lawn, NJ). The air in the chamber was vigorously mixed with two 80 mm diameter fans (ASAF-B83, Cooler Master, Fremont, CA, USA) and three 40 mm diameter fans (EC4010M12C, Evercool Thermal Corp., LTD., San-Chung City, Taiwan, ROC). The top of the test chamber was covered with a 5 mm thick Plexiglas sheet above which water-cooled lamps provided a photosynthetic photon flux density at 1062 μmol $m^{-2}$ $s^{-1}$ (±4.8 SE) at plant level. Temperature and relative humidity were measured every 10 min inside the chamber by two pocket humidity/temperature pens (Extech Instruments, League City, TX). Temperature in the test chamber was maintained at 34–35 °C (temperature amplitude: up to 1.1 °C). For soybean, it has previously been shown that significant effects of temperature on photosynthetic rates or heat shock proteins accumulation occur above this range, typically in the 36–40 °C range (Key *et al.*, 1985; Campbell *et al.*, 1990; Hsieh *et al.*, 1992; Vu *et al.*, 1997).

Experiments were initiated by acclimatizing the plants for approximately 80 min in the test chamber until water loss rates were at steady-state. Following acclimation, beakers containing the plants were weighed every 20 min on a balance with a resolution of 0.01 g (Model XP-300, Denver Instrument, Denver, CO). After four consecutive weighings, the plants were quickly transferred and stems sealed into beakers containing a solution of an AQP inhibitor. The new beakers with the plants were immediately weighed and placed back into the test chamber. The whole process typically lasted from 120–180 s.

Three inhibitors were applied: cycloheximide (CHX), mercury chloride ($HgCl_2$) and silver nitrate ($AgNO_3$). Four concentrations were tested for each inhibitor (10, 50, 100, and 200 μM for CHX and 10, 100, 200, and 500 μM for $HgCl_2$ and $AgNO_3$). In most cases, each concentration for each inhibitor was tested simultaneously on three plants of each genotype. The solutions were prepared 1–5 d before the experiments and stored in darkness at 4 °C. CHX solutions were obtained by dissolving in a 0.05% (v/v) aqueous dimethyl sulphoxide solution, according to the protocol of Cochard *et al.* (2007). Tests showed that dimethyl sulphoxide did not affect the TR of either genotype (data not shown). Silver nitrate solutions were placed in opaque, dark brown 30 ml glass bottles (Fisher Scientific, Suwanee, GA) to prevent silver precipitation. Since it has previously been shown that $NO_3^-$ ions can reduce TR in de-rooted plants (Wilkinson *et al.*, 2007), the effects of $KNO_3$ solutions were tested at 10, 100, 200, and 500 μM on 3–6 replicate plants on genotype N01-11136 which exhibited TR sensitivity to $AgNO_3$. There was no sensitivity to $KNO_3$ except for a TR decrease (29%) as a result of the 500 μM $KNO_3$ treatment (data not shown).

Simultaneously with the 20 min-step weighings, conditions in the test chamber were recorded. Weighings were stopped when the water loss rates reached a plateau in water loss rate for 4–6 consecutive measurements. Overall, the AQP inhibitor treatments lasted approximately 180 min, 270 min, and 390 min for CHX, $AgNO_3$, and $HgCl_2$, respectively. The leaf area of each plant was measured using an area meter (Model LI-3100, Li-Cor, Lincoln, NE) and TR of each plant was expressed per unit leaf area.

### Data analysis

To facilitate comparison between genotypes, a normalized transpiration rate (*NTR*) following the inhibitor treatment was calculated for each plant as the ratio between the TR value and the average of the four TR values before inhibitor treatment ($TR_0$).

For each inhibitor and concentration, a Boltzmann sigmoid equation (Equation 1) was fitted to the averaged TR to describe the decrease in TR following inhibitor treatment;

$$TR = TR_P + \frac{TR_0 - TR_P}{1 + \exp\left(\frac{v50 - \text{Time}}{C}\right)} \quad (1)$$

where $TR_P$ is the value of the eventual plateau in TR following the inhibitor treatment, $V50$ is the time at which the TR has decreased halfway between $TR_0$ and $TR_P$, and $C$ is a coefficient which represents the steepness of the curve.

The fraction of the total transpiration rate associated with exposure to AQP inhibitors was calculated for each individual from the drop in TR (*DTR*, %):

$$DTR = (1 - \frac{TR_P}{TR_0}) \times 100 = \frac{TR_0 - TR_P}{TR_0} \times 100 \quad (2)$$

The value of *DTR* assumed for $TR_P$ in PI 416937 when it was insensitive to an inhibitor was calculated based on the measurements during the same 80–120 min period when a plateau in the response for N01-11136 plants was observed. For each genotype, *DTR* and *NTR* values were the average of 3–6 individuals. All statistical analysis and fittings were carried out on GraphPad Prism (GraphPad Software Inc., San Diego, CA, 1996).

## Results

In total, 15 experiments were performed to document for each genotype the TR response to four concentrations of three different aquaporin (AQP) inhibitors under high *VPD* conditions (Table 1). In all experiments except one, three replicate plants per genotype (i.e. six plants) were studied simultaneously. In the remaining case (E11), two replicate plants per genotype (i.e. four plants) were studied. As a check for the consistency of the responses, some experiments have been replicated (E1, E3, and E11). Overall, for each inhibitor, the number of replicate plants varied from three to six for both genotypes (Table 1).

### Experimental conditions

A first condition for analysing the data across 15 experiments was that the chamber environment was similar across experiments. Overall, the average temperature of all experiments was 34.4 °C (±0.06 SE). There was less than 1 °C difference between the lowest and highest values (33.9 °C for E13 to 34.8 °C for E9; Table 1). *VPD* values were slightly more variable, averaging 3.8 kPa (±0.09 SE) across experiments with 1.15 kPa difference between the lowest and highest average values (3.4 kPa for E2 to 4.6 kPa for E12; Table 1). Photosynthetic photon flux density values were the most stable, averaging 1062 μmol $m^{-2}$ $s^{-1}$ (±4.8 SE) at canopy level.

A second condition to allow comparison among experiments was a similarity in leaf areas among genotypes since the AQP inhibitors effect may depend on plant size. In only two CHX experiments were leaf areas significantly different between PI 416937 and N01-11136 (E3 and E5 at $P < 0.05$ and $P < 0.01$, respectively) out of a total of 15 experiments. Except for the CHX treatment, pooling leaf areas across all experiments for a given inhibitor treatment did not result in significant differences in leaf areas between genotypes (Fig. 1).

**Table 1.** Summary of the experiments including sowing and measurement dates, inhibitor, and vapour pressure deficit (*VPD*) and temperature during measurement

| Experiment | Replicate/ genotype[a] | Sowing date | Measurement date | Inhibitor (μM) | *VPD* (kPa)[b] | Temperature (°C)[b] |
|---|---|---|---|---|---|---|
| E1 | 3 | 19/9/2008 | 7/10/2008 | CHX (100) | 3.5±0.03 | 34.3±0.07 |
| E2 | 3 | 19/9/2008 | 8/10/2008 | CHX (200) | 3.4±0.02 | 34.4±0.06 |
| E3 | 3 | 28/9/2008 | 15/10/2008 | CHX (50) | 3.6±0.02 | 34.6±0.05 |
| E4 | 3 | 28/9/2008 | 16/10/2008 | CHX (10) | 3.6±0.02 | 34.7±0.05 |
| E5 | 3 | 5/10/2008 | 3/11/2008 | CHX (50) | 3.6±0.02 | 34.6±0.06 |
| E6 | 3 | 5/10/2008 | 4/11/2008 | CHX (100) | 3.6±0.02 | 34.4±0.06 |
| E7 | 3 | 23/10/2008 | 17/11/2008 | $HgCl_2$ (200) | 4.2±0.02 | 34.3±0.05 |
| E8 | 3 | 23/10/2008 | 18/11/2008 | $HgCl_2$ (100) | 4.3±0.03 | 34.3±0.06 |
| E9 | 3 | 10/12/2008 | 7/1/2009 | $HgCl_2$ (10) | 3.6±0.03 | 34.8±0.06 |
| E10 | 3 | 4/1/2009 | 3/2/2009 | $HgCl_2$ (500) | 4.3±0.04 | 34.5±0.08 |
| E11 | 2 | 4/1/2009 | 4/2/2009 | $AgNO_3$ (100) | 4.5±0.02 | 34.3±0.11 |
| E12 | 3 | 4/1/2009 | 6/2/2009 | $AgNO_3$ (100) | 4.6±0.04 | 34.0±0.13 |
| E13 | 3 | 12/1/2009 | 20/2/2009 | $AgNO_3$ (500) | 3.9±0.06 | 33.9±0.14 |
| E14 | 3 | 12/1/2009 | 23/2/2009 | $AgNO_3$ (200) | 3.8±0.05 | 34.1±0.12 |
| E15 | 3 | 26/3/2009 | 21/4/2009 | $AgNO_3$ (10) | 3.5±0.02 | 34.5±0.05 |

[a] Number of replicate plants per genotype.
[b] Average and standard errors of the values measured during the experiments.

A third condition for these experiments was insensitivity of TR in N01-11136 and PI 416937 to de-rooting. This is necessary to avoid drastic changes in (i) their TR response to high evaporative demand or (ii) the differences between genotypes in TR response to high *VPD*. The average $TR_0$ value of de-rooted PI 416937 was 43.5 (±0.92 SE) mg $H_2O$ $m^{-2}$ $s^{-1}$, matching the maximum TR value previously reported on intact plants of this genotype by Fletcher *et al.* (2007) of 42 mg $H_2O$ $m^{-2}$ $s^{-1}$ (Fig. 2, inset b). TR of de-rooted N01-11136 (average of 58 mg $H_2O$ $m^{-2}$ $s^{-1}$ ±0.99 SE) was significantly higher ($P$ <0.0001) than that of de-rooted PI 416937 (Fig. 2). The higher TR for N01-11136 is consistent with the expected differences in TR values at high *VPD* of intact plants between genotypes. Overall, the fulfilment of this condition indicates little or no involvement of the root system in the leaf TR response under high evapourative demand for both genotypes at least for the duration of the experiments.

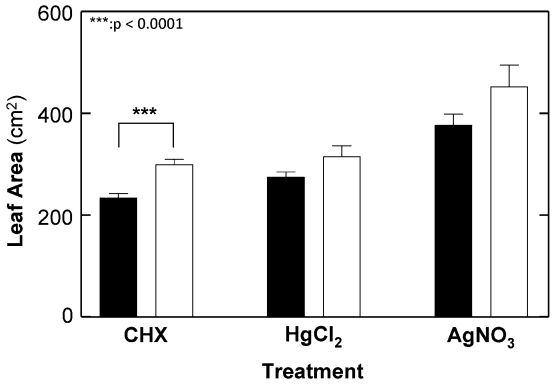


**Fig. 1.** Leaf area differences between genotypes N01-11136 (closed bars) and PI 416937 (open bars) during the three AQP inhibitors treatments. Data are the mean of the 18, 12 and 14 observations for CHX, Hg, and Ag treatments, respectively. Error bars are the standard error of the mean. ***$P$ <0.0001.

### *TR response to the AQP inhibitors*

The analysis of the TR response to CHX treatments, regardless of concentration and genotype, revealed that, after about 40 min of exposure to the inhibitor, TR began to decrease. Transpiration rate decreased ($V$50=132 min ±2.6 SE; Fig. 3a) in a dose-dependent way (Fig. 4) and eventually established a plateau TR, as exemplified in Fig. 5a for a CHX concentration of 100 μM. The resulting *DTR* values were non-significantly different between PI 416937 and N01-11136 with the marginal exception of the 100 μM treatment, for which the difference was significant at $P$ <0.05 (Figs 4, 5d). Overall, across a 10–200 μM CHX concentration range, *DTR* values were similar between genotypes, ranging from 39.4% ±3.8 SE to 57.9% ±1.2 SE for PI 416937 and 44.6% ±0.6 SE to 60.1% ±2.1 SE for N01-11136 (Fig. 4). For both genotypes, *DTR* did not significantly change when concentrations were increased from 100 μM to 200 μM CHX.

Similar to the CHX response, Hg treatments did not result in significant differences in *DTR* between PI 416937 and N01-11136 for any of the four concentrations in the 10–500 μM $HgCl_2$ range (Fig. 4). However, as exemplified by TR in Fig. 5b and e and compiled in the dose–response curve in Fig. 4, Hg treatments resulted in different effects on TR when compared to CHX, on the basis of three observations. First, *DTR* values induced by Hg treatments ranged to higher and lower values than those caused by CHX. Second, the maximum *DTR* values were reached at a concentration of 200 μM $HgCl_2$, twice the CHX concentration for which the maximum *DTR* was observed

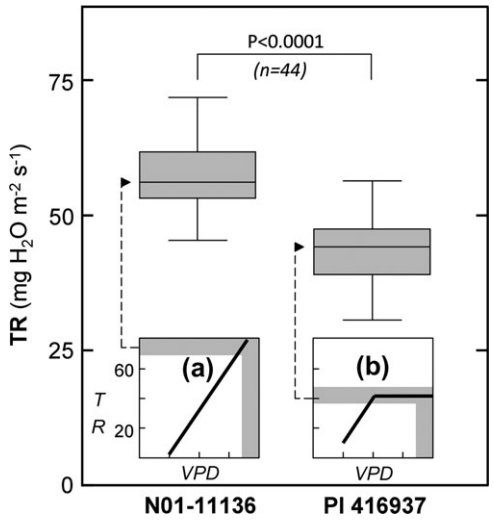


**Fig. 2.** The differences in transpiration rates (TR) of the two studied de-rooted genotypes observed before the treatments match those previously observed on intact whole plants under high *VPD* conditions [insets (a) and (b)]. The box and whiskers represent TR values that were measured for 80 min, before the AQP inhibitors treatments, under high *VPD* conditions (3.8 kPa±0.4 SD). Each value (*n*) represents the average of the four consecutive TR measurements of a single individual. Insets (a) and (b): TR versus *VPD* regressions previously established for the studied genotypes by Sadok and Sinclair (2009a) and Fletcher *et al.* (2007), respectively (see Materials and methods for details). Horizontal grey areas in insets (a) and (b) represent the TR values that can be inferred from the corresponding formalisms for high *VPD* values in the 3.5–4 kPa range (vertical grey areas).

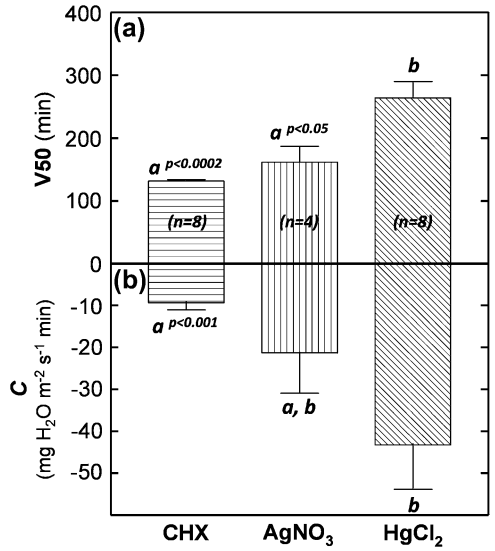


**Fig. 3.** Differences in the TR decrease induced by the three AQP inhibitors (averaged across all concentrations) as expressed by *V*50 (a) and the *C* (b) parameters of the Boltzmann fits. Bars with horizontal, vertical, and inclined lines refer to CHX (both genotypes), $AgNO_3$ (N01-11136 only), and $HgCl_2$ (both genotypes) treatments. Standard errors are indicated for each bar (*n* ranging from 4–8). Bars with different letters are significantly different at the indicated *P*-values.

(Fig. 4). Third, the time from $TR_0$ to $TR_P$ was much longer for Hg (*V*50=264 min ±26.2 SE; Fig. 3a) with a *C* value almost four times less than for the TR kinetics observed under CHX treatments (Fig. 3b).

In contrast to the above inhibitors, Ag induced a striking difference in *DTR* between PI 416937 and N01-11136 for all four tested concentrations in the 10–500 μM range (Fig. 4). For the lower $AgNO_3$ concentrations, a TR of PI 416937 was insensitive to silver (non-significant slopes). At the highest concentration of 500 μM $AgNO_3$, TR of PI 416967 exhibited a small but significant linear decrease (slope at 0.018±0.004 SE, Fig. 5c). By contrast, the TR of N01-11136 exhibited a sharp decrease across all Ag concentrations (*V*50=162 min ±25.4 SE, Fig. 3a) that started only 40 min after the treatment and stabilized at 120 min with a $TR_P$ of 42.5% ±3.6 SE (Fig. 4). Compared with the effects of CHX and Hg, Ag treatments induced markedly lower *DTR* values for N01-11136 which ranged from 15.9% ±2.5 SE to 42.5% ±3.6 SE. These values were lower than those of the other metallic ion, Hg, indicating an inhibition by Hg roughly twice as strong as that resulting from the Ag treatments. The decrease time from $TR_0$ to $TR_P$ of Ag treatments for N01-11136 was significantly shorter ($P$ <0.05) than that observed for Hg treatments, indicating a faster mode of action for silver.

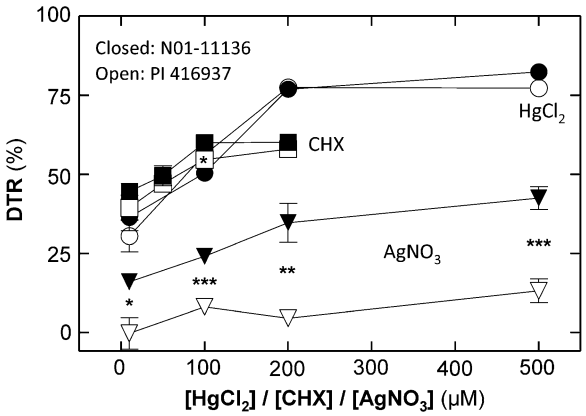


**Fig. 4.** Dose–response curves of the drop in transpiration (*DTR*) induced by different AQP inhibitors for soybean genotypes N01-11136 and PI 416937. Closed/open symbols refer to genotypes N01-11136 and PI 416937, respectively. Circles, squares, and inverted triangles correspond to $HgCl_2$, CHX, and $AgNO_3$ treatments, respectively. Data are the mean of 3–6 observations (see Table 1 for details) and error bars represent standard error of the mean. Error bars for some points are invisible when they are smaller than the size of the symbols (*, **, and ***: $P$ <0.05, $P$ <0.02, and $P$ <0.005, respectively).

## Discussion

This study showed that de-rooted plants under high *VPD* conditions could be used to study the TR response to AQP inhibitors. Side-by-side comparisons of de-rooted plants of two soybean genotypes with different sensitivities to *VPD* showed similar responses when treated with two AQP inhibitors (CHX and Hg). However, a major difference between genotypes was observed in their response to a third

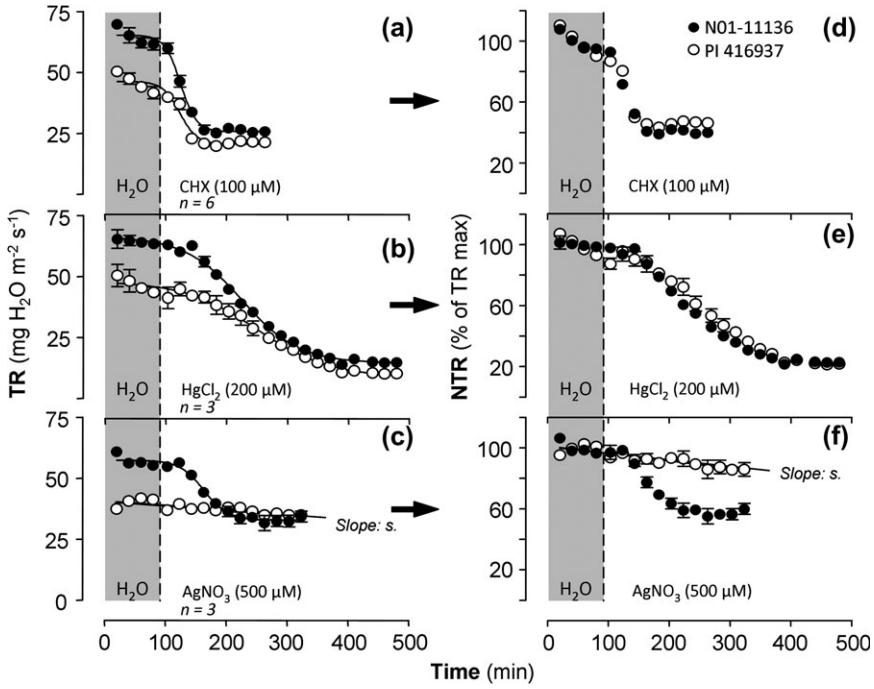


**Fig. 5.** Examples of time-courses of transpiration rate (TR) response to the three different AQP inhibitors: 100 µM CHX (a, d), 200 µM $HgCl_2$ (b, e), and 500 µM $AgNO_3$ (c, f) under constant, high *VPD* conditions for two de-rooted soybean genotypes (N01-11136: closed circles and PI 416937: open circles). Grey areas highlight the 80 min sequence where maximum TR values ($TR_0$) were measured under de-ionized water treatment. (a–c) Time-courses of absolute TR. (d, f) Time-courses of the corresponding normalized TR values (*NTR*, expressed in % of the average of TR maximum values, see Materials and methods for details). The number of replicate plants ($n$=3–6 ±SE) are indicated. Error bars for some points are invisible when they are smaller than the size of the symbols. Curves on (a), (b) and (c) are Bolzmann sigmoidal fittings.

inhibitor, Ag. The TR of a slow-wilting line (PI 416937) was virtually insensitive to $AgNO_3$ exposure of concentrations from 10–500 µM.

### *Cycloheximide inhibition*

Possible side-effects resulting from long exposure to CHX may be marginal in our study for two main reasons. First, root exposure to 1 mM CHX for 265 min in aspen roots (Voicu and Zwiazek, 2004) did not result in changes in $O_2$ uptake indicating that the effect of CHX was not the result of metabolic disruption. In this study with soybean leaves, the response to CHX treatment stabilized after 80 min, a duration that is comparable to that of the study by Cochard *et al.* (2007) to examine AQPs in walnut leaves (≈60 min). Second, the swiftness of the effect of CHX on TR observed in all experiments, reflected by (i) the early start of the decrease in TR (40 min after the inhibitor is added) or (ii) the steep slope and low *V*50 values (Fig. 3a, b) is consistent with the possibility of an inhibition of protein synthesis directly involved in the water flow within the leaf.

The *DTR* of both genotypes caused by the four concentrations of CHX indicates an inhibiting effect on a protein-mediated, symplastic/transcellular water pathway in the leaves that would require *de novo* synthesis. The involvement of such a process in the protein-mediated water pathway on whole-plant hydraulics has been established by Voicu and Zwiazek (2004), and its specific involvement in the leaf-based water flow was demonstrated by Cochard *et al.* (2007), who established that such involvement was dependent on the PPFD. Under high-light conditions (≈1400 µmol $m^{-2}$ $s^{-1}$), Cochard *et al.* (2007) found that 100 µM CHX reduced leaf hydraulic conductance by approximately 65%. The present study with soybean extends the range of environmental variables responsible for such responses to high *VPD* conditions and indicates that symplastic water pathway terminating in the guard cells may account for up to ≈60% (at 100 µM CHX) of the overall hydraulic pathway in the leaf (Fig. 4). The response to CHX was not significantly different between PI 416937 and N01-11136 indicating that the CHX-sensitive pathway may not be linked to their differences in TR responses to high *VPD* (Fig. 2).

Inhibition from CHX is reported to be a consequence of its negative effects on peptide initiation and extension (O'Brig *et al.*, 1971). Therefore, toxic doses of CHX or high exposure times to this inhibitor may result in a metabolic disruption (Zhang *et al.*, 1995). In our study, three CHX treatments were at concentrations equal to or lower than 100 µM, a concentration that was used by Cochard *et al.* (2007) as a physiological concentration on single leaves of walnut to study the involvement of AQPs in modulating water flow inside the leaf in response to light. These CHX concentrations were also much lower than those used in the

study of Voicu and Zwiazek (2004) on aspen seedlings (1 mM) and that of Moshelion *et al.* (2002) carried out on protoplasts (2 mM). The speed of the response to the CHX treatment is consistent with the fact that transcriptional regulation of AQP genes can operate in less than 60 min (Kawasaki *et al.*, 2002). This indicates that CHX could act by decreasing turnover of AQPs and/or of different elements essential to AQP activity, at the transcript and/or protein levels as suggested by the complex regulation of AQP (reviewed by Javot and Maurel, 2002).

### *Mercury inhibition*

Mercurials are the most commonly used AQP inhibitors in the literature. They act through covalent modification of cysteine residues within the water pore and in other regions of the protein causing either block or conformational changes leading to inhibition of water transport (Niemietz and Tyerman, 2002). However, Hg has been reported to have a large array of side-effects depending on the dose and the duration of the treatments (Tyerman *et al.*, 1999; Javot and Maurel, 2002; Niemietz and Tyerman, 2002; Kaldenhoff *et al.*, 2008; Maurel *et al.*, 2008). In our study, all $HgCl_2$ concentrations were equal or less than 500 μM, a concentration at which inhibition on sap flux of tomato roots were reversed partially by mercaptoethanol (Maggio and Joly, 1995). At 200 μM $HgCl_2$, Nardini *et al.* (2005) showed that the effects of mercury on the hydraulic conductance of sunflower leaves were completely reversed by 30 mM mercaptoethanol. Further, Levin *et al.* (2007) found that concentrations below 50 μM $HgCl_2$ were ineffective on leaf hydraulic conductance and suggested the use of higher concentrations. North *et al.* (2004) showed the decrease in conductance of *Agave deserti* roots caused by 25 μM $HgCl_2$ and it was reversed by 20 mM mercaptoethanol. Consequently, previous studies indicate the use of $HgCl_2$ at concentrations below 200 μM would involve minimal side-effect damages, especially the 10 μM $HgCl_2$ treatment in our study with soybean.

Overall, *DTR* values as a result of Hg treatment indicated that a symplastic/transcellular pathway involving proteins sensitive to Hg account for 36% (10 μM) to 82% (500 μM) of the leaf TR under high *VPD*. Interestingly, these values are consistent with previous studies reporting mercurials reducing hydraulic conductivities in plants. For instance, hydraulic conductivities of roots from different plant species were reduced by mercurials by values ranging from 32–90% (reviewed in Javot and Maurel, 2002). Further, a non-significant difference in *DTR* values between PI 416937 and N01-11136 indicated that putative Hg-sensitive AQP populations are not responsible for the difference in TR response to *VPD* observed between the two genotypes.

### *Silver inhibition*

Silver inhibits AQPs by a different mechanism than that of Hg, which may result from the difference in the sizes between $Hg^{2+}$ and $Ag^{+}$ ions, the specific interaction between Ag and a histidine (in addition to cysteine), and the structure of the AQP pores (Niemietz and Tyerman, 2002). Exposure of de-rooted soybean plants to $AgNO_3$ resulted in a striking difference in the *DTR* response of genotypes PI 416937 and N01-11136. In contrast to N01-11136, TR of PI 416937 was virtually insensitive to $AgNO_3$ when exposed to 10–500 μM $AgNO_3$. This study offers the first demonstration of an intraspecies difference in response to Ag at least in the 10–200 μM range.

Since Sinclair *et al.* (2008) have shown that the constant TR of PI 416937 when subjected to *VPD* greater than 2 kPa resulted from a limited hydraulic conductance within the leaf, the results of this current study indicate that this TR limitation may be the result of a lack a specific silver-sensitive AQP in the leaves of PI 416937. Extending this conclusion to intact plants is supported by the fact that the TR of untreated, de-rooted plants under high *VPD* of both genotypes closely matched those previously reported on intact plants.

The results of N01-11136 revealed a dose-dependent silver inhibition of leaf AQPs of this genotype, which is a type of inhibition infrequently reported in the literature. The only direct reference of plant tissue water permeability being affected by silver treatment was in the study of Niemietz and Tyerman (2002) carried out with soybean peribacteroid membrane and sugar beet plasma membrane vesicles. Based on the corresponding *DTR* values (Fig. 4, black inverted triangles), an inhibition caused solely by $Ag^{+}$ may account for up to 35% (200 μM $AgNO_3$) of the hydraulic pathway. In this case it is highly unlikely that the silver concentrations tested may have had significant side-effects, given that, in the same concentration range, TR of PI 416937 was not affected. Further, TR response to Ag in N01-11136 as described by the *V*50 parameter was as swift as that of the CHX treatment (non-significant difference; Fig. 3a), reinforcing the idea that silver probably inhibited proteins directly involved in leaf water flow under high *VPD*.

The results of this study indicated that, without the influence of the root system, soybean leaves under high *VPD* conditions may have active Hg/Ag-sensitive AQPs that require *de novo* synthesis, and may account for up to 82% of the hydraulic pathway terminating in the guard cells. Although previous studies indicated the importance of the symplastic pathway in the leaf hydraulics for different species under different developmental and environmental conditions (Sack *et al.*, 2004; Nardini *et al.*, 2005; Tyree *et al.*, 2005; Cochard *et al.*, 2007; Ye *et al.*, 2008), this study offers direct evidence that the symplastic pathway could be involved in the response of TR to high *VPD*. Given the fact that PI 416937 has restricted hydraulic flow at high *VPD* (Sinclair *et al.*, 2008) and displays slow-wilting capability in the field, the current results now indicate that this may be the result of a lack of Ag-sensitive symplastic pathway in PI 416937.

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