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Long-distance signals regulating stomatal conductance and leaf growth in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying

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Abstract

Tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) plants were grown with roots split between two soil columns. After plant establishment, water was applied daily to one (partial root-zone drying—PRD) or both (well-watered control—WW) columns. Water was withheld from the other column in the PRD treatment, to expose some roots to drying soil. Soil and plant water status were monitored daily and throughout diurnal courses. Over 8 d, there were no treatment differences in leaf water potential (ψ_{leaf}) even though soil moisture content of the upper 6 cm (θ) of the dry column in the PRD treatment decreased by up to 70%. Stomatal conductance (g_s) of PRD plants decreased (relative to WW plants) when θ of the dry column decreased by 45%. Such closure coincided with increased xylem sap pH and did not require increased xylem sap abscisic acid (ABA) concentration ([X-ABA]). Detached leaflet ethylene evolution of PRD plants increased when θ of the dry column decreased by 55%, concurrent with decreased leaf elongation. The physiological significance of enhanced ethylene evolution of PRD plants was examined using a transgenic tomato ($ACO1_{AS}$) with low stress-induced ethylene production. In response to PRD, $ACO1_{AS}$ and wild-type plants showed similar xylem sap pH, [X-ABA] and g_s, but $ACO1_{AS}$ plants showed neither enhanced ethylene evolution nor significant reductions in leaf elongation. Combined use of genetic technologies to reduce ethylene production and agronomic technologies to sustain water status (such as PRD) may sustain plant growth under conditions where yield would otherwise be significantly reduced.

Key words: Abscisic acid, $ACO1_{AS}$, ethylene, leaf growth, long-distance signalling, partial root-zone drying, stomatal conductance, tomato, xylem pH.

Introduction

When plants are exposed to drying soil, stomatal conductance (g_s) and leaf growth can be regulated by long-distance chemical signals travelling from root to shoots, independently of shoot water status (reviewed in Davies and Zhang, 1991). One way of demonstrating this non-hydraulic limitation of g_s and leaf expansion was provided by growing maize (*Zea mays*) plants with roots split between two pots of soil (Blackman and Davies, 1985). By keeping one pot well-watered and allowing the roots to dry the soil in the other, partial stomatal closure occurred in the absence of changes in leaf water status. Further evidence that roots can control shoot responses was provided by a split-root experiment showing that excising those roots that were in drying soil restored expansion of apple (*Malus×domestica*) leaves (Gowing *et al*., 1990). Root excision cannot make more water available to the shoot, but it removes a potential source of chemical growth inhibitors. Experiments such as these have stimulated the search for the identity of chemical regulators controlling shoot processes.

Due to the potent antitranspirant effect of abscisic acid (ABA), it is not surprising that researchers have addressed the possibility that increased ABA delivery to shoots could account for changes in g_s. Several comprehensive data sets from field and glasshouse studies, in a diverse range of species, showed an excellent correlation between g_s and xylem sap ABA concentration ([X-ABA]) when xylem sap

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was collected from the same leaves in which g_s was measured (Tardieu *et al*., 1992, 1996; Borel *et al*., 2001). Importantly, the relationship between g_s and [X-ABA] for a given species is commonly unified across different growing conditions, from leaf to leaf on individual plants, and from day to day as the plant develops (Tardieu *et al*., 1992), providing good evidence of a causal relationship. Feeding synthetic ABA to well-watered plants generates the same relationship between g_s and [X-ABA] as seen in drying soil (Tardieu *et al*., 1996). However, several reports indicate that variation in [X-ABA] alone cannot always explain the extent of drought-induced stomatal closure. Detached leaf transpiration assays suggested that other antitranspirant compounds are present in wheat (*Triticum aestivum*) xylem sap, as the antitranspirant effect of the sap could not be explained in terms of its ABA concentration (Munns and King, 1988). The importance of [X-ABA] in mediating drought-induced stomatal closure has been addressed by reciprocal grafting of wild-type (WT) and ABA-deficient genotypes and comparing the stomatal responses and ABA concentrations of the graft combinations (Holbrook *et al*., 2002). Irrespective of whether WT shoots were grafted on WT or ABA-deficient roots, stomatal closure occurred in both graft combinations, despite a 4-fold difference in [X-ABA]. Experiments such as these suggest that chemical regulators other than ABA can act as signals of the degree of soil drying and can be important in influencing stomatal behaviour.

Alkalization of xylem sap is a common response to various edaphic stresses (Wilkinson *et al*., 1998) and increasing the alkalinity of buffers supplied to detached leaves via the transpiration stream restricted transpiration (Wilkinson and Davies, 1997; Wilkinson *et al*., 1998). These buffers were believed to increase apoplastic pH, which was demonstrated to decrease sequestration of ABA by mesophyll cells. The resultant increase in apoplastic ABA concentrations ultimately closed the stomata (Wilkinson and Davies, 1997). Stomatal closure in response to xylem-supplied buffers was ABA-dependent, as leaves detached from an ABA-deficient tomato (*Lycopersicon esculentum*) mutant (*flacca*) did not show stomatal closure when fed pH 7 buffers (Wilkinson *et al*., 1998). In WT plants, it is possible that increased xylem sap pH could elicit ABA-dependent stomatal closure without any need for increased xylem ABA delivery to the shoot. Temporal changes in xylem sap pH and ABA concentration during soil-drying episodes have been measured, and increased xylem sap pH preceded (Bahrun *et al*., 2002) or followed (Liu *et al*., 2003) increased [X-ABA]. Both studies collected xylem sap from the shoot base, and since xylem sap composition can vary with the site of sap collection (Jokhan *et al*., 1999), there is a need to measure xylem sap pH and [X-ABA] in leaves actually responding to drying soil.

The role of ABA in controlling leaf expansion of plants grown in drying soil is less certain. Xylem ABA concentrations of field- and glasshouse-grown droughted maize plants (Ben Haj Salah and Tardieu, 1997) are high enough to partially inhibit leaf growth when fed via the xylem to detached maize shoots (IC Dodd, unpublished observations). ABA was more effective as a growth inhibitor as prevailing evaporative demand increased (Ben Haj Salah and Tardieu, 1997). However, in some cases maize leaf growth was restricted before [X-ABA] increased (Munns and Cramer, 1996). Also, xylem sap collected from droughted wheat and barley (*Hordeum vulgare*) plants contained 2–8 nM ABA, and inhibited growth in detached shoots by 60%, yet 10 μM synthetic ABA was required to inhibit leaf growth to the same extent (Munns, 1992). This suggested that [X-ABA] was not a major regulator of leaf growth in these species. However, subsequent investigations have revealed a key role for xylem or apoplastic pH in determining the apparent sensitivity of leaf growth to ABA.

Increased xylem sap pH can also correlate with drought-induced leaf growth inhibition, and increasing the alkalinity of buffers fed via the xylem to detached barley shoots inhibited leaf elongation (Bacon *et al*., 1998). Increasing buffer pH from 6 to 7 did not inhibit leaf growth of an ABA-deficient barley mutant (*Az34*), unless an ABA concentration typical of well-watered WT plants was also present in the buffer. These responses are directly analogous to the effect of alkaline buffers on detached leaf transpiration (discussed earlier). It was suggested that increased sap alkalinity allowed ABA access to sites of action within the leaf elongation zone, inhibiting growth. In well-watered plants with a more acidic, apoplastic pH, ABA is presumably partitioned into alkaline compartments in the symplast and away from sites of action regulating leaf growth. These results suggest that there is always enough ABA present in the leaf to regulate both leaf growth and stomatal behaviour in WT plants, but whether or not this occurs depends on the role of xylem or apoplastic pH in partitioning ABA away from or towards sites of action in the leaf.

Work with ABA-deficient mutants suggests that an important function of ABA is to restrict synthesis of another potential inhibitor of leaf expansion, ethylene (Sharp and LeNoble, 2002). Although soil compaction (Hussain *et al*., 1999) and decreased N availability (Lege *et al*., 1997) can increase ethylene evolution, an influential report indicated that water stress did not increase ethylene evolution of intact plants (Morgan *et al*., 1990). However, soil drying can also increase soil strength and change nutrient availability to roots, and it therefore seems possible that some soil-drying episodes can perturb ethylene evolution, thus modifying leaf growth. Hence, the possibility that ethylene might be involved in the regulation of leaf growth when the soil dries was re-examined. To minimize the effects of increasing soil strength as the soil dried, an organic-based potting compost of low soil bulk density was used deliberately.

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To investigate the role of different chemical regulators (ABA, pH, and ethylene) in controlling shoot physiology of plants exposed to drying soil, independently of leaf water status, tomato plants with roots split between two soil columns were grown. In half the plants, water was added daily to one column, while the roots in the other column were allowed to dry the soil. This technique maintained leaf water status of several species subjected to drying soil (Blackman and Davies, 1985; Stoll *et al*., 2000), and similar maintenance of leaf water status was achieved here. It was desirable to work with tomato since single leaves yielded sufficient xylem sap for analysis, and mutants and transgenics deficient in some of the chemical regulators described above are available.

Materials and methods

Plant material and culture

Isogenic wild-type (cv. Ailsa Craig) and $ACO1_{AS}$ (Hamilton *et al*., 1990) genotypes of tomato (*Lycopersicon esculentum* Mill.) were used. The $ACO1_{AS}$ genotype has decreased aminocyclopropane-1-carboxylic acid (ACC) oxidase activity and is thus less able to convert the ethylene precursor, ACC, to ethylene. During germination and subsequent growth, plants were maintained in a single controlled environment cabinet under a 12 h photoperiod, 25/22 °C day/night temperature, and 220 μmol m^{-2} s^{-1} photosynthetic photon flux density.

Seeds were sown in a well-watered peat-based compost (Levingtons M3, Levington Horticulture Ltd, Ipswich, UK) in seedling trays, with a single seed in each separate compartment (3 cm deep×2 cm×2 cm). The volumetric water content of this compost at field capacity was 0.56 cm^3 cm^{-3}, and the bulk density when dry was 0.18 g cm^{-3}. After 14 d, when the first true leaf had emerged, seedlings were transferred to split-pots containing the same compost, comprising two plastic rectangular columns (23 cm deep×6.5 cm×6.5 cm) that were taped together. Where the two individual columns adjoined, part of the plastic wall of the pots (3 cm deep×2 cm wide) had been removed to allow each seedling compartment to be inserted into the potting compost, minimizing seedling disturbance. Immediately following seedling transfer and daily thereafter, pots were watered to the drip point to prevent the development of plant water deficit. Plants were staked when the fifth leaf emerged. Side-shoots were removed daily and floral trusses were removed at the first sign of petal colour in the first flower.

Five or six weeks after germination, two different watering regimes were imposed. At the start of the photoperiod, one (partial root-zone drying—PRD) or both (well-watered control—WW) columns were watered to the drip point. Water was withheld from the other column in the PRD treatment to expose some of the root system to drying soil. In experiments with only one genotype, treatments were randomly arranged in the growth cabinet. In experiments with two genotypes, one plant of each treatment combination was randomly assigned to a block, and blocks of four randomly distributed plants were arranged in the growth cabinet. Following physiological measurements in which leaf tissue was removed, these replicates were removed from the cabinet and the remaining undisturbed plants were re-randomized with respect to position in the cabinet.

Physiological measurements

While leaf length was always measured only once during the middle of each photoperiod, other measurements were made every 1 h (Experiment 3), every 3 h (Experiment 2), or once during the middle of the photoperiod (Experiments 1, 4, 5). Elongation of an entire young, expanding leaf (from the nodal junction to the tip of the terminal leaflet) and of the terminal leaflet only (from leaflet base to tip) was measured, using a piece of graph paper photocopied onto acetate. Abaxial stomatal conductance of 3–5 leaflets per fully expanded leaf was measured using a diffusion porometer (AP4, Delta-T, Cambridge, UK). Replicated measurements of leaf elongation and g_s (n=5 plants per treatment) from several leaves on the one plant showed no systematic effects of the node of leaf insertion on responses to drying soil (data not shown).

The apparent dielectric constant of the upper 6 cm of compost was measured using a soil moisture probe (Type ML2X attached to a HH2 Moisture Meter, Delta-T Devices, Burwell, UK). Readings were converted from microvolts to volumetric soil moisture content (θ), based on a two-point calibration (field capacity and oven-dried soil) with the same potting compost.

Leaf water potential (ψ_{leaf}) was determined using a Scholander-type pressure chamber (Plant Moisture Systems, Santa Barbara, CA, USA) where the chamber was lined with moistened filter paper. The pressure chamber was located adjacent to the controlled environment cabinet to minimize the time between leaf excision and sealing the leaf into the pressure chamber.

Following measurement of ψ_{leaf}, an overpressure of 0.2–0.4 MPa was applied to leaves to collect xylem sap. Sap samples were stored on ice in the dark prior to pH determination using a microelectrode (Lazar Research Laboratories, Los Angeles, CA, USA). After pH measurement, saps were frozen in liquid nitrogen and stored at −20 °C prior to determination of ABA concentration with a radioimmunoassay (Quarrie *et al*., 1988), using the monoclonal antibody AFRC MAC 252 (kindly provided by Dr S Quarrie). Replicated measurements of [X-ABA] (n=4 plants for both node and overpressure experiments) from several leaves on the one plant showed no systematic effects of the node of leaf insertion (Leaves 5, 8, and 11 numbering from the base of the plant) or the overpressure applied (0.3 and 0.5 MPa) on [X-ABA] (data not shown).

Leaf ethylene evolution was measured as described previously (Hussain *et al*., 1999). Entire leaflets were removed from expanding leaves and placed in 14 cm^3 glass vials containing saturated filter paper. Vials were then immediately sealed with screw caps with a silicone septum and incubated for 1 h. A 1 cm^3 sample of air was extracted through the septum and injected into a gas chromatograph (Pye Unicam 204) fitted with a 1.5 m Poropak N (80–100 mesh) column maintained at 120 °C, and fitted with a flame ionization detector. The rate of ethylene evolution was calculated as a function of tissue fresh weight (FW), which was measured immediately after injection of the head-space sample into the gas chromatograph. Preliminary experiments showed stable ethylene evolution rates for up to 1 h from detachment.

Statistics

Student's unpaired t-tests were used to determine significant PRD effects within a genotype. In experiments with the two genotypes, analysis of variance (ANOVA) was used to detect significant effects of genotype, treatment, and their interaction. Linear regression analysis was used to determine the influence of soil moisture content on leaf growth rate.

Results

Diurnal courses of shoot physiology and chemical regulators in WT plants (Experiments 1–3)

An initial experiment (Experiment 1), in which physiological parameters were measured on a daily basis, showed

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that stomata began to close and leaf elongation slowed, on the third and fifth days, respectively, after imposing PRD (data not shown). Further experiments in which some variables were measured during diurnal cycles thus concentrated on Days 2 and 3 (Fig. 2) and Days 4–9 (Fig. 1) after imposing PRD, to determine temporal correlations between leaf growth, g_s, ψ_{leaf}, and changes in chemical regulators.

Following imposition of PRD, the volumetric water content of the upper 6 cm of compost (θ) of the drying column of PRD plants declined reasonably quickly until Day 4 (Fig. 1a) and more slowly thereafter. Measurements of θ from columns of WW plants (not shown in Fig. 1a) were statistically indistinguishable from measurements of θ from the well-watered column of PRD plants, which maintained an average θ of 0.43 cm^3 cm^{-3}. After 9 d of PRD, θ of the drying column of PRD plants was only 30% of well-watered soil columns.

The elongation rate of both entire leaves and terminal leaflets of Leaf 11 (numbering from the base of the plant) of PRD plants decreased on Day 5 (Fig. 1b). On this day, leaf ethylene evolution of PRD plants was significantly greater than WW plants throughout the photoperiod, and continued to increase (relative to WW plants) for the remainder of the experiment (Fig. 1c). The ψ_{leaf} did not differ significantly between WW and PRD plants at any time throughout the experiment (Fig. 1d).

At the end of the experiment (after 8 complete days of PRD), the mean ($\pm$SE) area of Leaf 11 of PRD plants (154 ± 8 cm^2, n=4) was 15% less than WW plants (181 ± 16 cm^2, n=4), although these differences were not statistically significant (P=0.18). Similar effects on total plant leaf area were found: the mean ($\pm$SE) area of PRD plants (1815 ± 92 cm^2, n=4) was 12% less than WW (2067 ± 79 cm^2, n=4) plants, although again these differences were not statistically significant (P=0.08).

At the time that measurements of g_s were initiated on Day 4, g_s of PRD plants was only 64% of WW plants. This difference was maintained throughout the experiment (Fig. 1e). PRD plants had more alkaline xylem sap than WW plants on Days 4 and 5, but this difference was not seen on Days 7 and 9 (Fig. 1f). When measured, [X-ABA] did not significantly differ between WW and PRD plants during the first 7 d of the experiment. On Day 9, the mean ($\pm$SE) [X-ABA] of PRD plants (430 ± 88 nM, n=4) greatly exceeded that of WW plants (173 ± 27 nM, n=4) (Fig. 1g).

To more closely study the physiological changes occurring when stomatal closure began, measurements were made hourly throughout two diurnal cycles (Fig. 2). Reductions in g_s caused by PRD were first seen during the third photoperiod following the imposition of PRD (Fig. 2c). There were no treatment differences in ψ_{leaf} at this time (Fig. 2b). There were no treatment differences in xylem sap pH during the second photoperiod following the imposition of PRD, but xylem sap of PRD plants was more alkaline than WW plants during the photoperiod of Day 3 (Fig. 2d). Xylem ABA concentration did not significantly differ between WW and PRD plants during this experiment (Fig. 2e).

Shoot physiology and chemical regulators in WT and ACO1$_{AS}$ plants (Experiments 4, 5)

On the fourth day of PRD in Experiment 4, WT plants showed a significant inhibition of elongation of both the entire leaf and the terminal leaflet (Fig. 3a, b), which was sustained throughout the experiment (although not always statistically significant). Growth of $ACO1_{AS}$ leaves was not inhibited (Fig. 3h, i). Despite this genotypic variation in leaf growth response to PRD, g_s of both genotypes decreased similarly (Fig. 3e, l).

At the end of the experiment, θ of dry columns of PRD plants had decreased by 63% and both genotypes showed a statistically similar θ in both watering regimes (data not shown). There was no difference in ψ_{leaf} between WW and PRD plants in either genotype over a 7 d period (Fig. 3d, k), indicating that changes in plant water status could not explain either growth inhibition or stomatal closure.

Xylem sap pH became more alkaline (Fig. 3f, m) and [X-ABA] increased (Fig. 3g, n) in both genotypes. Xylem sap pH peaked on the third day of PRD. On no measurement occasion were there significant effects of genotype, or genotype$\times$treatment interaction, on xylem sap pH. Although there were no significant (P<0.05) genotype$\times$treatment interactions affecting [X-ABA], on the third day of PRD there was a significant (P=0.016) genotypic effect. On this day, the mean ($\pm$SE) [X-ABA] (combining both watering regimes) of WT plants (77 ± 7 nM, n=8) exceeded that of $ACO1_{AS}$ plants (51 ± 10 nM, n=8). Additional experiments failed to show any genotypic difference in [X-ABA] (data not shown), as shown previously (Hussain *et al*., 1999).

Leaf ethylene evolution was at least 2-fold higher in WT plants compared with $ACO1_{AS}$ plants (Fig. 3c, j) as described previously (Hussain *et al*., 1999). In WT plants, ethylene evolution significantly increased in response to PRD (Fig. 3c). Ethylene evolution of $ACO1_{AS}$ plants did not change in response to PRD (Fig. 3j).

A further experiment was conducted to confirm the genotypic differences in leaf growth response to soil drying. WT plants showed a significant inhibition of elongation of both the entire leaf (data not shown) and the terminal leaflet (Fig. 4a) on the eighth day of PRD and thereafter. Under PRD, growth of $ACO1_{AS}$ leaves was not inhibited. To determine whether genotypic differences in leaf growth response were a function of differences in soil moisture depletion, daily increments of leaf elongation were plotted against θ of the dry column (PRD plants) and the corresponding wet column (WW plants) at the start of the 24 h

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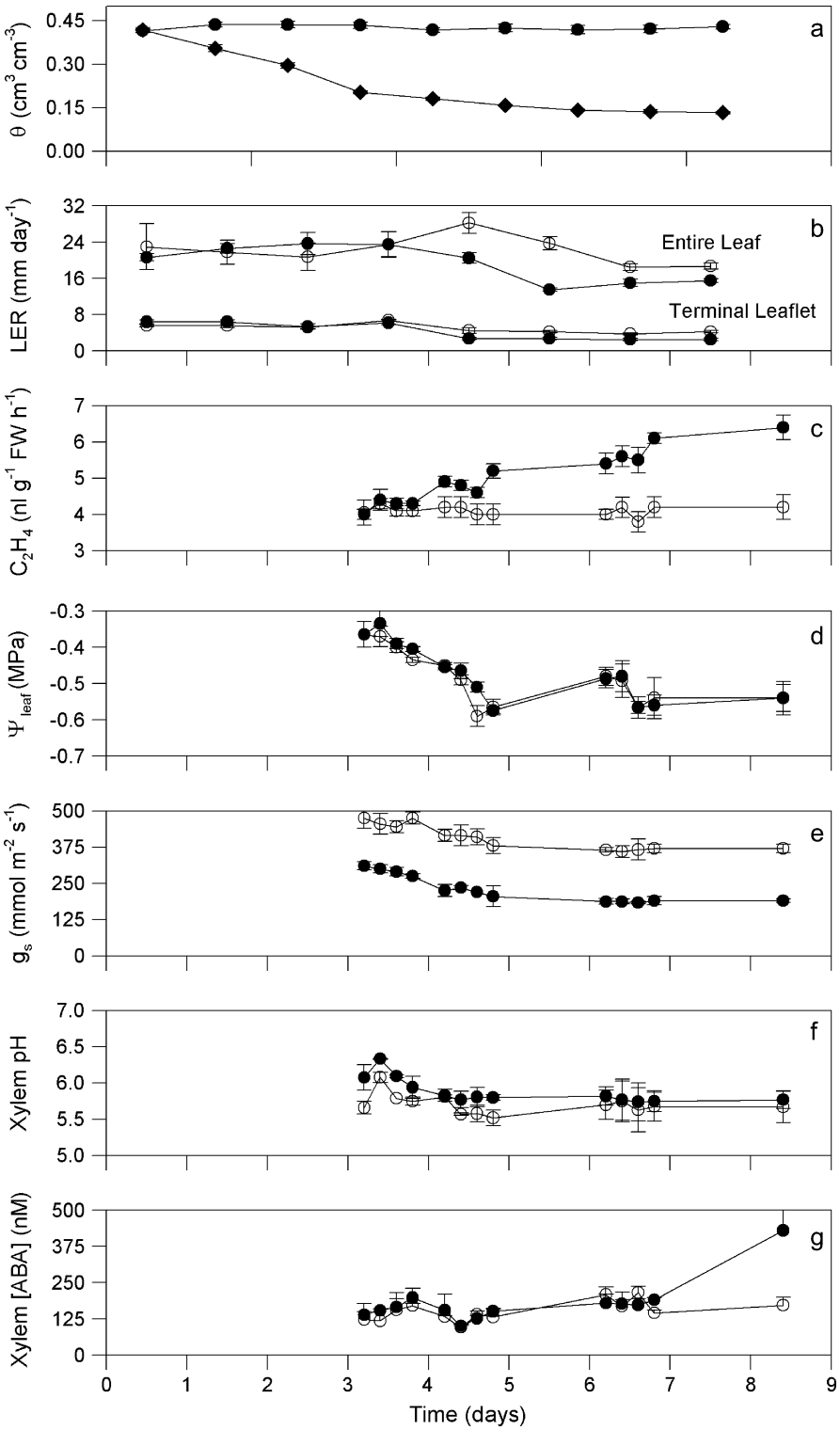

Fig. 1. (a) Moisture content of the upper 6 cm of potting compost from the watered (closed circles) and drying (closed diamonds) sides of plants watered daily on one side of the split-pot. (b) Growth rate of the entire leaf and terminal leaflet of leaves at Node 11. (c) Detached leaflet ethylene evolution of leaves at Node 11. The mean (±SE) of initial leaf length was 120±10 mm. (d) Leaf water potential, (e) stomatal conductance, (f) xylem sap pH, and (g) xylem ABA concentration, of fully expanded leaves at Node 6. On Days 4, 5, and 7, measurements in (c–g) were taken every 3 h during the photoperiod. Points are the means±SE of 4 (a, b) and 2–4 (c–g) replicates of WT plants watered daily on one (closed circles) or both (open circles) sides of the split-pot.

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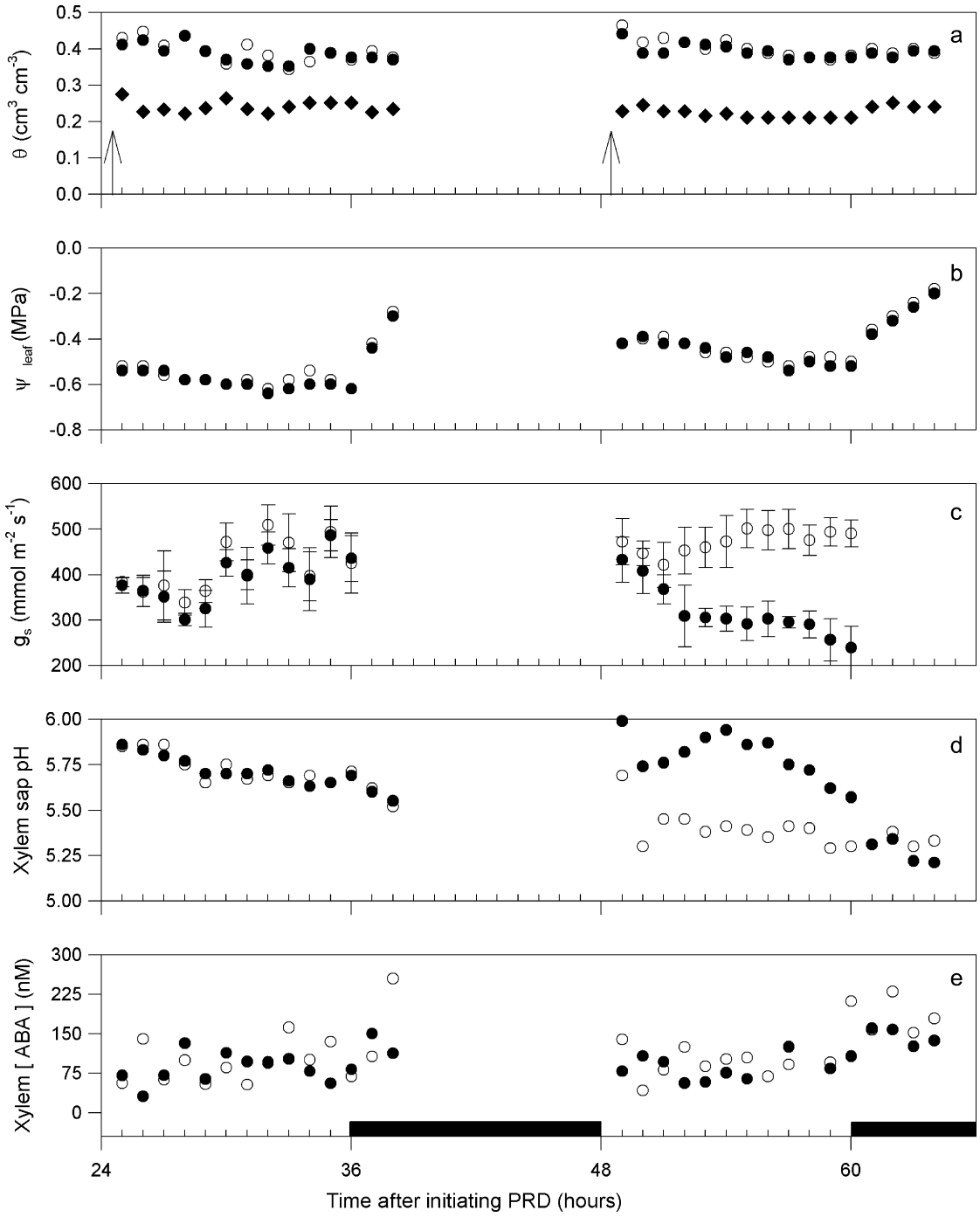

Fig. 2. (a) Moisture content of the upper 6 cm of potting compost from pots watered daily (indicated by arrows) on both sides of the split-pot (open circles), and from the watered (closed circles) and drying (closed diamonds) sides of plants watered daily on one side of the split-pot. (b) Leaf water potential, (c) stomatal conductance, (d) xylem sap pH, and (e) xylem ABA concentration, of fully expanded leaves at Node 9. Points are from individual WT plants watered daily on one (closed circles) or both (open circles) sides of the split-pot (b, d, e). In (c), points are means ± SE of five leaflets per leaf. Dark shading on the time axis indicates the night period.

measurement period. Representative figures (Days 10–11) are shown (Fig. 4b, c). WT plants showed a significant reduction in leaf elongation as soil moisture content decreased, while leaf growth of $ACO1_{AS}$ plants was relatively insensitive to changes in θ.

Leaf water potential and ethylene evolution were measured at the end of this experiment (Table 1). Again, there were no genotypic or treatment differences in ψ_{leaf}. WT plants showed a significant increase in leaflet ethylene evolution while $ACO1_{AS}$ plants did not.

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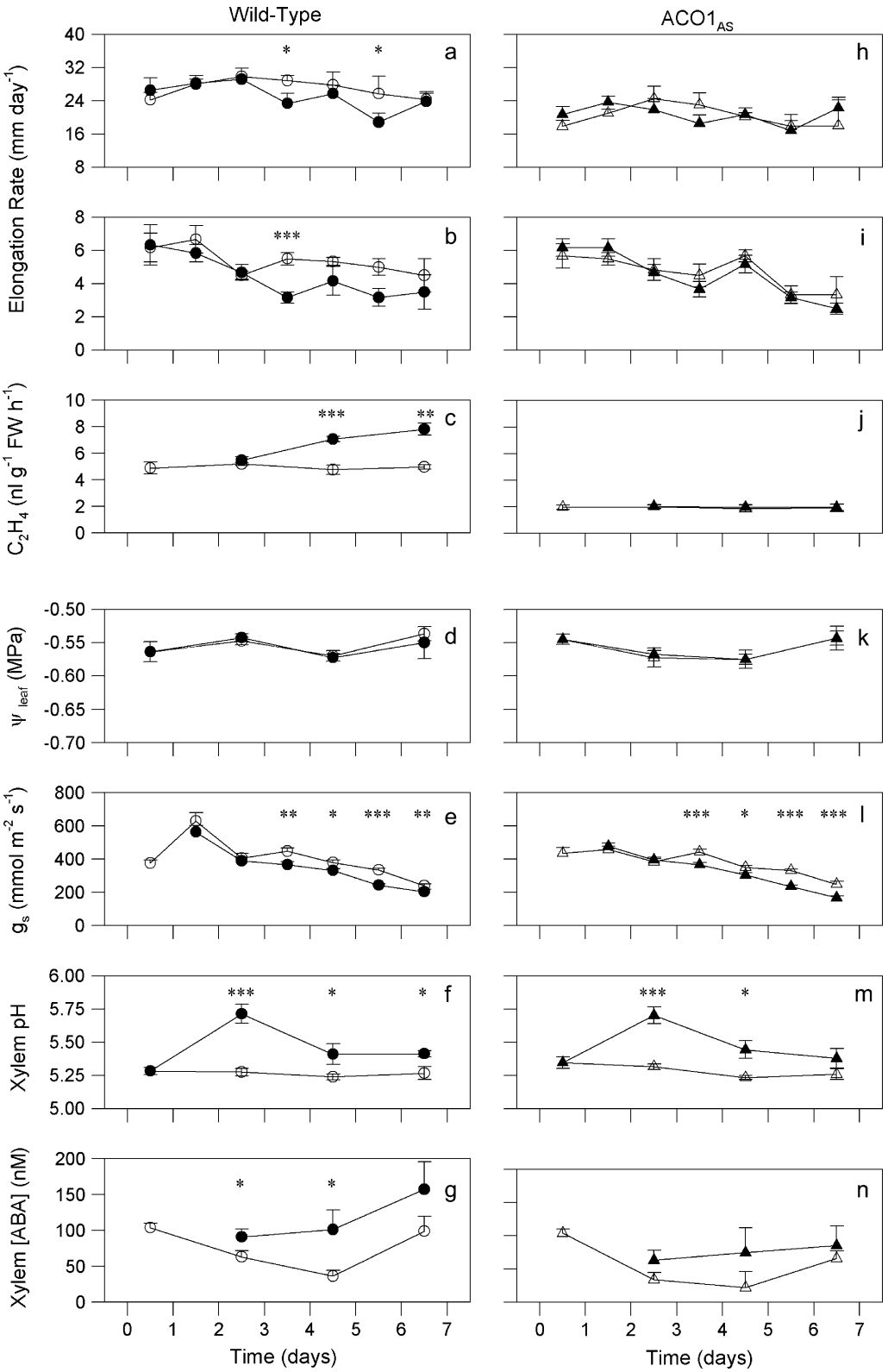

Fig. 3. (a, h) Elongation rate of the entire leaf of leaves at Node 12. (b, i) Elongation rate of the terminal leaflet of leaves at Node 12. (c, j) Detached leaflet ethylene evolution of leaves at Node 12. The mean±SE of initial leaf length was (a) 104±6 mm and (h) 105±7 mm. (d, k) Leaf water potential, (e, l) stomatal conductance, (f, m) xylem sap pH, and (g, n) xylem ABA concentration, of fully expanded leaves at Node 6. Points are the means±SE of six (a, b, e, h, i, l) and four (c, d, f, g, j, k, m, n) replicates taken from WT plants watered daily on one (closed circles) or both (open circles) sides of the split-pot (a–g), and $ACO1_{AS}$ plants watered daily on one (closed triangles) or both (open triangles) sides of the split-pot (h–n). Differences between watering regimes, as determined by the Student's *t*-test, are indicated thus: **P*<0.05, ***P*<0.01, ****P*<0.001.

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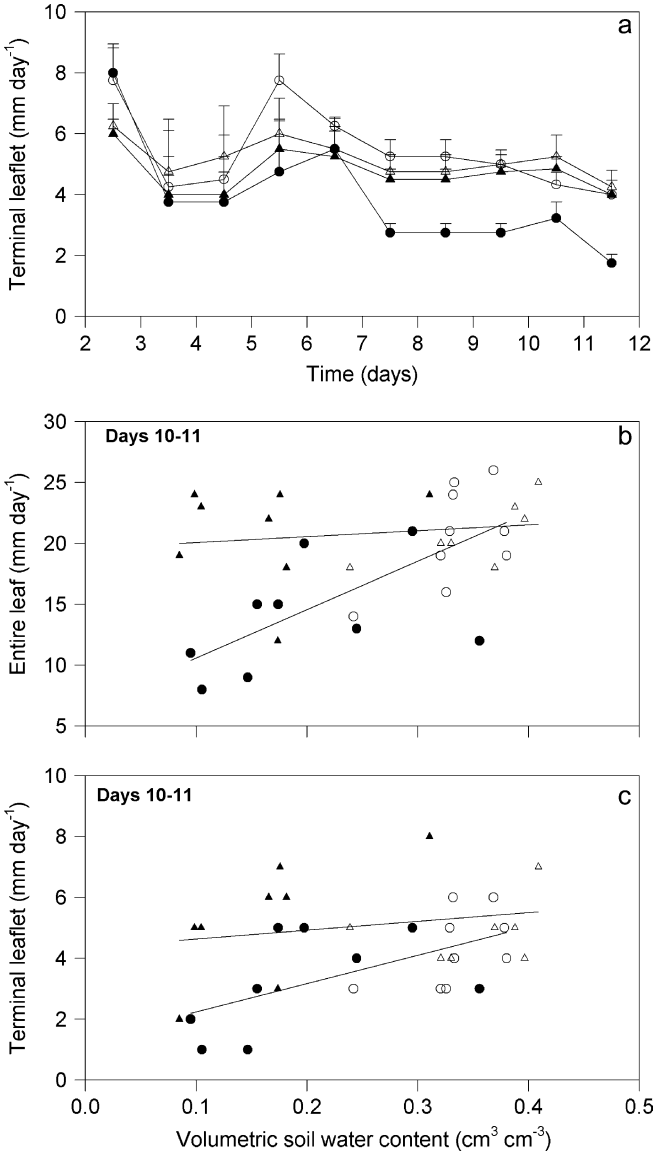

Fig. 4. (a) Terminal leaflet elongation rates of leaves at Node 12 from WT (closed, open circles) or $ACO1_{AS}$ plants (closed, open triangles) watered daily on one (closed circles, triangles) or both (open circles, triangles) sides of the split-pot. Data are the means±SE of 7–9 replicates. (b) Entire leaf elongation rate, and (c) terminal leaflet elongation rate (Days 10–11) plotted against the prewatering volumetric water content of the upper 6 cm of soil on Day 10. Linear regressions were fitted to each genotype in SigmaPlot for Windows 2.01, giving *P* values of 0.56 ($ACO1_{AS}$) and 0.0009 (WT) in (b), and 0.98 ($ACO1_{AS}$) and 0.009 (WT) in (c).

Discussion

To allow the effects of chemical signals on shoot physiology to be studied independently of hydraulic influences, it was important that plants exposed to drying soil maintained a similar ψ_{leaf} to WW plants throughout the experiments. Implementation of a partial root-zone drying treatment proved to be extremely effective in this regard. Since PRD plants were watered only once a day, it was necessary to demonstrate that ψ_{leaf} did not differ between PRD and WW plants throughout the diurnal cycle (Figs 1d, 2b). Even though PRD plants received half the water of WW plants, water uptake from roots in wet soil was sufficient to maintain ψ_{leaf}. Partial stomatal closure of PRD plants (Figs 1e, 2c) would have decreased daily transpiration rate and thus may be critical to the success of PRD in regulating leaf water status. Therefore it becomes important to understand the regulation of stomatal conductance by chemical signals.

A large body of literature suggests that increased [X-ABA] causes stomatal closure of plants grown in drying soil (reviewed in Dodd, 2003). However, this work showed that the response of [X-ABA] to soil drying was rather variable between experiments (cf. Fig. 1g and 3g). By contrast, increased xylem sap pH of PRD plants was consistently observed (Figs 1f, 2d, 3f). Bioassay studies have shown that supplying more alkaline buffers to detached tomato leaves restricted transpiration (Wilkinson *et al*., 1998), suggesting that increased xylem sap pH can act as a signal to close stomata without [X-ABA] necessarily increasing (as in Fig. 2). Further studies with detached leaves showed that increased xylem sap pH acts via an ABA-dependent mechanism, as leaves detached from an ABA-deficient tomato mutant (*flacca*) did not show stomatal closure when fed pH 7 buffers (Wilkinson *et al*., 1998). This emphasizes that the responsiveness of leaves to a pH signal is dependent on leaf ABA concentration, and can be an important variable in determining stomatal sensitivity to drying soil.

Stomata remained partially closed (Fig. 1e) even though the xylem sap pH of PRD plants approached that of well-watered plants after 6–7 d of PRD (Figs 1f, 3f, m); this implied that an additional mechanism maintained this stomatal response. In Experiment 4, increased [X-ABA] may be responsible for prolonged stomatal closure (Fig. 3g). Leaf ethylene status is unlikely to account for such stomatal closure, as stomatal behaviour of WT and $ACO1_{AS}$ plants was very similar (Fig. 3e, l) despite large differences in leaf ethylene evolution (Fig. 3c, j). Increased guard-cell ABA concentration or apoplastic ABA concentration around the guard cells (Zhang *et al*., 2001) (neither of which could be measured here due to technical limitations) is a likely explanation for stomatal closure in cases where xylem sap pH has returned to WW values and increased [X-ABA] has not yet been detected.

By contrast with the central role the present authors are proposing for ABA as a stomatal regulator when plants are exposed to drying soil, it seems much less likely that ABA is limiting leaf growth. In one experiment, PRD inhibited leaf growth several days before [X-ABA] increased (cf. Fig. 1b, g). When increased [X-ABA] preceded leaf growth inhibition (Fig. 3), there were similar relative increases in [X-ABA] in both WT and $ACO1_{AS}$ plants (Fig. 3g, n), despite contrasting responses of leaf growth to soil drying (Fig. 3a, h). Taken together, these observations suggest that

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Table 1. *Responses of WT and ACO1$_{AS}$ plants to PRD*

For each genotype and treatment, measurements of the two variables were made on the same leaves after 12 d of treatment. Data are the means±SE of four replicates. Values followed by different letters are significantly different at the 0.05 level according to Tukey's HSD test.

Parameter	Wild-type (Ailsa Craig)		Transgenic (ACO1$_{AS}$)	
	WW	PRD	WW	PRD
ψ_{leaf} (MPa)	−0.61±0.05 a	−0.61±0.03 a	−0.62±0.04 a	−0.62±0.03 a
Leaf C_2H_4 (nl g^{-1} FW h^{-1})	4.5±0.3 b	6.9±0.3 c	2.1±0.1 a	2.2±0.2 a

increased [X-ABA] is not directly limiting leaf growth of tomato plants exposed to PRD.

Feeding more alkaline buffers via the xylem to detached barley shoots inhibited leaf growth when shoot ABA concentration was high enough (Bacon *et al*., 1998), although leaf growth of an ABA-deficient mutant was similar when fed both pH 6 and pH 7 buffers. It seems that this mechanism did not operate here, as increased xylem sap pH did not temporally coincide with leaf growth inhibition (cf. Fig. 1b, f and Fig. 3a, f). Furthermore, during PRD xylem pHs of WT and ACO1$_{AS}$ plants were very similar (cf. Fig. 3f, m) despite divergent leaf growth responses (e.g. Fig. 4a).

In these studies, leaf growth inhibition by PRD was temporally correlated with increased ethylene evolution from expanding leaflets, suggesting that ethylene was limiting leaf growth. More compelling evidence for the involvement of ethylene was the divergent responses of leaf growth and ethylene evolution in WT and ACO1$_{AS}$ plants exposed to drying soil. WT plants showed increased ethylene evolution (Fig. 3c; Table 1) and leaf growth inhibition (Figs 3a, b, 4a), while ACO1$_{AS}$ plants showed no increase in ethylene evolution (Fig. 3j; Table 1) and no leaf growth inhibition (Figs 3h, i, 4a).

Enhanced ethylene evolution by droughted plants is often reported in the literature (reviewed in Yang and Hoffman, 1984). However, influential re-analyses of much of these data suggest that this enhancement is an artefact where drought is imposed by rapid desiccation of detached leaves (Morgan *et al*., 1990; Morgan and Drew, 1997). Although the present study measured ethylene evolution in detached leaves, after excision all leaves (from both WW and PRD plants) were rapidly enclosed in a saturated atmosphere to limit water loss during incubation. A further caveat is that most measurements of leaf ethylene evolution reported in the literature have measured head-space ethylene accumulation from detached leaves (due to technical limitations). Consequently, enhanced ethylene evolution of droughted plants has been dismissed as an interaction between 'leaf detachment stress' (excision, gravitropic disorientation, and in some cases desiccation) and leaf physiology (exposure of plants to drying soil) (Morgan *et al*., 1990). The few measurements of whole droughted plants or attached leaves of those plants show no increase, and usually a decrease, in ethylene evolution (Morgan *et al*., 1990; Narayana *et al*., 1991).

It has been further suggested that ethylene production by water-stressed plants depends on the rate at which ψ_{leaf} falls (Morgan and Drew, 1997). The study being reported here demonstrated an increase in ethylene evolution caused by soil drying that did not change leaf water status. Further examination of the literature shows that instances of mild soil drying (ψ_{leaf} declined at −0.1 MPa d^{-1}) stimulated ethylene production in tomato leaves (Kalantari *et al*., 2000) and ACC oxidase gene expression in sunflower (*Helianthus annuus*) leaves (Ouvrard *et al*., 1996). More severe water stresses decreased ethylene production measured in detached tomato leaves (ψ_{leaf} declined at −0.3 MPa d^{-1} [Kalantari *et al*., 2000]), and in intact cotton (ψ_{leaf} declined at −0.6 to −1.2 MPa d^{-1} [Morgan *et al*., 1990]) and wheat (ψ_{leaf} declined at −1.2 MPa d^{-1} [Narayana *et al*., 1991]) plants. The present study suggests that plants exposed to mild soil drying (in which ψ_{leaf} is maintained, or very slowly declines) show a stimulation of ethylene synthesis while plants with leaf water deficit show a suppression of ethylene synthesis.

It is yet to be determined whether, in the system used in the present study, increased root export of the ethylene precursor ACC acts as a long-distance signal of drying soil, increasing leaf ethylene evolution and thus inhibiting leaf growth. Soil drying can increase root and xylem ACC concentrations (Tudela and Primo-Millo, 1992). Root ACC export can quantitatively account for shoot ethylene evolution of well-drained and flooded tomato plants (Else and Jackson, 1998). Furthermore, feeding ACC via the xylem to detached barley shoots can inhibit leaf growth (IC Dodd, unpublished observations). However, the possibility that processes within the leaf are causing increased ethylene evolution in the absence of changes in root ACC export cannot be excluded. In sunflower plants that maintained leaf turgor when grown in drying soil (ψ_{leaf} declined at −0.1 MPa d^{-1}), a 2-fold up-regulation of ACC oxidase gene expression was observed in the leaves (Ouvrard *et al*., 1996), which might be expected to enhance ethylene evolution. Enhanced ethylene evolution of flooded *Rumex palustris* plants was accompanied by both increases in root ACC export and increased shoot ACC oxidase gene expression (Voesenek *et al*., 2003). Only judicious graft combinations of transgenic lines antisensed for the enzymes ACC synthase and ACC oxidase are likely to resolve the question of the relative contributions of root processes and

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shoot processes to the increased leaf ethylene evolution observed in the present study.

Irrespective of the causes and mechanisms of enhanced ethylene evolution by WT PRD plants, it is important to highlight the genotypic differences in leaf growth response between WT and $ACO1_{AS}$ plants. When comparing water stress responses of different genotypes, it is necessary to subject lines to a comparable degree of water stress. WT and $ACO1_{AS}$ plants subjected to PRD showed similar leaf (Fig. 3e, l; Table 1) and soil (Fig. 4b, c) water status as the soil dried. This was expected since unstressed plants of these genotypes show similar stomatal conductance (Fig. 3d, k; see also Hussain *et al*., 1999), leaf area development (Hussain *et al*., 1999), and whole-plant transpiration rate (data not shown). Root development is also similar in unstressed plants (Hussain *et al*., 1999), thus similar vertical profiles of soil water content were also expected. The authors are therefore confident that the genotypic differences in leaf growth response are real and attributed to differential ethylene evolution, and not an artefact of differing soil and/or shoot water status.

Since plants were grown with PRD to maintain shoot water status, the changes in leaf elongation were relatively small (e.g. 30% when averaged across all days for WT PRD plants in Fig. 4a), as is consistent with other PRD studies (Saab and Sharp, 1989 showed a 18% reduction in maize leaf growth rate). However, a response of this magnitude can still be extremely important in a young crop covering the ground, by minimizing soil evaporation and intercepting more radiation to accumulate more biomass.

In crops where leaves are the harvested portion, and when irrigation supplies are assured, it may be desirable to suppress the limitation of leaf growth caused by mild soil drying. When soil drying occurs with shoot water deficit, there is limited genotypic variation in leaf growth response to low tissue water potentials (Spollen *et al*., 1993). A better crop-improvement strategy may be to maintain leaf growth under mild soil drying by exploiting genotypic variation in the generation of, and response to, chemical signals. This may only be possible if stomata close partly to maintain leaf water status. Should ethylene prove to be a universal mediator of leaf growth of plants exposed to drying soil but maintaining leaf water status, antisense suppression of ACC oxidase activity seems to be a useful means to overcome leaf growth inhibition.

Conclusions

Long-distance chemical signalling in droughted plants can limit both growth and stomatal opening but different chemical regulators may dominate in the control of the two processes. It is likely that increased xylem sap pH limited stomatal opening, thus maintaining leaf water status of plants in drying soil. Since $ACO1_{AS}$ and WT plants showed similar changes in both xylem sap pH and [X-ABA] as the soil dried, it seems likely that the enhanced ethylene evolution of WT plants under PRD inhibits leaf growth.

In $ACO1_{AS}$ plants, a 'normal' stomatal limitation of water loss as the soil dries occurred without significant limitation of leaf growth. PRD plants received only half of the water applied to well-watered plants. Despite this, leaf growth rates of $ACO1_{AS}$ plants were comparable to those of the well-watered WT plants and greater than those of droughted WT plants. The suppressed leaf growth responses of ethylene-deficient plants to drying soil provides an opportunity to maximize biomass production when water supply is limited, as long as plants are grown using an agronomic system (e.g. PRD) that maintains leaf water status.

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