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RESEARCH PAPER

Anti-transpirant activity in xylem sap from flooded tomato (*Lycopersicon esculentum* Mill.) plants is not due to pH-mediated redistributions of root- or shoot-sourced ABA

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Abstract

In flooded soils, the rapid effects of decreasing oxygen availability on root metabolic activity are likely to generate many potential chemical signals that may impact on stomatal apertures. Detached leaf transpiration tests showed that filtered xylem sap, collected at realistic flow rates from plants flooded for 2 h and 4 h, contained one or more factors that reduced stomatal apertures. The closure could not be attributed to increased root output of the glucose ester of abscisic acid (ABA-GE), since concentrations and deliveries of ABA conjugates were unaffected by soil flooding. Although xylem sap collected from the shoot base of detopped flooded plants became more alkaline within 2 h of flooding, this rapid pH change of 0.5 units did not alter partitioning of root-sourced ABA sufficiently to prompt a transient increase in xylem ABA delivery. More shoot-sourced ABA was detected in the xylem when excised petiole sections were perfused with pH 7 buffer, compared with pH 6 buffer. Sap collected from the fifth oldest leaf of 'intact' well-drained plants and plants flooded for 3 h was more alkaline, by ~0.4 pH units, than sap collected from the shoot base. Accordingly, xylem [ABA] was increased 2-fold in sap collected from the fifth oldest petiole compared with the shoot base of flooded plants. However, water loss from transpiring, detached leaves was not reduced when the pH of the feeding solution containing 3-h-flooded [ABA] was increased from 6.7 to 7.1 Thus, the extent of the pH-mediated, shoot-sourced ABA redistribution was not sufficient to raise xylem [ABA] to physiologically active levels. Using a detached epidermis bioassay, significant non-ABA anti-transpirant activity was also detected in xylem sap collected at intervals during the first 24 h of soil flooding.

Key words: ABA, ABA-GE, pH, signalling, soil flooding, stomatal closure, tomato, xylem sap.

Introduction

The root- or shoot-sourced signal that triggers stomatal closure within 2–4 h of soil flooding has not yet been identified (Jackson, 2002) although several possibilities have been tested. For example, flooding-induced alterations to photosystem II photochemistry did not initiate closure via an accumulation of intracellular CO_2 (MA Else *et al*., unpublished data). Also, root-sourced hydraulic signals may be important in some species, but do not appear to be involved in tomato (Else *et al*., 1995*a*, 2001). However, the rapid effects of decreasing oxygen availability on root metabolic activity are likely to generate many potential root-sourced chemical signals that could regulate stomatal apertures. For instance, we have shown the ionic composition of xylem sap to be altered within 2 h of soil flooding (Jackson *et al*., 2003) and some component(s) of these changes could constitute a root-sourced signal. Perturbed hormone traffic between roots and shoots of flooded plants occurs equally rapidly (Janowiak *et al*., 2002), with abscisic acid (ABA) delivery to the shoot being reduced by 75% within the first 4 h of flooding in tomato and *Ricinus* (Else *et al*., 1996, 2001; Janowiak *et al*., 2002). It therefore seems probable that the transpiration stream in flooded plants carries one

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Abbreviations: ABA, abscisic acid; ABA-GE, glucose ester of abscisic acid; GC–MS, gas chromatography–mass spectrometry; SIM, selective ion monitoring.

or more chemicals that close stomata. This view has been supported experimentally by detecting anti-transpirant activity in xylem sap of flooded plants that was not attributable to ABA or free calcium (Else *et al*., 1996; Tiekstra, 1999).

Wild-type concentrations of shoot-sourced ABA are necessary to invoke complete stomatal closure in flooded plants (Jackson, 1991). Also, stomatal responses to drying soil were most strongly influenced by the capacity of the shoot to synthesize ABA, rather than the root (Holbrook *et al*., 2002). The signal that prompted the enrichment of xylem sap with shoot-sourced ABA was not identified (Holbrook *et al*., 2002), but one possibility is an increase in the pH of xylem sap. ABA is a weak acid and its *in planta* distribution between membrane-bound compartments is governed by pH gradients (Kaiser and Hartung, 1981; Hartung and Radin, 1989). Increased xylem sap alkalinity following soil drying may promote pH-mediated redistributions of shoot-sourced ABA that enhance xylem sap ABA concentrations *en route* to the guard cells (Wilkinson *et al*., 1998; Sauter *et al*., 2002). Increased xylem sap pH can also reduce the ability of leaf cells to remove xylem- and leaf-sourced ABA from the apoplast (Wilkinson and Davies, 1997). Deactivation of plasma membrane H^+-ATPases in oxygen-deficient roots would be expected to alter the pH of xylem sap in flooded plants (Netting, 2000; Felle, 2005). Furthermore, perturbations in the ionic composition of xylem sap may also influence its pH (Kirby and Armstrong, 1980; Gollan *et al*., 1992); reduced nitrate concentrations and associated changes in organic acid components in particular may cause the pH to shift towards alkalinity (Wilkinson and Davies, 2002).

Recently, Jackson *et al*. (2003) reported a marked alkalinization of xylem sap collected from the shoot base of tomato within 3 h of soil flooding. Whether this pH change encouraged redistribution of apoplastic ABA within the shoot to concentrations that close stomata is not yet known. Similarly, the alkalinization of xylem sap could promote the redistribution of existing root-sourced ABA into the apoplast of the roots. Carriage in the transpiration stream to the shoots and the resultant short-lived 'pulse' of ABA arriving at the exterior of the guard cells may initiate stomatal closure. Hitherto, time-courses of ABA delivery following soil flooding have not been sufficiently detailed to test this hypothesis. The glucose ester of ABA (ABA-GE) has also been implicated in long-distance signalling; Hartung and co-workers have suggested that β-glucosidases could liberate ABA from ABA-GE in the leaf apoplast (Dietz *et al*., 2000; Sauter *et al*., 2002). The effect of soil flooding on the transport of ABA-GE in xylem sap is not known. There may also be a role for as yet unidentified conjugates of ABA that may release free ABA under certain conditions (Netting, 2000).

In this report, two different bioassays were used to detect significant anti-transpirant activity in xylem sap exported from roots within the first few hours of flooding. The strength of the notion that this activity arises from xylem sap alkalinization induced by soil flooding, that prompts redistribution of root- or shoot-sourced ABA in favour of the apoplast, is tested. The possibility that increased ABA-GE output from oxygen-deficient roots acts as a long-distance signal to close stomata is also considered.

Materials and methods

Plant material and growth conditions

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) were sown in a Levington F2 compost–sand mix in a heated glasshouse (minimum temperature, 20 °C). Fully emerged seedlings were potted individually into pots (90×90×100 mm) containing Richmoor compost and a slow-release fertilizer (Osmocote, 1 kg 225 l^{-1}, Sierra Chemical Europe BV, Heerlen, The Netherlands). Plants were maintained in a glasshouse with a light/dark temperature of 25/20 °C and a 16 h photoperiod; when necessary, day length was extended by 400 W SONT lamps (Phillips Lighting, Surrey, UK). Relative humidity was uncontrolled. Plants were watered automatically via capillary matting; side shoots were removed regularly. Plants were used for experiments at the 7–8 leaf stage and were divided into well-drained and soon-to-be flooded treatments at random. Plant root systems were flooded at 09.00 h by placing the pots of compost into larger pots filled with tap water warmed to 25 °C and maintained 10 mm above compost level.

Seeds of *Commelina communis* L. were sown in John Innes No. 2 compost and, after emergence, seedlings were grown under the conditions described by Trejo *et al*. (1993). The third oldest, fully expanded leaf was used as a source of experimental material.

Viscous flow porometry and measurement of transpiration

Differences in stomatal aperture of well-drained and flooded plants were estimated by measuring leaf resistances with a viscous flow porometer (Allaway and Mansfield, 1969). Two leaflets on each of six plants were sealed individually into porometer cups; an open cup was used to measure the lowest pressure (zero), and the highest pressure (std) was measured by connecting the inflow and outflow tubes together. On the second day, three plants were flooded at 09.00 h and leaf resistances were measured for a further 48 h.

Gravimetric measurements of whole-plant transpiration, corrected for evaporation from the soil surface, were made at hourly intervals during the first 6 h of soil flooding using an electronic balance. Leaf areas were measured destructively using a leaf area meter (Li-Cor, Lincoln, NE, USA) after plants were detopped for xylem sap collection.

Xylem sap collection

Stems of well-drained or flooded plants were cut through just below the cotyledonary node with a sharp razor blade, and root pressure chambers were used to express sap from detopped roots at flows that encompassed earlier gravimetric measurements of whole-plant transpiration rates (Else *et al*., 1994). Xylem sap was collected from root systems of well-drained and flooded plants at 2 h intervals during the first 6 h of soil flooding. The initial 200 mm^3 of sap from each root system was discarded. Sap for solute analysis was collected for 600 s in preweighed plastic scintillation vials kept on ice. Samples were weighed, frozen in liquid nitrogen, and stored at −78 °C.

Sap from the fifth oldest leaf (counting from the shoot base) of intact, transpiring plants was collected using split-top pressure

chambers as described previously (Tiekstra *et al*., 2000). Briefly, a balancing pressure was applied to the roots such that sap barely exuded into Tygon tubing placed over the cut end of the sixth oldest petiole. The terminal leaflet of the fifth oldest petiole was then excised to raise xylem hydrostatic pressure slightly; the expressed sap was left to drip for 20 min. Thereafter, four sequential 300 mm^3 sap samples were collected in Eppendorf tubes kept on ice. The shoot was then excised below the cotyledonary node and the pressure adjusted such that the expelled sap flowed from the roots at rates similar to those of whole plant transpiration. Sap samples were weighed, frozen in liquid nitrogen, and stored at −78 °C.

Sap solute analyses

Free ABA concentrations [ABA] in xylem sap and perfusates were quantified by gas chromatography–mass spectrometry [GC–MS; selective ion monitoring (SIM)]. A 10 ng aliquot of [$^2H_6$]ABA was added to xylem sap samples or perfusates and loaded onto pre-equilibrated (20% methanol) 'Isolute C_{18}' cartridges (100 mg sorbent bed, EC, Argonaut Technologies Ltd., Hengoed, UK). The Isolute C_{18} cartridges were washed with 20% methanol and the ABA eluted with 80% aqueous ethanol into autosampler vials (Chromacol, Welwyn Garden City, UK). The eluates were reduced to dryness *in vacuo*, redissolved in 50 mm^3 Aristar methanol, and methylated with an excess of ethereal diazomethane. After 30 min, any remaining diazomethane was removed under a stream of dry, O_2-free N_2. The samples containing ABA were taken to dryness *in vacuo* and redissolved in 15 mm^3 ethyl acetate for GC–MS analysis.

A 1 μl aliquot was injected into a Hewlett-Packard 5890 Series II gas chromatograph equipped with a split/splitless injector coupled to a ThermoQuest Trio-1 mass spectrometer linked by an A CP-SIL 5CB-ms column (BP1 equivalent) [Chrompack (UK) Ltd, London, UK] that was 30 m long, 0.25 mm in internal diameter, and with 0.25 mm film thickness. The carrier gas was helium and the linear flow rate 350 mm s^{-1}. Interface temperature, source temperature, and ionization voltage were 285 °C, 200 °C, and 70 eV, respectively, and the mass spectrometer operated under positive ion electron impact conditions. The MS was used in SIM mode. The injector temperature was 240 °C and oven temperature 60 °C. After 1 min, the split valve opened and, after a further 30 s, the oven temperature was increased at 35 °C min^{-1} to 210 °C, and 1 min later increased at 5 °C min^{-1} to 235 °C. The temperature was then increased to 275 °C for a further 5 min. The ions monitored were 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]ABA. Amounts of ABA were computed by the Lab-Base data system from calibration curves relating molar ratios to ion intensities of *m/z* 190 (Me-ABA) and *m/z* 194 (Me-[$^2H_6$]ABA).

Conjugated [ABA] in xylem sap was first hydrolysed to free ABA then quantified by GC–MS (SIM). Samples of xylem sap (1 ml) were hydrolysed with an equal volume of NaOH (1 M) for 1 h at 25 °C. At the outset, solutions were bubbled with N_2 then capped to minimize oxidative degradation of ABA. Samples were adjusted to pH 3.0 with 1.1 ml of 1 M HCl, 10 ng of [$^2H_6$]ABA added, and partitioned three times against 4 ml of dichloromethane. The organic fractions were combined and washed with 2 ml of pH 3 water. A 1 ml aliquot of water was added and samples reduced to aqueous *in vacuo*. Total ABA was then extracted and quantified as above. Conjugated [ABA] in xylem sap was calculated by subtracting free [ABA] from total [ABA].

The acidity of the xylem sap was measured in 15 mm^3 samples with a Camlab 'pH Boy' meter (Camlab Ltd, Cambridge, UK).

Petiole perfusion

Eight 60 mm long petioles were excised from leaves 4 and 5 of well-drained plants and connected via Tygon tubing to disposable syringes filled with potassium phosphate buffer (1 mol m^{-3} KH_2PO_4 and K_2HPO_4 in ratios generating the desired pH). After housing the syringes in syringe pumps (KDS 100, Royem Scientific Ltd., Luton, UK), petiole sections were perfused with pH 6 buffer at a flow rate of 60 mm^3 min^{-1}. This value was derived from preliminary experiments that determined the average rate of sap flow through intact petioles of leaves 4 and 5 of well-drained plants. The outflow (perfusate) was collected in Eppendorf tubes on ice every 10 min. After 30 min, the perfusing solution was changed to pH 7 buffer and the perfusate collected every 10 min for a further 50 min. Finally, the perfusing solution was changed back to pH 6 buffer and the perfusate collected every 10 min for a further 50 min. Perfusates were stored at −78 °C until analysed by GC–MS. Tests with apoplastic dyes indicated that the perfused solution travelled only through the xylem vessels and did not infiltrate non-vascular tissues (MA Else, unpublished data).

Detached leaf experiments

Leaflets were excised from well-drained plants under a stream of de-ionized water (Di H_2O) and transferred, under water, to Petri dishes containing Di H_2O. The ends of petioles were recut under water to give a length of 30 mm and then transferred quickly to glass vials containing potassium phosphate buffer (1 mol m^{-3}) of the desired pH. Vials and leaves were weighed on an electronic balance (Mettler ESSLAB, Essex, UK) then placed in a Sanyo growth cabinet (SCC 097.CPX.F, Sanyo Gallenkamp PLC, Leicester, UK) maintained at 25 °C. Relative humidity was 50% with a light intensity of 300 μmol m^{-2} s^{-1} at leaf height, provided by fluorescent tubes (PLL-58W/83/4P) and incandescent lamps. Water loss from transpiring leaves was determined gravimetrically every hour. After 2 h, leaves were transferred to vials containing either pH 6.2 phosphate buffer, sap from well-drained plants, or sap from plants flooded for 2 or 4 h. Water loss from each leaf was recorded at hourly intervals for a further 5 h. Finally, the growth cabinet lights were turned off and water loss measured after 1 h of darkness to check the functioning of stomata. Leaflet areas were determined with a Li-Cor leaf area meter.

In some experiments, after the first 2 h, half of the leaves were transferred to vials containing pH 7.1 phosphate buffer and (+)-ABA (12 or 20 μmol m^{-3}), and the other half were transferred to vials containing pH 6.7 phosphate buffer and (+)-ABA (12 or 20 μmol m^{-3}). Water loss from each leaf was recorded at hourly intervals for a further 5 h. Leaflet areas were determined with a Li-Cor leaf area meter.

In experiments where the effects of xylem sap on water loss from excised leaves were determined, sap samples were thawed and filtered through 0.2 mm nylon 66 membranes (Alltech Associates Inc., Deerfield, IL, USA) immediately before use in detached leaf tests.

Commelina *epidermal strip bioassay*

The epidermis was stripped from the abaxial surface of the third leaf from 4-week-old *Commelina* plants and divided into 5×5 mm strips. Each strip was floated in plastic 50 mm diameter Petri dishes containing 5×10^3 mm^3 of 10 mol m^{-3} MES buffer and 50 mol m^{-3} KCl adjusted to pH 6.15 with 100 mol m^{-3} KOH. The Petri dishes were incubated on a water bath for 3 h at 25 °C under a photosynthetic photon flux density (PPFD) of 280 μmol m^{-2} s^{-1}. CO_2-free air (ambient air passed through a column of 3–9 mesh Sodalime) was bubbled through hypodermic needles into the buffer in each Petri dish at 5×10^3 mm^3 min^{-1}. After incubation, single epidermal strips were selected randomly, mounted on a microscope slide, and the apertures of 10 stomata from each strip measured under a light microscope.

Xylem sap samples were collected from flooded and well-drained tomato plants at intervals following inundation. Sap was collected from pressurized roots of detopped plants at rates of whole plant transpiration, diluted 4-fold in MES buffer and KCl, and then incubated for 30 min in a water bath under the conditions described

above. The sap was divided into six aliquots of 5×10^3 mm^3 which were then placed in separate 50 mm diameter Petri dishes. Seven epidermal strips were selected randomly and transferred to each of the dishes containing xylem sap. Two Petri dishes containing epidermal strips in KCl, MES buffer served as a control. Single strips were removed at hourly intervals and the apertures of 10 individual stomata per strip were measured.

Results

Flooding-induced effects on stomatal apertures and water loss

Leaf resistances (marker for stomatal apertures) in well-drained plants followed a distinct diurnal pattern; resistances were high during the night, began to fall around day break, and reached minimum values between mid-day and early afternoon (Fig. 1A). Leaf resistances then increased gradually during the late afternoon and evening, reaching maximum values between 22.00 h and midnight. Following soil flooding, leaf resistances began to increase within 3 h and diverged further from well-drained values throughout the rest of the day (Fig. 1A). The following morning, leaf resistance in flooded and well-drained plants was initially similar, but the normal diurnal fall was attenuated in flooded plants (Fig. 1A).

Gravimetric determinations of water loss during the first 6 h of inundation confirmed the flooding-induced effects on stomatal apertures inferred from the porometer data. Water loss from flooded plants was reduced within 1 h and remained suppressed, relative to well-drained values, for at least 5 h (Fig. 1B).

Effects of xylem sap in detached leaf transpiration tests

Rates of water loss from leaflets fed via the xylem with pH 6.2 potassium phosphate buffer or xylem sap from well-drained plants (pH 6.2) were similar throughout each experiment (Fig. 2A, B). When leaflets were transferred to vials containing xylem sap from plants flooded for 2 h, rates of water loss were significantly lower, compared with well-drained values, after 3 h, and were reduced by a further 20% after 5 h (Fig. 2A). Rates of water loss from leaflets fed with xylem sap collected from plants flooded for 4 h were reduced by up to 30%; again, statistically significant differences were detected after 3 h (Fig. 2B). Clearly, sap collected from plants flooded for 2 h and 4 h contains one or more factors that reduce water loss from detached transpiring leaves, presumably as a consequence of stomatal closure.

Flooding-induced xylem sap alkalinization

Sap was induced to flow from pressurized, detopped roots at rates that encompassed those of whole-plant transpiration (shown by arrows in Fig. 3). Xylem sap pH was not dependent on sap flow rates in either well-drained or flooded plants. The pH of xylem sap from well-drained plants averaged 6.2 at 10.00 h and midday, and increased gradually during the early afternoon (Fig. 3). The pH of xylem sap from flooded plants increased markedly within 2 h of inundation to 6.7, and remained more alkaline throughout the early afternoon (Fig. 3).

Free and conjugated ABA in xylem sap

Concentrations of free ABA in xylem sap, exported at whole-plant transpiration rates from detopped roots, were reduced by 85% within 2 h of soil flooding, and ABA delivery rates were reduced by 91% (Table 1). After 6 h flooding, ABA delivery was only 5% of that from well-drained roots. Concentrations and deliveries of conjugated ABA in xylem sap were unaffected by soil flooding (Table 1).

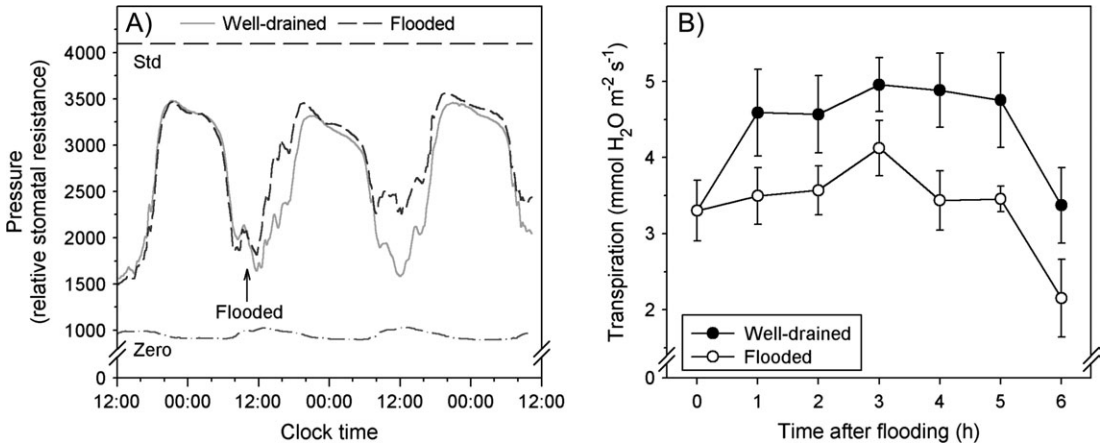

Fig. 1. Effect of soil flooding on (A) leaf resistance to air flow determined by viscous flow porometry and (B) whole-plant transpiration rates. Twelve individual leaflets from six plants were sealed into the porometer cups and readings were taken from each cup for 10 s every minute and logged using a PC. Whole-plant transpiration rates were determined gravimetrically after correction for evaporative losses from the soil surface. Vertical bars are means of six replicate plants with associated standard errors.

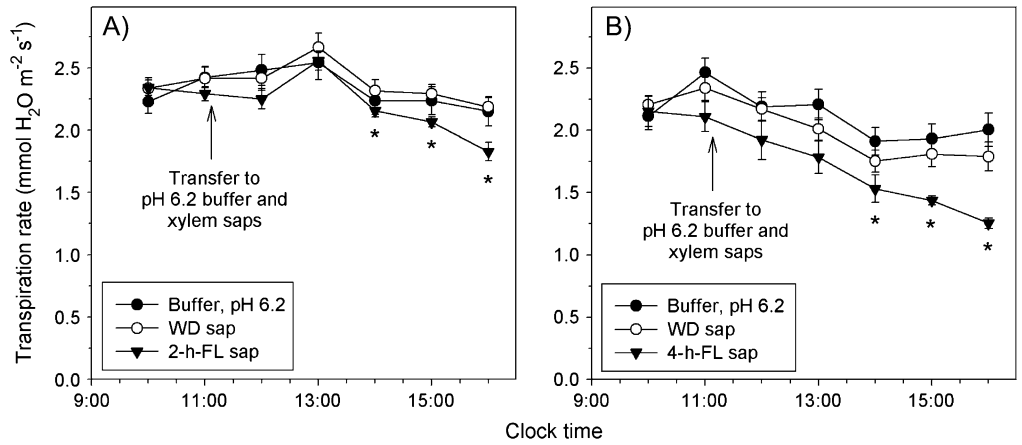

Fig. 2. Effect of xylem sap collected at realistic flow rates from (A) well-drained plants and plants flooded for 2 h, and (B) well-drained plants and plants flooded for 4 h on water loss from detached, transpiring leaves. Initially, all leaves were fed 1 mol m^{-3} potassium phosphate buffer (pH 6.2) via the xylem. After 2 h, leaves were quickly transferred to new vials containing either phosphate buffer (pH 6.2) or filtered sap from well-drained plants, and were plants flooded for 2 or 4 h. Each data point is a mean of six replicate leaves with associated standard errors. Asterisks indicate statistically significant differences (P <0.05) between sap from well-drained and flooded plants.

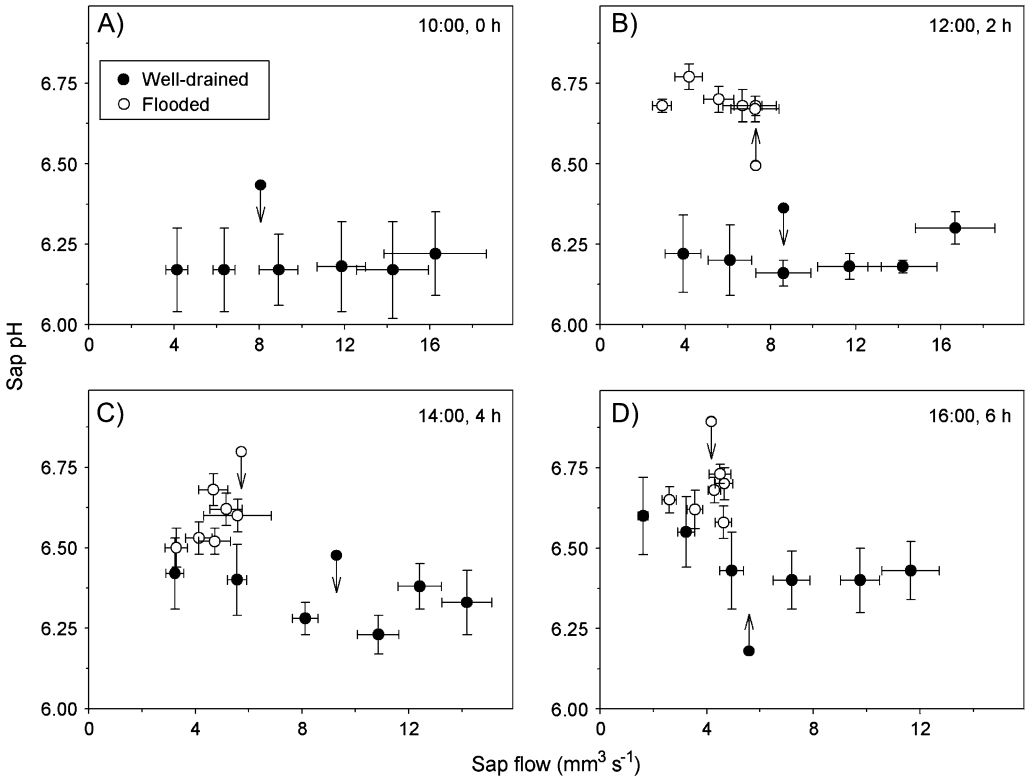

Fig. 3. Effect of soil flooding on the pH of xylem sap expressed from detopped, pressurized roots at flows that encompassed those of whole-plant transpiration. pH was measured in 15 mm^3 subsamples with a 'pH Boy' meter. Each data point is a mean of six replicate plants with associated standard errors; arrows indicate rates of whole-plant transpiration.

pH-mediated redistribution of shoot-sourced ABA

Concentrations of ABA in perfusates from petiole sections perfused only with ABA-free acidic buffer declined gradually with time (Fig. 4). When the perfusion solution was changed to pH 7 buffer, [ABA] in the perfusate increased by 12% within 10 min (data not shown) and remained elevated until the petioles were again perfused with pH 6 buffer (Fig. 4).

Xylem sap pH was increased in both well-drained and flooded plants by passage through stem and petiole tissue, compared with values measured at the shoot base (Tables 1, 2). In well-drained plants, despite the increased sap pH,

Table 1. *Effect of soil flooding on free and conjugated ABA in xylem sap flowing at rates of whole-plant transpiration*

[ABA] was determined by GC–MS (SIM); conjugated ABA was first hydrolysed to free ABA before quantification. Delivery rates were obtained by multiplying sap flow rates and free ABA or conjugated ABA concentrations. Results are means of six replicates with associated standard errors.

Time (h)	[ABA] (μmol m^{-3})		ABA delivery (fmol s^{-1})		Conjugated [ABA] (μmol m^{-3})		Conjugated ABA delivery (fmol s^{-1})	
	Well-drained	Flooded	Well-drained	Flooded	Well-drained	Flooded	Well-drained	Flooded
0	34.7±10.4	–	327.8±102.7	–	23.04±10.51	–	193.57±106.35	–
2	40.2±7.1	5.8±1.3	461.4±85.7	42.3±13.8	15.36±4.24	19.95±5.71	165.07±45.53	166.73±45.47
4	43.9±2.6	8.9±3.6	344.8±27.5	57.7±24.9	12.62±1.89	12.55±3.16	91.64±12.53	99.05±37.77
6	89.9±20.9	4.9±1.2	434.7±120.2	21.1±5.1	34.09	29.75±9.61	174.62	119.09±36.35

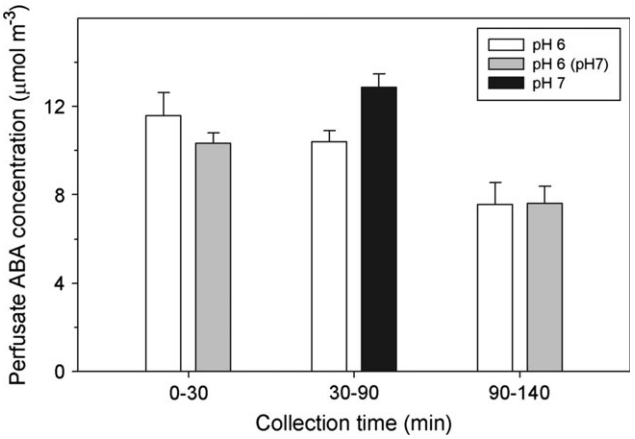

Fig. 4. Effect of perfusion buffer pH on the extent of ABA redistribution into the xylem of excised 60 mm long petiole sections. Initially, potassium phosphate buffer (1 mol m^{-3}) pH 6 was perfused through all petiole sections at 60 mm^3 min^{-1}. Perfusates were collected on ice, weighed, and frozen in liquid N_2. After 30 min, the perfusion solution was changed to potassium phosphate buffer pH 7 in half of the petioles; the other half continued to be perfused with pH 6 buffer. After 50 min, all petioles were perfused with pH 6 buffer and the perfusates collected for a further 50 min. [ABA] in the perfusates was determined by GC–MS (SIM).

[ABA] in sap expelled from the fifth oldest petiole was reduced compared with values measured in sap collected at the shoot base (Tables 1, 2). However, xylem sap collected from the fifth oldest leaf of 'intact' plants flooded for 3 h was augmented by shoot-sourced ABA (Tables 1 and 2).

ABA dose response

An [ABA] of 15 μmol m^{-3} (+)-ABA only slightly reduced transpirational water loss from detached leaves compared with those fed with pH 6.7 buffer alone (Fig. 5). However, rates of water loss were not limited further when leaves were fed with a pH 7.1 buffer containing 12 μmol m^{-3} (+)-ABA (Fig. 5). A concentration of at least 25 μmol m^{-3} (+)-ABA was necessary to limit water loss from detached, transpiring leaves (data not shown).

Commelina *bioassay*

When epidermal strips were incubated for 1 h on sap from plants flooded for 4 h, stomatal apertures decreased by 27% compared with those incubated on sap from well-drained plants (Fig. 6). Stomatal apertures were further reduced by up to 65% when sap collected from plants flooded for up to 24 h was tested in the assay (Fig. 6). These effects on stomatal apertures were maintained for the remaining 2 h of the assay (data not shown). Apertures of strips floated on sap from well-drained plants were similar to those of strips floated on MES buffer (Fig. 6).

Discussion

The signal(s) that prompts and maintains stomatal closure in tomato plants following soil flooding has yet to be identified. Although a hydraulic signal was generated within the first few hours of soil flooding, earlier work demonstrated that it was not sufficient to trigger stomatal closure in tomato (Else *et al.*, 1995*a*). Stomata continued to close even though a balancing pressure was applied at the roots to prevent the transient leaf water deficits triggered by a flooding-induced suppression of the normal daily rise in root hydraulic conductivity (Else *et al.*, 1995*a*). Rapid reductions in the delivery of xylem sap solutes, including calcium, nitrate, potassium, and other ions from flooded roots, have been reported (Jackson *et al.*, 2003). However, reducing or eliminating the delivery of potassium in detached leaf tests failed to invoke stomatal closure.

The detached leaf transpiration bioassays indicate that sap collected from tomato plants flooded for 2 h and 4 h contained one or more factors that reduced foliar water loss. Sap was filtered immediately prior to use in bioassays to remove any particulate matter that could have occluded xylem vessels and all leaves remained turgid throughout the experiments. Thus, unlike some other studies, the anti-transpirant activity in sap from flooded plants was not an artefact of storage at −70 °C (Munns *et al.*, 1993; Sinclair *et al.*, 1995) or occlusion of xylem vessels by large proteins (Zhu and Zhang, 1997).

It has already been reported that xylem sap ABA deliveries fall after 4 h of inundation (Else *et al.*, 1996; Janowiak *et al.*, 2002), an expected outcome of declining oxygen availability in flooded soils coupled with the dependency of ABA biosynthesis on molecular oxygen. However, ABA is a weak acid and its *in planta* distribution is governed by pH gradients between different

Table 2. *Effects of soil flooding for 3 h on pH and ABA concentration in xylem sap collected from leaf 5 of 'intact' tomato plants*

Sap samples were collected, at realistic flow rates, using split-top pressure chambers. Results are the means of 24 (pH) or 6–8 (ABA) replicates with associated standard errors.

Treatment	Fifth oldest leaf	
	pH	ABA (μmol m^{-3})
Well-drained	6.7±0.01	15.4±3.9
3 h flooded	7.1±0.02	12.5±3.7

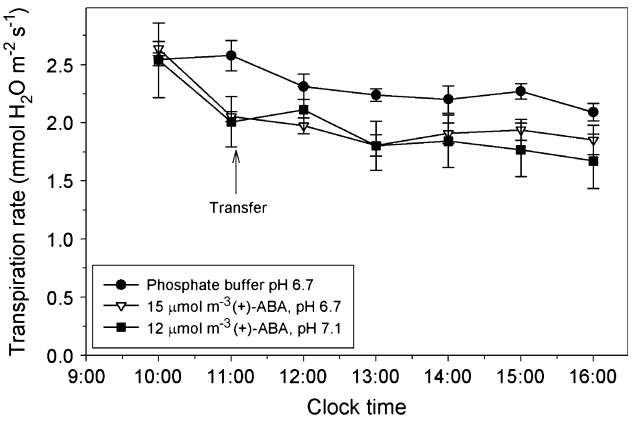

Fig. 5. Effect of buffer pH on water loss from detached, transpiring leaves fed with 12 and 15 μmol m^{-3} (+)-ABA. Initially, all leaves were fed 1 mol m^{-3} potassium phosphate buffer (pH 6.7) via the xylem. After 2 h, leaves were quickly transferred to new vials containing either pH 6.7 phosphate buffer, pH 6.7 phosphate buffer with 15 μmol m^{-3} (+)-ABA, or pH 7.1 phosphate buffer with 12 μmol m^{-3} (+)-ABA. Each data point is a mean of six replicate leaves with associated standard errors.

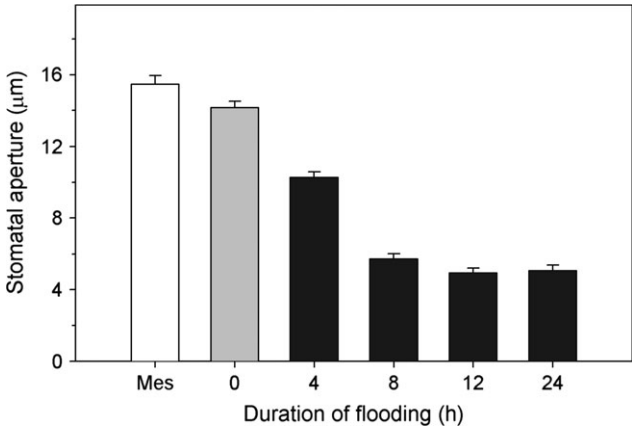

Fig. 6. Stomatal apertures of epidermal strips of *Commelina communis* floated on MES or xylem sap collected from roots of flooded and well-drained plants flowing at rates of whole-plant transpiration. Sap was diluted 4-fold prior to assay. ABA concentrations in xylem sap of plants flooded for 4 h and well-drained plants as determined by GC–MS were 8.9 and 43.9 μmol m^{-3}, respectively, before dilution. Results are the means of 10 replicates with associated standard errors.

membrane-bound compartments (Kaiser and Hartung, 1981; Slovik and Hartung, 1992; Hartung *et al*., 2002). Xylem sap becomes more alkaline within 3 h of soil flooding (Jackson *et al*., 2003), and the present results show a rise of 0.5 pH units within 2 h of inundation (Fig. 3). Tests were conducted to determine whether this rapid rise in sap pH altered the partitioning of existing root-sourced ABA and enriched xylem [ABA] within the first few hours of flooding. The GC–MS analyses showed that xylem sap [ABA] was already reduced by 80% within 2 h of soil flooding and declined further during the next few hours. Therefore, the rapid reductions in stomatal apertures were not triggered by an increased flux of redistributed root-sourced ABA in the minutes immediately following flooding.

An investigation was also conducted to determine whether soil flooding increased the loading and transport of conjugated ABA in the xylem. Hartung and co-workers have suggested that xylem-borne ABA-GE may play a role in regulating apoplastic [ABA]; β-glucosidase enzymes can liberate ABA from ABA-GE in the leaf apoplast, and glucosidase activity increased 7-fold following salt stress (Dietz *et al*., 2000). The mechanism by which ABA-GE is loaded into the root xylem is not clear; the low membrane permeability coefficient and hydrophilic nature of ABA-GE (Baier *et al*., 1988) necessitates the involvement of an, as yet, unidentified carrier (Sauter and Hartung, 2000). In flooded plants, root cell integrity is quickly compromised (Everard and Drew, 1989; Else *et al*., 1995*b*) and could facilitate the unmediated entry of ABA-GE into the xylem. However, the GC–MS analyses indicated that xylem sap concentrations of conjugated ABA, including ABA-GE, were not altered by soil flooding. Thus, the early stomatal closure was not triggered by increased output of ABA-GE from flooded roots. Whether glucosidase activity increases following soil flooding and liberates ABA into the leaf apoplast is not yet known. However, the epidermal strip bioassay suggests that flooding-induced stomatal closure is triggered by other means (see below).

Sap issuing from the base of flooded plants clearly contains one or more substances that initiates some stomatal closure in detached leaf tests. En route to the leaves, this anti-transpirant activity may be modified further by other signals extruded into the sap from xylem parenchyma cells; Fromard *et al*. (1995) reported that vessel-associated cells acidified sap through extrusion of H ions as it passed through *Robinia* wood. Also, xylem sap can be enriched with ABA sourced from xylem parenchyma cells as sap flows through stems, petioles, and leaf tissue (Sauter *et al*., 2002). Xylem [ABA] was increased by 4 μmol m^{-3} after passage of ABA-free buffer solution through 60–88 mm long maize mesocotyl sections (Sauter and Hartung, 2002). A role for shoot-sourced ABA in the stomatal response to soil flooding was also suggested by grafting experiments with the ABA-deficient tomato mutant *flacca* (Jackson, 1991). Therefore, tests were conducted

to determine whether the stomatal response to soil flooding was triggered by a pH-mediated redistribution of shoot-sourced ABA (Hartung *et al*., 1998) that more than compensates for the loss of root-sourced ABA.

Tests were performed to determine whether sap pH was modified en route to the shoots and whether any associated enrichment of xylem sap [ABA] would be sufficient to initiate stomatal closure. The petiole perfusion experiments revealed that a pH change from 6 to 7 increased xylem [ABA] by 3 μmol m^{-3} when solutions were perfused at realistic flow rates through 60 mm long petiole sections (see also Sauter and Hartung, 2002). This higher concentration was sustained until the perfusate pH was lowered again. Assuming a total distance of 120–300 mm between the hypocotyl and the leaves, xylem sap [ABA] could be enriched by 6–15 μmol m^{-3}. The direct measurements of [ABA] in sap entering the leaves of flooded plants confirmed that xylem sap [ABA] was enriched by 7 μmol m^{-3} (Table 2). However, amidst the 80–90% reduction in ABA output from flooded roots, the contribution of pH-mediated ABA redistribution to the apoplastic concentrations was insignificant. In well-drained plants, ABA was removed from the sap as it flowed through the shoots since xylem [ABA] entering the leaves was lower than that measured at the shoot base. A similar reduction was reported in *Ricinus communis* by Jokhan *et al*. (1999).

The nature of the flooding-induced xylem sap alkalinization is not yet known (Felle, 2005). Reduced ATP levels arising from limited oxygen availability must quickly impact on the activity of H^+-ATPases with subsequent limited extrusion of H^+ into the apoplast (Netting, 2000). Schurr and co-workers have argued that altered ionic composition can affect sap pH via a strong ion difference (Schurr *et al*., 1992; Gerendas and Schurr, 1999). The ionic composition of xylem sap is altered markedly in the first few hours after inundation (Jackson *et al*., 2003). Nitrate and phosphate concentrations are strongly depressed and output of potassium, calcium, and magnesium is also reduced within 2 h of flooding (Jackson *et al*., 2003; MA Else *et al*., unpublished data). Depletion of nitrate and phosphate can increase the apparent sensitivity of stomata to xylem ABA (Radin *et al*., 1982; Radin, 1984). However, given the substantial reductions in xylem ABA within the first few hours of flooding, it is questionable whether such changes in sensitivity underlie the flooding-induced stomatal responses. Kirkby and Armstrong (1980) proposed that xylem sap [malate] can influence sap pH, and Pantonnier *et al*. (1999) reported a pH-mediated effect of xylem sap malate on stomatal apertures. However, preliminary experiments suggested that xylem malate deliveries were unaffected during the first few hours after soil flooding (F Janowiak *et al*., unpublished data).

In the *Commelina* bioassay, stomatal apertures were reduced by exposure to diluted xylem sap collected from plants flooded for 4 h. Apertures were further reduced by sap collected after 8, 12, and 24 h of soil flooding. GC–MS analyses indicated that the ABA concentrations in these sap samples were only 5% of those from well-drained plants. The tests reported here compared the activity of sap taken from well-drained and flooded plants flowing at their respective rates of whole-plant transpiration. Thus, concentrations would be similar to those present in the transpiration stream of intact plants (Else *et al*., 1995*b*). Tiekstra (1999) characterized further the anti-transpirant activity in sap from plants flooded for 24 h and found that it was reversible, non-proteinaceous, and non-calcium based. Solvent partitioning of xylem sap with ethyl acetate removed ABA, but substantial stomatal closing activity remained in the aqueous fraction (Tiekstra, 1999).

In summary, both the detached leaf transpiration tests and *Commelina* epidermal bioassay suggest that soil flooding quickly causes changes in the anti-transpirant activity in xylem sap that cannot be attributed either to ABA content or to pH. Whether oxygen deficiency promotes the output of ABA precursors from flooded roots in a manner analogous to that of aminocyclopropane-1-carboxylic acid (ACC) is not yet known. Alternatively, the anti-transpirant activity could be attributable to a flooding-induced increase in hydrogen peroxide, an important signalling intermediate in guard cell closure (Zhang *et al*., 2001). These possibilities are currently being investigated.

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