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Root hydraulic conductivity in plants (Lp) exhibits large variations in response to abiotic stimuli. In this study, we investigated the impact of dynamic, aquaporin-mediated changes of LP on leaf growth, water potential, and water flux throughout the plant. For this, we manipulated LP by subjecting roots to four independent treatments, with aquaporin inhibitors applied either to transpiring maize (Zea mays) plants grown in hydroponics or to detopped root systems for estimation of LP. The treatments were acid load at pH 6.0 and 5.0 and hydrogen peroxide and anoxia applied for 1 to 2 h and subsequently reversed. First, we established that acid load affected cell hydraulic conductivity in maize root cortex. LP was reduced by all treatments by 31% to 63%, with half-times of about 15 min, and partly recovered when treatments were reversed. Cell turgor measured in the elongating zone of leaves decreased synchronously with LP, and leaf elongation rate closely followed these changes across all treatments in a dose-dependent manner. Leaf and xylem water potentials also followed changes in LP. Stomatal conductance and rates of transpiration and water uptake were not affected by LP reduction under low evaporative demand. Increased evaporative demand, when combined with acid load at pH 6.0, induced stomatal closure and amplified all other responses without altering their synchrony. Root pressurization reversed the impact of acid load or anoxia on leaf elongation rate and water potential, further indicating that changes in turgor mediated the response of leaf growth to reductions in LP.

Leaf growth is an essential process for crop production and is subject to large temporal fluctuations with environmental conditions. There is accumulating evidence that a large part of the changes observed in leaf growth depends on water transport within the plant (Sperry et al., 1998; Bouchabke et al., 2006). It has also been shown that changes in leaf water potential induced by root pressurization can trigger rapid variations of leaf elongation rate in wheat (Triticum aestivum) and barley (Hordeum vulgare; Passioura and Munns, 2000). This raises the question of whether and to what extent low hydraulic conductivity within the plant can limit leaf growth.

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After the stomata, the root system represents the largest resistance to water flow in the soil-plant atmosphere continuum (Steudle and Peterson, 1998). Root hydraulic conductivity (Lp) is affected by environmental stimuli such as drought, salinity, anoxia, low temperature, and nutrient availability (Zhang and Tyerman, 1991; Azaizeh et al., 1992; Birner and Steudle, 1993; Boursiac et al., 2005; Vandeleur et al., 2009). This ability to respond rapidly to fluctuating conditions suggests that LP may participate in plant adaptation to diverse environments (Steudle, 2000). Aquaporins, a large family of water channel proteins located in plasma and intracellular membranes, are the main determinants of water flow across plant cells and tissues (Javot et al., 2003; Maurel et al., 2008). The dynamic changes in LP in response to chemical or environmental stimuli may result from modifications of aquaporin abundance or activity (Carvajal et al., 1996; Tournaire-Roux et al., 2003; Boursiac et al., 2005). In particular, aquaporin regulation by phosphorylation, protonation, and relocalization in intracellular compartments has been reported in response to extracellular stimuli (Guenther et al., 2003; Tournaire-Roux et al., 2003; Vera-Estrella et al., 2004; Boursiac et al., 2008).

The first insights into the involvement of aquaporins in physiological processes such as cell enlargement, tissue differentiation, and organ movement have been obtained at the cell or tissue level (Hukin et al., 2002;
Moshelion et al., 2002; Wei et al., 2007). It is still unknown to what extent changes in root aquaporin activity impact integrated physiological processes such as shoot growth of intact plants. The importance of aquaporins in controlling physiological processes in adult, transpiring plants is assumed to be rather limited; this is because the proportion of water transport controlled by aquaporins is believed to be much lower than that in slowly transpiring plants (Steudle and Frensch, 1996; Steudle and Peterson, 1998).

The manipulation of aquaporin activity offers the possibility to address this question. A classical approach is to alter the expression of aquaporin genes. Down-regulation of genes encoding aquaporins of the Plasma membrane Intrinsic Protein1 (PIP1) and PIP2 subfamilies in Arabidopsis and NtAQP1 in tobacco (Nicotiana tabacum) reduced the ability of these plants to recover after a water deficit treatment (Martre et al., 2002; Siefriz et al., 2002). However, genetic studies have been hindered by phenotypic compensation due to the functional redundancy of aquaporin isoforms in plants (Hachez et al., 2006b). The use of aquaporin inhibitors is a useful tool to investigate the role of root aquaporins in controlling leaf growth. Mercuric chloride (HgCl₂), which blocks aquaporins by binding of Hg²⁺ ions to Cys residues, has been widely used to evaluate the contribution of aquaporins to root water transport (Maggio and Joly, 1995; Carvajal et al., 1996). For instance, Lu and Neumann (1999) have observed that root exposure to 0.5 mM HgCl₂ immediately inhibited leaf growth in water-stressed rice (Oryza sativa) seedlings, thereby suggesting a role for aquaporins in controlling leaf growth. However, the signaling mechanisms involved in leaf growth inhibition remained unclear. Side effects of HgCl₂ application, such as the reduction of membrane potential in root cortex cells or an impaired cell respiration, restrict its usefulness in physiological studies (Wan and Zwiazek, 1999; Zhang and Tyerman, 1999). Manipulating the root environment is an alternative strategy to efficiently alter Lp, and to assess the significance of such changes on leaf and/or shoot growth. The effects of varying Lp by root chilling or anoxia are correlated to leaf growth responses (Malone, 1993; Else et al., 1995, 2001), but the interpretation of these results remains controversial in the absence of precise hydraulic measurements.

The goal of this study was to determine whether alterations of root aquaporin activity can influence leaf growth in intact, adult plants via effects on Lp and cell turgor in the leaf elongation zone. In this work, we compared three chemical treatments that target aquaporin inactivation in roots via different mechanisms. Each of them could exert side effects, but provided that all treatments resulted in common responses, the role of aquaporins on leaf growth could be established. We have followed, with a high temporal definition, the consequences of experimentally induced changes in Lp on water flux, leaf water potential, and leaf elongation rate under different scenarios (three evaporation demands and pressurized or nonpressurized root systems). In addition, we have measured cell turgor in growing leaves using a pressure probe to investigate whether cell turgor responds to changes in root hydraulic conductivity and whether such changes could account for the control of leaf growth.

The first treatment used to alter Lp was acid loading of the solution surrounding the roots, which causes cytosolic acidification in root cortex cells. This triggers the closure of aquaporins due to the protonation of a conserved His residue (Tournaire-Roux et al., 2003). The second treatment was hydrogen peroxide (H₂O₂) application to the roots, which results in the inhibition of Lp in maize (Zea mays) by oxidative gating of aquaporins and/or their internalization (Ye et al., 2004; Aroca et al., 2005; Ye and Steudle, 2006; Boursiac et al., 2008). The third treatment was anoxia, an environmental stress that induces an inhibition of Lp in a large array of species through proton-induced closure of aquaporins (Zhang and Tyerman, 1991; Birner and Steudle, 1993; Else et al., 1995; Tournaire-Roux et al., 2003).

RESULTS

Cell Hydraulic Conductivity in Maize Root Cortex Was Decreased by Acid Loading

Figure 1 shows typical examples of the time course of hydraulic relaxation in root cortical cells following a change in turgor imposed by a cell pressure probe. Cells from the first and second outer cortical layers of untreated roots exhibited a short half-time of hydrostatic relaxation (τ₁/₂) of 2.9 ± 0.9 s (n = 9 cells; ±sD; Fig. 1B). These values were similar to τ₁/₂ values previously reported for maize root cortex cells (Hose et al., 2000). Acid load treatment of the root segments with proton acid load at pH 6.0 on pressure relaxation of cortical cells in maize roots. A, Typical examples of pressure probe recordings in root cortex cells of control and acid-loaded roots. A stationary turgor was reached within a few minutes after cell impalement with a cell pressure probe. The probe was adjusted manually to maintain a constant volume until turgor had returned to a constant value. Hydrostatic relaxation was monitored in conditions where water exchange can mostly be accounted for by passage through the plasma membrane of the measured cell. B, Mean τ₁/₂ for cortex cells in control (n = 9) and acid-loaded (n = 8) root segments. Error bars indicate sD.
pionic acid at pH 6.0 for 1 h significantly increased the half-time to 7.4 ± 2.0 s (n = 8 cells; P < 0.05; Fig. 1B). This represents a decrease in cell hydraulic conductivity (Lp$_c$) from 7.7 ± 2.5 × 10$^{-7}$ m s$^{-1}$ MPa$^{-1}$ (control) to 2.7 ± 1.2 × 10$^{-7}$ m s$^{-1}$ MPa$^{-1}$ (acid load). The cell elastic modulus was not affected by acid loading (control, 4.4 ± 0.8 MPa; acid load, 4.1 ± 0.4 MPa). Turgor varied among measured cells and, on average, exhibited slightly but not significantly higher values upon acid loading (0.38 ± 0.09 MPa compared with 0.27 ± 0.04 MPa before acid load).

The Hydraulic Conductivity of Seminal Root Systems Was Reduced in Response to Acid Load, H$_2$O$_2$, and Anoxia Treatments

The effects of root treatments on Lp$_r$ were studied using the suction technique applied to excised seminal root systems in hydroponics (Fig. 2). In a first series of experiments, a constant suction force (–0.02 MPa) was maintained at the upper section of the excised root system, and the resulting water flow was continuously monitored before and after root exposure to acid load, H$_2$O$_2$ or oxygen deprivation. All treatments induced a rapid reduction in root water flow, with half-times of approximately 15 min (Fig. 2, A–D). Experiments were replicated with different suction forces applied to the root systems. The resulting water flow per unit root area was linearly related to the applied suction force ($r^2$ ranging from 0.59 to 0.82). Lp$_r$ was calculated as the slope of the regression line. Statistically significant reductions in Lp$_r$ by 51%, 61%, and 54% were observed during the 1-h period following acid loading at pH 6.0, acid loading at pH 5.0, and H$_2$O$_2$ treatment, respectively (Fig. 2E). In the same manner, bubbling the nutrient solution with N$_2$, which induced a decrease in oxygen partial pressure from 95% to 10% in less than 10 min, caused a reduction in Lp$_r$ by 31% (Fig. 2E). These effects were partially reversed when treatments were reversed after 60 min (Fig. 2E). No change in the osmotic potential of the xylem sap was detected after any of the treatments (data not shown). We conclude that the treatments were appropriate to induce rapid and appreciable reductions in Lp$_r$.

Reduction in Lp$_r$ Had No Effect on Transpiration Rate, Stomatal Conductance, and Water Uptake under Low Evaporative Demand But Affected Xylem and Leaf Water Potentials

Under low evaporative demand (vapor pressure deficit (VPD) maintained at 1.35 kPa and photosynthetic photon flux density at 150 μmol m$^{-2}$ s$^{-1}$), neither plant transpiration nor stomatal conductance was affected by the acid-loading treatment at pH 6.0 (Fig. 3). The mean transpiration rate was 11 ± 1.38 mg s$^{-1}$ m$^{-2}$ before and 12.7 ± 1.4 mg s$^{-1}$ m$^{-2}$ at 60 min after the onset of root treatment (n = 6 plants; ±SD). Similar results were obtained for the H$_2$O$_2$ treatment (data not shown). Root water uptake was not affected either (11.9 ± 2.2 mg s$^{-1}$ m$^{-2}$ before and 11.7 ± 1.9 mg s$^{-1}$ m$^{-2}$ after the acid-loading treatment; n = 6 plants). Therefore, the reduction in Lp$_r$ induced by acid loading was not associated with any significant change in water uptake or water loss. Consistently, the ratio of uptake to transpiration rate, calculated for each plant immediately before and 60 min after the onset of acid loading, was also unaffected (1.08 ± 0.07 and 1.05 ± 0.04, respectively). The ratios of greater than unity indicated that there was a slight accumulation of water in the plant, which is possibly related to plant growth. All root treatments reduced xylem and leaf water potentials progressively and in parallel during the first 60 min of treatment: reduced by 0.44, 0.5, 0.6, and 0.24 MPa in response to acid loading at pH 6.0, acid loading at pH 5.0, H$_2$O$_2$, and anoxia treatments, respectively (Fig. 4). Therefore, all treatments markedly affected plant water relations not only at the root.

Figure 2. Changes in root water flow (Jv, A–D) and in Lp$_r$ (E) of hydroponically grown maize seedlings in response to various root treatments: acid load at pH 6.0 (AL6; A), acid load at pH 5.0 (AL5; B), 2 mM H$_2$O$_2$ (C), and anoxia (D). Jv and Lp$_r$ measurements were made on individual excised maize root systems kept in nutrient solution. Water flow was induced across root systems by applying tensions between −0.02 and −0.06 MPa below atmospheric pressure to the detopped section. A to D, Typical time courses of Jv changes at a constant tension of −0.02 MPa. The treatments were applied at 0 min (black arrows) and reversed at 60 min (white arrows) either by washout of acid load and H$_2$O$_2$ (A–C) or rebubbling with air (D). During the root treatment, changes in Jv were induced by varying the applied tension. E, Lp$_r$ was deduced from the slope of the Jv versus tension relationship; means with ±SD (error bars; n = 5–6 plants) were calculated before treatment (control), 60 min after the onset of treatment, and 60 min after its reversion.
but also at the shoot level without alteration in plant transpiration. However, a constant difference between xylem and leaf water potentials of about 0.15 MPa was maintained in all cases, consistent with the maintenance of water flow rate shown in Figure 3.

Increased Evaporative Demand, When Combined with Acid Load at pH 6.0, Induced Stomatal Closure and Amplified Xylem and Leaf Water Potential Responses

High evaporative demand (VPD maintained at 2.8 kPa and photosynthetic photon flux density at 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) induced much higher transpiration rates in control plants compared with plants maintained under low evaporative demand (Fig. 3). In this case, acid loading at pH 6.0 dramatically decreased the transpiration rate and the water potentials (Fig. 4A). Xylem and leaf water potential stabilized at approximately −1.3 and −0.8 MPa, respectively, after 60 to 80 min of acid load.

Synchronous Fluctuations in Leaf Elongation Rate and in Turgor of Growing Cells Occurred after Acid Loading, \( \text{H}_2\text{O}_2 \), or Anoxia Treatment

Following acid loading or \( \text{H}_2\text{O}_2 \) treatment, the elongation rate of leaf 6 decreased for 30 to 40 min, with half-times of approximately 15 to 20 min when measured under low or high evaporative demand (Fig. 5, A–C). Anoxia induced a more gradual response of leaf elongation rate, which decreased for more than 100 min. Leaf elongation then stabilized at a significantly lower value (reductions of 48%, 79%, 75%, 58%, and 40% in response to acid loading at pH 6.0 and low evaporative demand, acid loading at pH 6.0 and high evaporative demand, acid loading at pH 5.0, \( \text{H}_2\text{O}_2 \), and anoxia treatments, respectively). The inhibition of leaf elongation rate by acid loading at pH 6.0 or anoxia treatment was partially reversed within 60 and 120 min after return to a standard nutrient solution or rebubbling with air, respectively. By contrast, no reversal was observed following acid loading at pH 5.0 or \( \text{H}_2\text{O}_2 \) treatment. We tested whether the decrease in leaf elongation rate observed after acid-loading treatments could be due to a propagation of propionate in the leaf elongation zone. To address this possibility, we used \(^{13}\text{C}\)-labeled propionic acid and analyzed via mass spectroscopy the abundance of \(^{13}\text{C}\) in root and leaf tissues (Fig. 6). No accumulation of \(^{13}\text{C}\) was observed.

Figure 3. Typical examples of the time course of changes in transpiration rate of intact maize plants in response to acid loading of the roots under two evaporative demands. Hydroponically grown maize plants were transferred to plastic containers with standard nutrient solution and placed on balances. The evaporative demand in the culture chamber was maintained either low (dashed line; 1.3 kPa VPD and 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) photosynthetic photon flux density) or high (solid line; 2.8 kPa VPD and 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) photosynthetic photon flux density). The container was continuously weighed, and weight loss data were stored in a data logger. Plant transpiration rate was calculated at intervals of 15 min and related to the leaf area. Acid load at pH 6.0 was applied to the roots at 0 min, as indicated by the dashed vertical line.

Figure 4. Effects of root treatments on xylem (crosses, white circles, and gray symbols) and leaf water potentials (black and white squares) of maize plants grown in hydroponics. Treatments were acid load at pH 6.0 (A), acid load at pH 5.0 (B), 2 \( \text{mM} \) \( \text{H}_2\text{O}_2 \) (C), and anoxia (D). Xylem and leaf water potentials were measured on leaves 2 and 4 of plants kept at low evaporative demand in B to D and on plants kept at low (black symbols) or high (white symbols and dashed line) evaporative demand in A (as described in Fig. 3). Leaf 2 was wrapped in aluminum foil down to the base of the plant (including first leaves) at the end of the day preceding experiments to prevent transpiration and to reach water balance with the xylem at the root-leaf junction. For the sake of clarity, SD is not presented in A for plants subjected to high evaporative demand; mean SD was approximately 0.1 MPa. The treatments were applied at 0 min (black arrows) for 120 min. Data at 0 min correspond to xylem and leaf water potentials of untreated plants. Data represent mean values of \( n = 4 \) plants. Error bars indicate SD.
in the elongation zone of leaf 6, suggesting that reductions in leaf elongation rate following acid-loading treatments were not due to significant accumulation of propionic acid molecules in the leaf elongation zone.

A decrease in turgor of growing cells paralleled that of leaf elongation rate in all treatments under low evaporative demand, with larger reductions for acid loading at pH 5.0 (45%) than for H2O2 and acid loading at pH 6.0 treatments (30% and 34%; Fig. 5, E–G). Anoxia had the most gradual effects on both cell turgor (18% reduction) and leaf elongation rate (Fig. 5, D and H), while leaf elongation rate and cell turgor remained constant in nontreated plants (data not shown). The osmotic potential of growing tissues of leaf 6, as determined by psychrometry, was not affected during the 60 min of all treatments (Table I). After the end of each treatment, turgor and leaf elongation rate exhibited similar time courses of response (i.e. a clear recovery in response to acid loading at pH 6.0 and anoxia and no recovery in response to acid loading at pH 5.0 and H2O2 treatment).

Leaf elongation rate and turgor responses to acid loading at pH 6.0 were further analyzed under three transpiration regimes (Fig. 5, A and E). Both leaf traits were unaffected by root treatment in dark conditions. By contrast, the responses of leaf elongation rate and turgor were amplified by an increase in evaporative demand.

Overall, the synchrony between leaf elongation rate and turgor was remarkably conserved in all transpiration regimes and root treatments tested. Therefore, these data suggest that reductions in leaf elongation rate in response to chemical treatments on roots were mediated by hydraulic signals from the root to the shoot and were a consequence of reductions in turgor of growing leaf cells.

To further investigate the synchrony between hydraulic processes and the inhibition of leaf elongation rate in response to the decrease in $L_{pr}$, we examined the relationships between mean reductions in $L_{pr}$ and...
changes in water potential gradients, turgor, and leaf elongation rate (Fig. 7). A surprising result was that the gradient of water potential between the nutrient solution and the mature leaves (as measured with a pressure chamber) increased more with root treatments than the root hydraulic resistance (experimental points were located above the 1:1 line in Fig. 7A), while an Ohm’s law analogy would suggest simultaneous variations. However, this result did not apply to the water potential of the expanding zone. This water potential can be estimated by the sum of measured cell turgor and osmotic potential presented in Table I. Changes in total water potential 60 min after the start of the treatments were much lower in the expanding zone (decreased by 0.17–0.25 MPa depending on treatments) than in mature tissues (Fig. 4). This suggests that our treatments have triggered an increase in the resistance between the xylem and mature tissues, which did not apply to the growing zone located at the base of the leaf. Turgor of growing cells and $L_{pr}$ followed a common relationship in all treatments, supporting a physiological link between them (Fig. 7B). The same was true for the relationship between turgor and leaf elongation rate, which were linked by a common relationship throughout all treatments (Fig. 7C). Thus, there was a marked similarity between the effects of acid root loading, H$_2$O$_2$ treatment, and anoxia on $L_{pr}$, turgor, and leaf elongation rate, which only differed in amplitude. The greater the inhibiting effect of a treatment on $L_{pr}$, the greater the reduction in turgor and leaf elongation rate.

**Root Pressurization Reversed the Effects of Acid Loading or Anoxia**

Pressurization of the roots of an intact plant by means of a pressure chamber increases the water potential of the total medium-plant system. We used this method to test whether the observed reductions in leaf elongation rate were primarily caused by hydraulically mediated processes (Fig. 8). When root pressurization was applied to an intact plant that had received an acid load at pH 6.0 treatment for 30 min, the leaf elongation rate was restored to values recorded prior to the treatment (Fig. 8A). We measured water potentials in the mature, transpiring leaf 4 at 1 h after simultaneous pressurization and acid load treatment and obtained an average value of $2.03 \pm 0.13$ MPa, similar to those of nontreated plants ($n = 3$; SD). Similarly, no reduction in leaf elongation rate was observed when root pressurization was applied at the same time as anoxia to an intact plant (Fig. 8B).

**Table I. Osmotic potentials (MPa) measured on leaf tissue extracts using a vapor pressure osmometer (Wescor Vapro 5520)**

Pieces of leaf tissue were sampled on maize plants before treatment (control) or 60 min from the start of root treatment (described in the text). Leaf samples were frozen and then centrifuged to collect the liquid leaf extracts. Values are means ± SD of eight or more samples on different plants.

<table>
<thead>
<tr>
<th>Root Treatment</th>
<th>Leaf 6 (Growing Zone)</th>
<th>Leaf 6 (Mature, Emerged Zone)</th>
<th>Leaf 4 (Mature Zone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (control)</td>
<td>$-0.93 \pm 0.05$</td>
<td>$-0.74 \pm 0.11$</td>
<td>$-0.73 \pm 0.09$</td>
</tr>
<tr>
<td>Acid load, pH 6.0</td>
<td>$-0.95 \pm 0.08$</td>
<td>$-0.78 \pm 0.08$</td>
<td>$-0.79 \pm 0.09$</td>
</tr>
<tr>
<td>Acid load, pH 5.0</td>
<td>$-0.96 \pm 0.04$</td>
<td>$-0.77 \pm 0.07$</td>
<td>Not determined</td>
</tr>
<tr>
<td>Anoxia</td>
<td>$-0.87 \pm 0.04$</td>
<td>$-0.73 \pm 0.05$</td>
<td>$-0.82 \pm 0.1$</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>$-0.94 \pm 0.04$</td>
<td>$-0.76 \pm 0.05$</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

Figure 7. Relationships between hydraulic variables and leaf elongation rate across treatments. A. Reduction in $L_{pr}$ plotted against the gradient of water potential between the nutrient solution and the leaf ($\Delta W_{leaf-solution}$). B. $L_{pr}$ plotted against turgor in growing leaf cells. C. Turgor in growing leaf cells plotted against leaf elongation rate (LER). Changes in $L_{pr}$ or in water potential gradients are normalized by the initial values at 0 min (e.g. a value of 2 means a doubling at 60 min from the start of treatment compared with the initial value). Black diamonds, control (before treatment); gray circles, acid load at pH 6.0 under low evaporative demand; white circles, acid load at pH 6.0 under high evaporative demand; gray triangles, acid load at pH 5.0; gray squares, H$_2$O$_2$ treatment; crosses, anoxia. A and B, $n = 4$ plants; error bars indicate SD. C. Each point corresponds to the reductions in turgor and in leaf elongation rate measured on the same plant.
DISCUSSION

Changes in $L_p$, Induce Cessation of Leaf Elongation by Decreasing Turgor of Growing Cells

In this study, four treatments altering $L_p$, applied at two levels of evaporative demand had similar effects on the sequence of events leading up to a decrease in leaf elongation rate. All treatments induced reductions in water potential and cell turgor in the leaf growth zone. The similar patterns of the time course of the response of $L_p$, cell turgor, and leaf elongation rate to these treatments, and the consistency of the dose-dependent response, suggest that processes mediating the response of leaf elongation rate were of a hydraulic nature. Regardless of the cause of changes in $L_p$, its inhibition modulated water potentials in roots and shoots, with strongly correlated changes in turgor of growing cells and leaf elongation rate. This also provides evidence for the highly debated role of cell turgor in mediating short-term leaf growth responses to water-challenging treatments (Munns et al., 2000; Bouchabke et al., 2006). According to a recent study in parenchyma cells of maize leaf midribs (Kim and Steudle, 2007), a 4-fold decrease in $L_p$ could follow from a drop in cell turgor by about 0.2 MPa. This may amplify the consequences of changes in $L_p$, on the water potential gradient in the growing zone. However, at the tissue scale, we did not detect any change in water potential gradient between xylem and bulk leaf upon $L_p$, reduction in our experiments (Fig. 4), whereas the gradient between the solution and xylem (at the root-shoot junction) dramatically increased.

This indicated that most of the changes in hydraulic conductivity occurred across the roots but not along the leaves.

Our results are consistent with the general view that leaf elongation rate is more sensitive to hydraulic challenges than stomatal conductance, photosynthesis, or the growth of other organs (Boyer, 1970; Westgate and Boyer, 1985; Saab et al., 1990). The sensitivity of leaf elongation to changes in $L_p$, suggests that, under natural conditions, down-regulation of root aquaporins might contribute to leaf growth responses to stimuli sensed in the soil. Nevertheless, we considered the possibility that inhibition of leaf elongation rate was induced in our experiments by events other than a reduction in $L_p$. Metabolic changes in root cells due to weak acid or $H_2O_2$ treatment or a transport of these compounds via the xylem into the leaf might have directly affected leaf elongation rate. However, there are several arguments against this possibility. First, we used acid load, $H_2O_2$, and anoxia treatments, rather than $HgCl_2$, a common aquaporin inhibitor reported to affect membrane electrical potential or cell respiration (Wan and Zwiazek, 1999; Zhang and Tyerman, 1999). Second, acid loading and anoxia as well as $H_2O_2$ treatments rely on different mechanisms to reduce $L_p$ (Tournaire-Roux et al., 2003; Ye and Steudle, 2006; Boursiac et al., 2008); therefore, the very similar responses induced by the treatments were probably not the result of toxic side effects of either cytosolic acidification or oxidative stress but rather a direct consequence of the effects on $L_p$. Third, we performed experiments that ruled out the possibility of an appreciable transport of the weak acid or its metabolites into the leaf following acid load treatment. In particular, no accumulation of $^{13}$C-labeled propionic acid was found in the elongation zone of leaf 6, whereas diffusion of this compound into the roots was clearly observed. Measurements with pH microelectrodes also showed that acid load and anoxic treatments of roots did not induce any change in apoplastic pH in the leaf elongation zone (data not shown). Fourth, neither leaf elongation rate nor turgor was affected by acid load in the dark. This remains consistent with our hydraulic signal hypothesis and excludes the presence of other side effects of acid load. Finally, the reversion by root pressurization of the effects of acid load at pH 6.0 and of anoxia on leaf water potential, cell turgor, and leaf elongation provided evidence that these changes were primarily due to hydraulic signals. In our experiments, these signals were specifically triggered by treatment-induced reductions in $L_p$.

Aquaporin Inhibitors Induced Rapid Changes in Hydraulic Conductivity in Maize Roots

Cell and root hydraulic conductivities were reduced by acid load in maize, as they are in Arabidopsis (Gerbeau et al., 2002; Tournaire-Roux et al., 2003). These decreases are mediated by cell acidosis and subsequent proton-dependent gating of PIP aquaporins.
showed that, in Arabidopsis, the H2O2-induced de-
interfere with the activity of aquaporins by oxidative
demand and in stomatal conductance, because sto-
the plant primarily follows changes in evaporative
depressurization or pressurization (Lu and Neumann,
using special devices allowing the control of root
subjects to a stabilized gradient of water potential
had no effect on the hydraulic conductivity of the root
measured in the intact root. This indicates that the H2O2
treatment did not directly inhibit aquaporin activity,
which induced a dramatic decrease in leaf water po-
tential. As a consequence, transpiration was steady and the
water flux across maize roots under all evaporative
demands tested.

We also confirmed the previously reported de-
creases of maize Lp, in response to H2O2 treatment
(Aroca et al., 2005; Ye and Steudle, 2006). Ye and
Steudle (2006) suggested that millimolar concentra-
tions of H2O2, as applied in this work, may directly interfere with the activity of aquaporins by oxidative gating. Conversely, Bourssiac et al. (2008) recently showed that, in Arabidopsis, the H2O2-induced decreases in Lp, were mediated by a signaling cascade that involved the relocation of PIP aquaporins into intracellular compartments. The amplitude and speed of Lp, inhibition in maize roots, together with the inhibition of water transport at the cell level in our work and the work of Ye and Steudle (2006), support the idea that the H2O2 treatments result in an inhibi-
tion of aquaporins.

A composite transport model of water in the root
has been proposed (Steudle and Freisch, 1996; Steudle
and Peterson, 1998). This model accounts for variable
contributions of transmembrane (where aquaporins
may exert a control) and apoplastic (independent of
aquaporin activity) pathways to the overall root water
uptake, depending on the nature and the intensity of
the driving force. In agreement with previous studies
(Aroca et al., 2005; Ye and Steudle, 2006), our results
suggest that aquaporins mediate a large proportion of
water flow across maize roots under all evaporative
demands tested.

Why Did Water Potentials Change with Hydraulic
Conductivity without Changing Water Flux throughout
the Plant?

Despite large reductions in Lp, in response to root
 treatments, no associated changes in water uptake or
plant transpiration rate were observed in this study
under low evaporative demand. This contrasts with the
assumption that the rate of water flow follows
fluctuations in Lp,. Concomitant changes in water flow and
Lp, were observed when excised roots were subjected to a stabilized gradient of water potential
using special devices allowing the control of root
depressurization or pressurization (Lu and Neumann,
1999; Quintero et al., 1999; Martinez-Ballesta et al.,
2003). In intact transpiring plants, the water flux across
the plant primarily follows changes in evaporative
demand and in stomatal conductance, because sto-
matas represent by far the dominant resistance barrier.
The hydraulic conductivity of inner tissues can affect
water flux either by becoming markedly limiting or by
indirectly interfering with stomatal behavior. This was
clearly not the case in our experiments, in which the
low evaporative demand was maintained steady and the
stomatal conductance was not affected by the
application of aquaporin inhibitors on roots. Conse-
sequently, transpiration was steady and the water po-
tential gradient between the root medium and the
xylem at the leaf base was increased and counteracted
the Lp, reduction, at least under low evaporative
demand.

This does not rule out the possibility that a reduction
in Lp, causes stomatal closure and thereby could reduce
plant transpiration rate under other circumstances. This
was observed in this study when Lp,-reducing treat-
ments were done under higher evaporative demand,
which induced a dramatic decrease in leaf water po-
tential, down to values similar to those observed in
maize in field conditions (Tardieu and Simonneau,
1998). Previous studies of aspen (Populus species; Wan
and Zwiazek, 1999) and pepper (Capsicum annuum;
Martinez-Ballesta et al., 2003) also reported that, on a
slightly longer term (2–2.5 h), exposure of the roots to
HgCl2 (0.05 and 0.1 mM HgCl2, respectively) induced a
significant decrease in stomatal conductance.

It seems surprising that water potential gradients
from solution to mature leaves were more sensitive to
root treatments than Lp,. For example, the 2-fold in-
crease in root hydraulic resistance (the reciprocal of Lp,)
induced by H2O2 treatment should have induced a no
more than 2-fold increase in water potential gradient
between leaf 4 and the nutrient solution. In the absence
of appreciable changes in osmotic potential, this sugges-
ts that a gradient of water potential developed
between the elongation zone at the base of the leaf 6,
where turgor was measured, and the mature leaves.
Cavitation may have developed in the xylem of mature
leaves, consistent with the long-lasting decreases in leaf
and xylem water potentials following root treatment.

Short-Term Effects of Anoxia on Leaf Elongation Follow
the Same Hydraulic Cascade as Induced by Chemical
Inactivation of Root Aquaporins

While an early and conserved response of plants to
anoxia is the down-regulation of Lp, (Birner and
Steudle, 1993; Else et al., 1995; Tounnaire-Roux et al.,
2003), rapid stomatal closure and reductions in leaf
elongation rate can also be observed, but the mecha-
nisms underlying these responses are still debated
(Reumann and Smit, 1991; Else et al., 1995, 2001; Dat
et al., 2004). In anoxia-susceptible plant species, in-
cluding maize, anoxia induces modifications in root
metabolism or photosynthesis (Geigenberger, 2003).
Smit et al. (1990) proposed along these lines that in
poplar, transport of a root chemical stimulus to the
shoot may be necessary to cause a reduction in leaf
elongation. Yet, neither abscisic acid nor cytokinin
concentrations were changed in growing leaf tissues. In tomato (*Solanum lycopersicum*), soil flooding resulted in a rapid reduction in leaf elongation and water potential, which could not be reversed by root presurization (Else et al., 1995). This was interpreted as evidence that chemical, rather than hydraulic, messages were involved in this response. In castor bean (*Ricinus communis*), by contrast, the same authors found that the reduction in leaf growth induced by oxygen deprivation was caused by a negative hydraulic signal generated by a $L_P$ reduction (Else et al., 2001).

In our study, in spite of the multitude of physiological alterations that are possibly triggered by anoxia, the cascade of hydraulic responses observed in oxygen-deprived maize plants was remarkably similar to that induced by aquaporin inhibitors. Although the effects of anoxia were less pronounced and slower than those of other root treatments, the synchrony of $L_P$ reduction, decrease in cell turgor, and inhibition of leaf elongation were conserved. Furthermore, root presurization reversed the short-term effect of anoxic stress on leaf elongation rate. These observations provide converging evidence that in maize, the reduction of $L_P$ induced by anoxia was propagated as a decrease in leaf water potential and cell turgor, which in turn resulted in an inhibition of leaf elongation. Finally, differences in the time scales of response may partly reconcile the discrepancy in leaf growth responses raised by studies published to date. In this work, we concentrated on short-term plant responses to $L_P$ changes. On this time scale, changes in turgor appear to determine most of the leaf elongation responses. In longer term studies, the hydraulic component may be overridden by osmotic adjustments (Van Volkenburgh and Boyer, 1985), growth-depressive effects of hormones such as cytokinins (Smit et al., 1990) or ethylene (Shiu et al., 1998), or changes in cell wall rheology.

In conclusion, this study provides evidence that changes in $L_P$ can be crucial determinants for rapid plant responses to environmental stresses. Changes in cytosolic pH and $H_2O_2$ have recently emerged as cellular signals triggered by various external stimuli and mediating pronounced and rapid changes in $L_P$ (Birner and Steudle, 1993; Tournaire-Roux et al., 2003; Lee et al., 2004; Aroca et al., 2005). Therefore, the cascade of hydraulic responses triggered by acid load or external $H_2O_2$, as described here, could be of general significance for the response of plants to various environmental stresses in the short term. The relevance of hydraulic signaling and leaf growth response resulting from $L_P$ reduction in a wider range of environmental stresses and on a longer term deserves further investigation.

### MATERIALS AND METHODS

#### Plant Growth Conditions

Maize seeds (*Zea mays ‘Dea’*) were germinated on a wet tissue in the dark at 24°C for 2 d. Seedlings were then placed in tubes with their roots bathing in a continuously aerated solution with the following composition: 0.25 mM CaSO$_4$, 0.8 mM KNO$_3$, 0.6 mM KH$_2$PO$_4$, 0.2 mM MgSO$_4$ (7H$_2$O), 0.4 mM NH$_4$NO$_3$, 2 $\times$ 10$^{-3}$ mM MnSO$_4$, 0.4 $\times$ 10$^{-3}$ mM ZnSO$_4$, 0.4 $\times$ 10$^{-3}$ mM CuSO$_4$, 0.2 $\times$ 10$^{-3}$ mM Na$_2$MoO$_4$·(2H$_2$O), 1.6 $\times$ 10$^{-3}$ mM H$_3$BO$_3$, 0.04 mM Fe-EDDHA, and 2.5 mM MES, pH 5.5 to 5.8. The hydroporic solution was renewed every 3rd to 4th d. Plants were grown in a growth chamber at a VPD of 0.8 kPa, with cycles of 14 h of light (400 μmol m$^{-2}$ s$^{-1}$) at 24°C and 10 h of dark at 20°C.

#### Acid Load, $H_2O_2$, and Anoxia Treatments and Manipulation of Evaporative Demand

All root treatments were applied, for 60 to 120 min, to the root system of intact plants, which, unless otherwise stated, had a leaf 6 of 15 to 20 cm in length. During the treatments, plants were kept under the same conditions as described above. The evaporative demand was manipulated by changes in VPD (0.8, 1.3, or 2.8 kPa) and/or photosynthetic photon flux density (0, 150, or 400 μmol m$^{-2}$ s$^{-1}$). Measurements of leaf elongation rate, turgor, and water flux were made under identical environmental conditions except that light intensity was at 150 μmol m$^{-2}$ s$^{-1}$. Acid load treatments consisted of applying a weak acid to the roots and were imposed by substitution at constant pH (pH 6.0 or 5.0); 20 mM propionic acid/potassium propionate replaced 20 mM KCl in the nutrient solution, as described by Tournaire-Roux et al. (2003). A more acidic pH (pH 5.0) was also used to enhance diffusion of propionic acid into root cells. $H_2O_2$ was applied to plant roots at a concentration of 2 mM $H_2O_2$ in the nutrient solution. Anoxia was induced by bubbling the nutrient solution with $N_2$, and the depletion of oxygen was monitored using an oxygen electrode.

#### Measurements of Cell Hydraulic Conductivity of Root Cortex Cells

Cell pressure probe measurements on root cortex cells were performed as described by Gerbeau et al. (2002) and Tournaire-Roux et al. (2003). Seminal and nodal root tip segments of a length of 30 to 35 mm were excised from dehydrated maize plants and preincubated in a solution containing either 20 mM KCl and 10 mM MES, pH 6.0 (control), or 20 mM propionic acid/potassium propionate and 10 mM MES, pH 6.0 (acid load). The segments were laid horizontally on a filter paper and partially submerged by perfusion with the same solution. Pressure probe measurements were made at a distance of 21 to 27 mm from the root apex. Cells were impaled with micropipettes with an external tip diameter of 3 to 4 μm. After impalement of a root cortex cell, the meniscus was stabilized at its initial position. For hydrostatic relaxation measurements, a pressure change was applied to the cell and the meniscus was rapidly moved forward and backward and kept at a constant position during the relaxation procedure. Turgor and $t_{1/2}$ could be continuously monitored in individual cortical cells over periods of up to 10 min. The cell volumetric elastic modulus ($c$) was obtained from pressure changes ($\Delta P$) recorded after experimentally imposed volumetric changes ($\Delta V$) according to the following equation (Javot et al., 2003):

$$c = \frac{\Delta P}{\Delta V}$$

where $V$ (m$^3$) is the mean cell volume.

Mean cell diameter and cell length values of 27.6 and 170 μm, respectively, were determined from longitudinal sections on three to four plants (Muller et al., 1998) and used to calculate mean cell volume ($V$) and cell area ($A$). The $L_P$ cell was calculated from the following equation:

$$L_P_{cell} = \frac{V}{A \ln \left(1 - c + P_{e} - P_{wa}\right)}$$

where $V$ (m$^3$), $A$ (m$^2$), $t_{1/2}$ (s), and $c$ (MPa) are as defined above and $P$ (MPa) and $P_{wa}$ (MPa) represent the cell turgor and the external osmotic water potential, respectively.

#### $L_P$, Measurements in Whole Seminal Root Systems

A xylem sap flow was triggered in excised root systems by applying vacuum-induced subatmospheric pressures between −0.02 and −0.08 MPa, as described by Freundl et al. (1998) and Hose et al. (2000). The seminal root system was excised by sectioning the mesocotyl, which was then tightly fixed to silicon tubing using low-viscosity dental paste (President Light; Coltene).
Whaledent). To determine the rate of water flow across the root system, a water trap, made of a 2-mL tube filled with dry cotton, was sealed onto the tubing. Water flow was determined by weighing the xylem sap absorbed by a piece of cotton every 10 min; the cotton piece was renewed after each measurement. Free exudation was determined at atmospheric pressure, starting 30 min after root excision. Then, the tubing was connected to a vacuum port equipped with a precision tension gauge. Tension data were averaged and stored every 60 s using a data logger (Campbell Scientific; LTD-CR10). A two-value system, driven by the data logger, automatically controlled the tension applied to the seminal roots. The water flux was subsequently measured for at least 60 min at three different suction forces ranging from −0.02 to −0.06 MPa. Acid load, H₂O₂, or anoxia treatment was then applied to the root system, and the solution was replaced by fresh nutrient solution after 1 h. Anoxia treatment was released by simply rebubbling the solution with air. The xylem sap was harvested for at least 60 min at the three different suction forces during and after the treatment. At the end of one experiment, the root system was dried from excess water using paper towels and weighed. The root surface was deduced from an independently determined relationship between the mass and surface of seminal root systems.

**Measurements of Plant Water Transport Rates and Stomatal Conductance**

Transpiration rate and stomatal conductance were measured on intact plants in a growth chamber under moderate evaporative demand (VPD of 1.3 kPa, photosynthetic photon flux density of 150 μmol m⁻² s⁻¹) and under high evaporative demand (VPD of 2.8 kPa, photosynthetic photon flux density of 400 μmol m⁻² s⁻¹). On the day before measurements were taken, individual maize plants were placed into 400-mL, 20-cm-high cylindrical containers filled with a nutrient solution containing 20 mM KCl, pH 6.0, and transferred into a growth chamber set at identical environmental conditions as described above. Transpiration measurements were obtained by recording changes in weight (of the cylinder containing the nutrient solution and the maize plant) on a balance (Precisa). Direct evaporation from the solution was avoided by covering the solution with a layer of air and bubbling with water-saturated air. Weight loss data were averaged and stored every 10 s using a data logger (Campbell Scientific; LTD-CR10). Weight loss was plotted against time, and leaf transpiration rate was calculated at intervals of 30 min by the slope of this relationship. Changes in weight caused by substitution of nutrient solution due to acid loading were excluded from calculations. Transpiration rates were divided by leaf area (A) calculated from leaf width (W) and length (L) as follows: \( A = 0.75 \times W \times L \). Additionally, a gas-exchange system (CIRAS; PPSystems) was used to continuously measure stomatal conductance on leaf 4 before and during the acid load treatments.

Measurements of root water uptake rate required a slight modification of this experimental setup. A maize plant was held at its leaf base by a metal holder, which was placed on a second balance of the same type. Thereby, one balance measured the weight loss of the solution container only while the other balance measured the changes in weight of the plant. Root water uptake was calculated from weight loss of the solution container, and plant transpiration was obtained from the total weight changes of both balances. Mean root water uptake and transpiration rates were obtained from calculations on six plants under moderate evaporative demand (VPD of 1.3 kPa).

**Measurements of Leaf Elongation Rate**

Leaf elongation rate and turgor were simultaneously monitored on the same plant. For this, linear displacement transducers (LVDTs; L100; Chauvin Arnoux) were connected to the tips of the growing leaf 6 with linen threads (Bouchabke et al., 2006). The LVDTs were connected to a data logger (Campbell Scientific; LTD-CR10), and data were averaged and stored every 1 min. Leaf elongation rate was calculated from the displacement of the LVDT during intervals of 20 min. To check that changes in leaf elongation rate were not induced by changes in meristem temperature, a fine copper-constantan thermocouple (0.2 mm diameter) was inserted between the sheaths of leaves 1 and 2 at the height of the meristem (Sadok et al., 2007).

**Cell Turgor Measurements**

A cell pressure probe was set up in the growth chamber to permit turgor measurements in the elongation zone of leaf 6 while simultaneously measuring leaf elongation rate. Two hours prior to measurements, a window of 12 mm length and 5 mm width was cut through the bases of leaves 2 to 5 to access the elongation zone of leaf 6. The exposed zone of this leaf was covered with a thin layer of silicon gel to avoid any water loss that could have altered cell water status. Cell turgor was measured as described previously (Bouchabke et al., 2006). Consistent with the report of Bouchabke et al. (2006), we observed that leaf elongation rate was decreased by approximately 50% after cutting a window through the outer leaf layers and that it remained stable afterward. For this study, we retained plants for leaf elongation rate and turgor measurements that exhibited a leaf elongation rate higher than 1.5 mm h⁻¹ after window opening.

Cells were impaled with a microcapillary filled with silicon oil and connected to a pressure transducer. Readings were repeated up to three times following several back-and-forth movements of the meniscus formed at the contact of the cell sap with the silicon oil. The pressure was read each time the cell sap was pushed back to its location within the cell prior to impalement. Only repeatable measurements with less than 0.05 MPa min⁻¹ deviation during the successive readings were kept. Before applying the root treatment, the initial average cell turgor was calculated for each plant from five to six measurements on different cells. Cell turgor was then measured for 1 to 2 h after applying the treatments. Mean turgor values at each time point correspond to three to seven measurements made in different cells of the same plant. The mean turgor values presented in the figures were calculated for five to seven plants per treatment. After treating the plants for 1 or 2 h, the treatment was reversed by applying control nutrient solution or rebubbling the solution with air, and turgor measurements were pursued as described above.

**Water Potential Measurements**

Water potentials of leaves 2 and 4 were measured with a pressure chamber (Soil Moisture Equipment). On the day before measurements, the back part of the plant including leaves 1 and 2 was wrapped in aluminum foil in order to prevent transpiration and to allow water potential equilibrium between leaf 2 and its insertion point (Supplemental Fig. S1). Thus, the water potential of leaf 2 was considered as indicative of the water potential within the xylem (Simonneau and Habib, 1991), whereas measurement on leaf 4 characterized the water potential of fully developed transpiring leaves. Prior to the root treatments, initial water potentials of leaves 2 and 4 were measured for five to six control untreated plants. To exclude possible diurnal fluctuations, water potentials were also measured in five to six control plants at 120 min (i.e. at the end of the measurement series).

**Osmotic Potential Measurements**

The osmotic potential of elongating (leaf 6) leaf tissue was measured using a vapor pressure osmometer (Vapro 3520; Wescor). Leaf tissue samples of 40 mm length were excised, placed in 1.5-mL reaction tubes, and immediately transferred to liquid nitrogen. The tissue sap was extracted by centrifugation (5 min at 10,000g). Filter paper discs (10 mm diameter) were soaked with 10 μL of the extracts and introduced into the chamber of the osmometer to determine the osmotic potential.

**Root Pressurization of Intact Plants**

The root systems of intact maize plants were inserted into a customized pressure chamber similar to previously described devices (Passiourea and Munns, 1984). Plants were tightly fixed to the chamber cap by silicon seals using a screw. Pressures between 0.08 and 0.1 MPa were applied to the root system either directly or 30 min after the onset of acid load at pH 6.0 or anoxia. The chamber was equipped with a precision pressure sensor connected to a data logger (Campbell Scientific; LTD-CR10). Pressure data were averaged and stored every 60 s. A two-value system, driven by the data logger, allowed the automatic control of the pressure applied to the root system. Leaf elongation rates were monitored before and during root pressurization as described above. After 60 min of acid loading, the water potential of leaf 4 was measured.

**Analytical Procedure for 13C Analysis in Root and Leaf Tissue by Mass Spectroscopy**

13C-labeled propionic acid (13CH₃CH₂CO₂H; Sigma) was used in an acid-loading experiment at pH 6.0 at a final concentration of 1 mM sodium propionate. Segments of 40 mm length were excised from the root apex, at a...
distance of 10 mm from the leaf 6 insertion point (elongating tissue) and at the tip of leaf 6 (nonelongating tissue). Tissue sampling was made before the treatment and 120 min after the application of the acid-loading treatment. Tissue samples from six plants at each time point were used for calculating the mean 13C content. The sample preparation for mass spectrometry analysis was done as described by Lejay et al. (1997). Briefly, the tissues were dried, ground, and combusted. The 13C content (δ13C/δ12C) of the released CO2 was then measured using an online mass spectrometer (GV Instruments). Isotopic signatures were expressed as the ratio δ13C/δ12C relative to carbon standards as described previously by Maros et al. (2006).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Comparison of water potentials measured on leaves 2 and 4 previously wrapped in aluminum foil to prevent transpiration.

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**LITERATURE CITED**


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