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Stomatal Closure in Flooded Tomato Plants Involves Abscisic Acid and a Chemically Unidentified Anti-Transpirant in Xylem Sap[1]

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We address the question of how soil flooding closes stomata of tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig) plants within a few hours in the absence of leaf water deficits. Three hypotheses to explain this were tested, namely that (a) flooding increases abscisic acid (ABA) export in xylem sap from roots, (b) flooding increases ABA synthesis and export from older to younger leaves, and (c) flooding promotes accumulation of ABA within foliage because of reduced export. Hypothesis a was rejected because delivery of ABA from flooded roots in xylem sap decreased. Hypothesis b was rejected because older leaves neither supplied younger leaves with ABA nor influenced their stomata. Limited support was obtained for hypothesis c. Heat girdling of petioles inhibited phloem export and mimicked flooding by decreasing export of [^{14}C]sucrose, increasing bulk ABA, and closing stomata without leaf water deficits. However, in flooded plants bulk leaf ABA did not increase until after stomata began to close. Later, ABA declined, even though stomata remained closed. *Commelina communis* L. epidermal strip bioassays showed that xylem sap from roots of flooded tomato plants contained an unknown factor that promoted stomatal closure, but it was not ABA. This may be a root-sourced positive message that closes stomata in flooded tomato plants.

There are numerous reports that show that flooding of the soil closes stomata (see Jackson, 1993, and refs. therein). This closure has been associated with increases in the concentration of bulk ABA in leaves (Hiron and Wright, 1973; Jackson and Drew, 1984; Jackson, 1985; Zhang and Davies, 1987; Castonguay et al., 1993; Zhang and Zhang, 1994). These measurements, coupled with reports of an attenuated stomatal response to flooding by ABA-deficient mutants of tomato (*Lycopersicon esculentum*) and pea (*Pisum sativum*) (Jackson, 1990, 1991), implicate ABA as the regulator. However, detailed time courses showing a close relationship between stomatal behavior and bulk leaf ABA are rare.

Several previous attempts have been made to characterize the message generated by oxygen-deficient roots that closes stomata during soil flooding. Leaves of plants with oxygen-deficient roots export less assimilate (Vartapetian et al., 1978; Castonguay et al., 1993). Accordingly Jackson (1985, 1991) proposed that stomatal closure is promoted by ABA that accumulates in leaves because of diminished phloem export resulting from depressed growth in root mass (Neuman and Smit, 1991; but see Reece and Riha, 1991). The notion of ABA as an accumulation message is supported by experiments that show that phloem sap contains ABA (Hoad, 1975) and that the hormone can move out of leaves into the phloem (Zeevaart and Boyer, 1984; Wolf et al., 1990). Furthermore, when sinks for assimilates are removed (Jackson and Hall, 1987) or the phloem pathway is interrupted (Setter et al., 1980; Setter and Brun, 1981), ABA concentrations increase and stomata close in the absence of leaf water deficits.

An alternative to the accumulation message hypothesis, first proposed by Zhang and Davies (1987), is that the additional foliar ABA originates in flooded roots and is transferred as a positive message to leaves via the transpiration stream. But subsequent work (Jackson et al., 1988) failed to confirm that oxygen-deficient roots of peas were a rich source of ABA (Castonguay et al., 1993) for the shoots. Moreover, flood-induced stomatal closure and increased foliar ABA were not prevented by grafting shoots of pea or tomato onto root systems of ABA-deficient mutants (Jackson, 1991), and ABA delivered from roots to shoots in xylem sap decreases rather than increases after 24 h of flooding (Smit et al., 1990; Else et al., 1995b). A third hypothesis was suggested by Zhang and Zhang (1994), who attributed increased levels of ABA in younger leaves of flooded pea plants to synthesis and export from older leaves in association with a loss of turgor and wilting caused by waterlogging the soil. They showed that ABA increased in old leaves coincidentally with the onset of stomatal closure in young leaves and before bulk ABA increased in these young leaves. A fourth possibility is that foliar water deficits, caused by decreased root hydraulic conductance (negative hydraulic message), increases leaf ABA biosynthesis through turgor loss. Although this may

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Abbreviations: ANOVA, analysis of variance; g_s, stomatal conductance; ψ_L, leaf water potential.

be true sometimes (Hiron and Wright, 1973), we have recently shown that it is not an essential component of the signaling system (Else et al., 1995a).

Clearly, there is little consensus about how oxygen shortage at the roots may close stomata and increase ABA supply to receptor sites. There is also a lack of detailed time courses linking changes in bulk leaf ABA with g_s. We address this shortcoming in the present paper and reassess three existing hypotheses for the source of ABA thought to close stomata of flooded plants: (a) increased delivery from roots in xylem sap, (b) accumulation within leaves due to diminished phloem export, and (c) increased export from old leaves to young leaves. We also examine the possibility that xylem sap flowing from flooded roots possesses stomatal closing activity that is not attributable to ABA.

MATERIALS AND METHODS

Seedlings of tomato (*Lycopersicon esculentum* Mill, cv Ailsa Craig) were transplanted into 95-mm-diameter plastic pots filled with general purpose Levington MS peat-based compost (Fisons, Ipswich, UK) containing 2 kg m^{-3} slow-release fertilizer (Osmocote, Grace Sierra UK, Nottingham, UK) and grown in a Fisons 1550 controlled environment room. Light and dark temperatures were 25 and 20°C, respectively, the photoperiod was 16 h at 400 μmol m^{-2} s^{-1} PPFD, and the RH was 50%. All pots were irrigated automatically, and side shoots were removed from plants regularly. Plants with seven or eight leaves were flooded for up to 72 h by placing their pots into 1.2 × 10^{-3}-m^3 containers filled with tap water.

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

Measurements of *gs* and ψ_L

Abaxial g_s of leaf 4 was determined at frequent intervals using an Mk3 diffusion porometer (Delta-T Devices, Cambridge, UK). ψ_Ls of individual leaflets were determined with a Scholander-type pressure chamber pressurized at a rate of approximately 0.02 MPa s^{-1}. Measurements were not adjusted for the influence of osmolites in xylem sap.

Collection of Xylem Sap and Leaf Samples

Xylem sap was collected from root systems of well-drained or flooded plants at 4-h intervals throughout the first photoperiod and less frequently thereafter. Plants were detopped and a 20-mm length of rubber sleeving was attached to the stump. The assembly was placed in a pressure chamber and pressurized using compressed air for well-drained plants or oxygen-free nitrogen for flooded plants. The pressures chosen generated flows from detopped root systems that were similar to whole-plant transpiration rates. Sap was collected for 10 min in Eppendorf tubes or in glass vials with screw lids. The initial 200 mm^3 of sap from each root system was discarded (Else et al., 1994). Samples were weighed, frozen in liquid nitrogen, and stored at −20°C for 7 d prior to analysis. Single leaflets were removed with a razor blade from leaf 4 (counting from the base) at intervals during a 72-h period. Samples were placed immediately in glass vials with screw lids, weighed, and frozen in liquid nitrogen before storing at −20°C.

ABA Analyses

Xylem ABA concentrations were measured by GC-MS under conditions described by Whitford and Croker (1991) but with the following modifications. Ten nanograms of [$^2H_3$]ABA (a level that approximately matched the endogenous ABA level in well-drained sap) were added to 1 × 10^{-6} m^3 xylem sap samples, which were evaporated to dryness under vacuum, redissolved in 50 mm^3 of methanol, and methylated with ethereal diazomethane. The methylated samples were dried and redissolved in 20 mm^3 of ethyl acetate. Samples (3 mm^3) were injected into a fused silica WCOT capillary column (25 mm × 0.22 mm × 0.15 μm) with a coating of BPX5 (SGE, Milton Keynes, UK). Ions at *m/z* 190 and 162 (methyl abscisate) and *m/z* 193 and 165 ([*methyl*-$^2H_3$]abscisate) were monitored, and the 190:193 ion ratio was used to calculate ABA concentration. ABA delivery rates in xylem sap issuing from roots were obtained by multiplying sap flow rates by concentrations (Else et al., 1995b).

ABA in leaves (about 0.5 g fresh weight) was measured by GC-MS. Before extraction, 100 ng of [$^2H_3$]ABA were added to frozen tissue samples. Samples were homogenized (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany) in 80% methanol (containing 20 g m^{-3} butylated hydroxytoluene) for 3 min and centrifuged at 4000 rpm, and the supernatant was decanted. The extraction was repeated. The methanol was taken off in vacuo, and the aqueous phase containing ABA was passed through filters (Nylon 66, Alltech, Carnforth, Lancashire, UK) and C_{18} Sep-Pak reverse-phase cartridges (Waters) eluted with 6% methanol. Filtering through Nylon 66 membranes removed the need for time-consuming purification by HPLC. Samples were then prepared for GC-MS in the same way as described for sap samples. Concentrations are expressed on a fresh weight basis.

Phloem Translocation Studies

[^{14}C]Suc (specific activity 370 MBq $mmol^{-1}$) was applied to well-drained and 6-h-flooded plants in water containing 0.1% (mass/volume) Tween 20. A 5-mm^3 droplet of [^{14}C]Suc solution (1629 Bq per droplet) was applied to an area devoid of major veins on seven leaflets of leaf 4, giving an average application of 11,403 Bq per leaf. Plants were harvested after an 18-h uptake period. Treated leaflets were washed with 20 × 10^{-6} m^3 of a 50% ethanol solution, and the washings were collected for scintillation counting. Plants were then divided into the lamina and petiole of the leaf receiving [^{14}C]Suc and all other shoot tissue. In plants in which the treated leaf was girdled, this amounted to sampling tissue distal and proximal to the girdle. Roots

were not sampled because of difficulties with separating them from the peat-based compost. Plant material was dried overnight at 100°C, weighed, and then burned in a biological oxidizer (Harvey OX-500; RJ Harvey, Hillsdale, NJ). $^{14}CO_2$ released by combustion was absorbed in Carbomax Plus (Lumac LSC, Groningen, The Netherlands) scintillant and counted on a 1217 liquid scintillation counter (LKB, Bromma, Sweden). Recovery of ^{14}C through oxidation and scintillation was 99%.

Petiole Girdling

Well-drained and 6-h-flooded plants were divided into two groups: half was petiole-girdled and the other half was not. The petiole of leaf 4 was girdled at 9 AM, 1 h after the start of the 16-h photoperiod, by passing a 10-V potential for 20 s across a 300-mm length of constantan wire looped around the petiole (Housley et al., 1977; Smith and Dale, 1988). Approximately 1 h after this treatment, a 2- to 3-mm ring of damaged tissue was clearly visible. The lamina of the girdled leaves remained turgid throughout the experiment, although they needed physical support from small props inserted in the soil. At 2 PM on the same day, [^{14}C]Suc was applied to the plants as described above. After an 18-h uptake period, plants were divided into two tissue samples: lamina and petiole distal to the petiole girdle and the rest of the plant, excluding roots. Plant material was dried and analyzed for radioactive content as described above.

Commelina Epidermal Strip Bioassay

The third leaf was detached from 4-week-old plants, and the epidermis was stripped from the abaxial surface using the method described by Weyers and Meidner (1990). Each strip was divided into 5 × 5-mm strips. Eighty epidermal strips were divided into groups of 10 and floated in eight plastic 50-mm-diameter Petri dishes containing 5×10^{-6} m^3 of 10 mol m^{-3} Mes buffer (Sigma) and 50 mol m^{-3} KCl (BDH Chemicals, Poole, UK) 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 PPFD of 280 μmol m^{-2} s^{-1}. CO_2-free air (ambient air passed through a column of three- to nine-mesh Carbosorb sodalime [BDH]) was bubbled through hypodermic needles into the buffer in each Petri dish at 5×10^{-6} m^3 min^{-1}. After incubation, single epidermal strips were selected randomly from each dish and mounted on a microscope slide, and the apertures of 10 stomata from each strip were measured under a light microscope.

Xylem sap samples were obtained from flooded and well-drained tomato plants flowing from roots of detopped plants at their respective rates of whole-plant transpiration. Sap was diluted 4-fold in Mes buffer and KCl and incubated for 30 min on a water bath under the conditions described above. The sap was divided into six aliquots of 5×10^{-6} m^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 controls. Single strips were removed at intervals, and the apertures of 10 individual stomata per strip were measured.

Statistical Analyses

Treatment effects on g_s, xylem ABA, and bulk leaf ABA of girdled and nongirdled plants were compared by ANOVA after $\log_e$ transformation to decrease variance heterogeneity. To obtain ^{14}C percentage export data, an angular transformation was required to give sufficient variance homogeneity before comparison by ANOVA. All other data were compared by ANOVA and LSDs were generated ($P < 0.05$).

RESULTS

g_s

g_s of well-drained plants increased rapidly at the beginning of each photoperiod, reaching a maximum between midday and early afternoon, and then declined (Fig. 1). However, after 4 h of flooding, stomata opened less than those of well-drained plants, and thereafter, g_s remained static and later declined to 48 to 67% of well-drained plant values. At the beginning of the second photoperiod, stomata of flooded plants failed to open fully, after which time closure increased progressively and was even more complete in the third and fourth photoperiods (Fig. 1).

Effect of Flooding on Foliar ABA

ABA concentrations in well-drained leaves remained constant throughout each photoperiod (Fig. 2A). For the first 8 h, concentrations of ABA in leaves of flooded plants were little different from controls. After 12 h, a 44% increase was seen, but this was sustained for only another

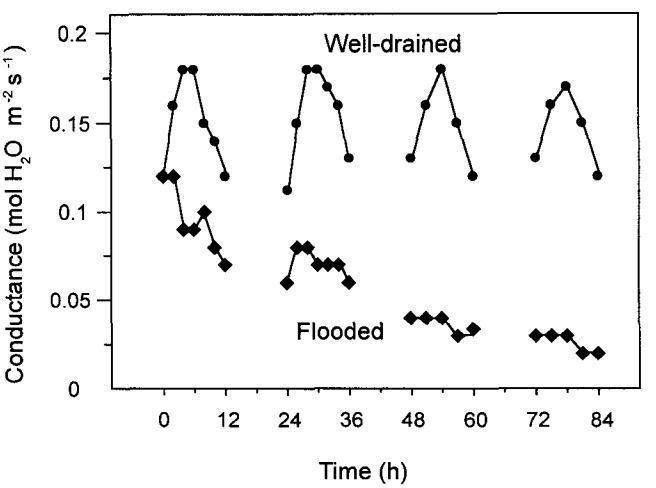

Figure 1. Effect of flooding over 84 h on abaxial g_s of the fourth-oldest leaf of tomato plants. All means from flooded plants are significantly different from well-drained plants except at 2 h (LSD of $\log_e$ transformed data = 0.217, $P < 0.05$, $n = 8$). Results for the first 36 h appear in Else et al. (1995a).

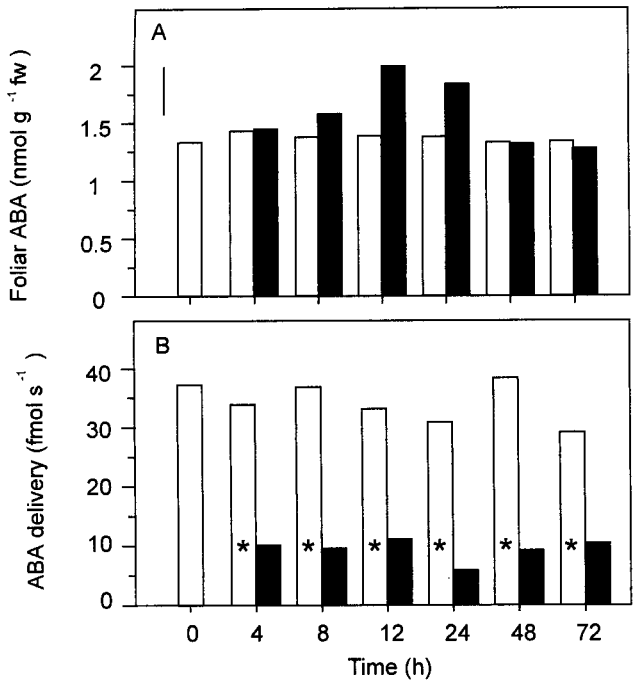

Figure 2. A, Effect of 72 h of flooding on ABA concentrations in leaf 4 (counting from base) of tomato plants, determined by GC-MS. The vertical line is the LSD ($P < 0.05$, $n = 5$). B, Effect of 72 h of flooding on ABA delivery in xylem sap expressed from detopped root systems pressurized to generate sap flow rates that were similar to whole-plant transpiration. ABA delivery rates were calculated by multiplying concentrations by sap flow rates. Asterisks indicate a statistically significant difference (LSD of $\log_e$ transformed data = 0.238, $P < 0.05$, $n = 8$). fw, Fresh weight. Open bars, Well-drained plants; black bars, flooded plants.

12 h. By the end of the second photoperiod (36 h), foliar ABA concentrations were again similar to those of well-drained controls, and this similarity persisted until at least 72 h (Fig. 2A), despite large differences in g_s.

Testing the Hypothesis of ABA as a Positive Message

Export of ABA in sap expressed from detopped oxygen-deficient roots decreased by 75% within 4 h of the start of soil flooding when compared with well-drained plant values (Fig. 2B). This large decrease was sustained for at least 72 h. At no time was there any increase in ABA delivery from flooded roots. Delivery of ABA from roots of well-drained plants remained constant throughout the experiment. There was no evidence of variation in pattern of export from well-aerated roots within a photoperiod, despite sap flow rates being adjusted to parallel the increase and decrease of transpiration brought about by the stomatal behavior shown in Figure 1.

Testing the Accumulation Hypothesis

Export of [^{14}C]Suc

Export of ^{14}C to other parts of the shoot system from the leaf, to which the radiolabeled Suc was applied (leaf 4), was measured by deducting the amount of ^{14}C found in the treated leaf from the total amount of ^{14}C present in the whole of the shoot 18 h after Suc application. This amount was expressed as a percentage of total ^{14}C content of the whole shoot. Uptake of ^{14}C into the plant was not influenced by flooding or girdling, since in all treatments uptake accounted for a similar proportion of the radioactivity supplied (i.e. 6.4–16.2% of that rinsed from the leaves after 24 h). However, distribution within the plant of absorbed ^{14}C was strongly affected by these treatments. In well-drained plants, 49% of total ^{14}C taken-up was exported from the source leaf lamina. Flooding reduced this export value to 20%. Petiole girdling also reduced translocation of ^{14}C out of treated laminae. It was equally effective in both flooded and well-drained plants, with only 1% of the total radioactivity taken up being exported past the girdle (data not shown).

g_s, ψ_L, and ABA Concentration of Girdled Leaves

Stomatal behavior of nongirdled well-drained and flooded plants is shown in Figure 3A and was similar to that shown in Figure 1. Girdling neither inhibited nor added to the effect of flooding, whereas girdling well-drained plants gave a pattern of closure resembling that of flooded plants (Fig. 3A). Ψ_L of well-drained plants remained approximately −0.45 MPa throughout each photoperiod (Fig. 3B). After girdling, ψ_L of leaf 4 was comparable to that of leaves of well-drained plants during the first photoperiod but was less negative during the second photoperiod (Fig. 3B). Ψ_L of flooded plants decreased transiently during the first 6 h of inundation, returning to well-drained plant values at the end of the first photoperiod. Girdling did not alter this pattern. During the subsequent photoperiod, flooded plants had significantly higher (less negative) ψ_L than their well-drained counterparts. Again, girdling the leaf under study did not alter the timing or extent of this flooding-induced reversal in ψ_L (Fig. 3B).

As shown in Figure 2A, bulk ABA concentrations in leaflets from flooded plants increased transiently at 12 and 24 h after the start of flooding but subsequently returned to control values (Fig. 3C). If this ABA transient was a consequence of hormone accumulation because of inhibition of export, then girdling should reproduce the flooding effect in well-drained plants. Girdling leaf 4 of a well-drained plant did increase its ABA concentration (Fig. 3C, compare girdled and nongirdled well-drained plants). In flooded plants, too, girdling increased ABA levels to above those obtained by flooding alone. There was no statistically significant interaction between flooding and girdling (Fig. 3C).

Testing Older Leaves as a Source of ABA

Leaf 4 of well-drained plants, each with eight visible leaves, showed the expected pattern of g_s during the two photoperiods, i.e. increasing toward midday and then decreasing gradually thereafter (Fig. 4A). This pattern was not materially altered by girdling the three oldest leaves

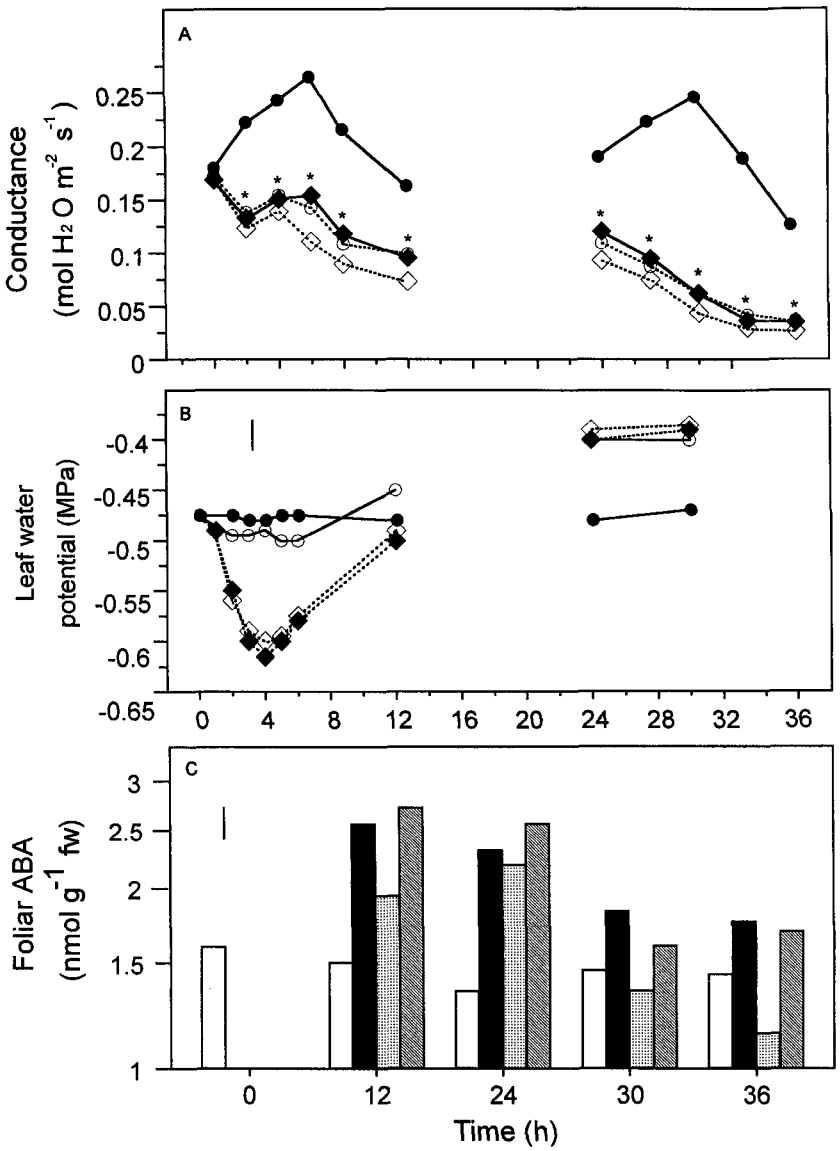

Figure 3. Effects on tomato plants with seven or eight leaves of soil flooding and/or girdling leaf 4 on g_s of leaf 4 (LSD of $\log_e$ transformed data = 0.245, $P < 0.05$) (A), ψ_L of leaf 4 (B), and ABA concentrations of leaf 4 (C). Plants were girdled at 9 AM and flooded at 10 AM. g_s was measured at intervals with a diffusion porometer, ψ_L was determined with a pressure chamber, and leaf ABA concentrations were measured by GC-MS. Results are means of eight replicates in A and B and five replicates in C. Asterisks in A indicate significant differences between means ($P < 0.05$). Vertical lines in B and C are LSDs ($P < 0.05$). A and B, ●, Well-drained plants; ○, well-drained plus girdled plants; ◆, flooded plants; ◇, flooded plus girdled plants. C, Open bars, Well-drained plants; black bars, flooded plants; dotted bars, well-drained plus girdled plants; shaded bars, flooded plus girdled plants. fw, Fresh weight.

below. As before, flooding decreased g_s of leaf 4 within 4 h. Girdling the lower leaves to prevent possible export of ABA from them to leaf 4 did not interfere with its flooding response during the first photoperiod (Fig. 4A). At the beginning of the second photoperiod, stomata of flooded plants failed to open fully, and closure developed gradually during the rest of the photoperiod (Fig. 4A). Again, girdling the lower leaves to inhibit possible ABA export did not interfere with this response to soil flooding (Fig. 4A). Similarly, flooding the soil for 24 h substantially increased leaf ψ_Ls (less negative) of leaf 4, the expected consequence of stomatal closure. Girdling the three oldest leaves did not prevent this increase in leaf ψ_L in the younger leaf 4 above (Fig. 4B). This lack of effect by girdling the oldest three leaves was repeated in terms of ABA accumulation in leaf 4 (Fig. 4C). These results suggest that, at least during the first 3 d of soil flooding, increased foliar ABA concentrations and attendant stomatal closure in young leaves of tomato plants are not reliant on synthesis and import of ABA or any other active factor from older leaves.

Commelina Bioassay

When epidermal strips were incubated for 1 h on Mes buffer containing sap from flooded plants, stomatal apertures decreased by nearly 39% compared with those incubated on Mes and sap from well-drained plants. The effect was maintained for at least 2 h more (Table I). Apertures of strips floated on Mes plus well-drained sap were similar to those of strips floated on Mes buffer alone. GC-MS analyses indicated that the ABA concentration in sap collected from roots of plants flooded for 48 h was only 15% of that from well-drained plants (Table I). It is instructive to assess the stomatal closing activity of xylem sap in terms of ABA equivalents. After 1 h of incubation stomatal apertures of epidermal strips supplied with xylem sap from well-drained plants (16.2 μm) were similar to those given by

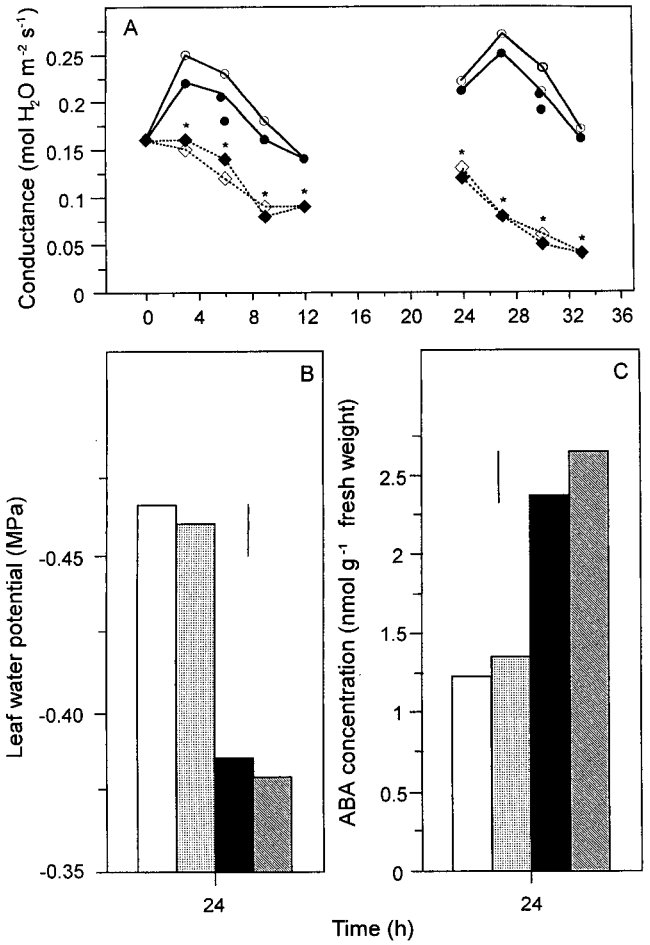

Figure 4. Effects in tomato plants with seven or eight leaves of flooding and/or girdling the three oldest leaves on g_s (LSD of $\log_e$ transformed data = 0.245, $P < 0.05$, $n = 8$) (A), ψ_L (B), and bulk ABA concentration of leaf 4 (C). Plants were girdled and/or flooded as for Figure 3. Vertical lines are LSDs ($P < 0.05$, $n = 8$). A, ●, Well-drained plants; ○, well-drained plus girdled plants; ◆, flooded plants; ◇, flooded plus girdled plants. B and C, Open bars, Well-drained plants; dotted bars, well-drained plus girdled plants; black bars, flooded plants; hatched bars, flooded plus girdled plants.

approximately 1 μmol m^{-3} of synthetic (+)-ABA. This concentration is very close to the actual concentration of ABA in the sap as measured by GC-MS (1.23 μmol m^{-3}). Thus, most of the activity in this sap from well-drained plants is attributable to its ABA content. In contrast, 25 μmol μm^{-3} (+)-ABA would be needed to reproduce the small stomatal apertures induced in the *Commelina* bioassay by xylem sap from flooded plants (9.9 μm). However, this sap contained only 0.18 μmol m^{-3} endogenous ABA. Thus, xylem sap from flooded plants possesses considerable anti-transpirant activity that cannot be attributed to ABA.

DISCUSSION

Link between Stomatal Closure and Bulk Leaf ABA

Previously published measurements of ABA in leaves of pea or tomato plants (Jackson, 1985; Jackson and Hall, 1987; Zhang and Davies, 1987; Jackson et al., 1988; Jackson, 1990, 1991) using electron capture-GC or immunoassay indicated a close relationship between patterns of stomatal closure and increasing bulk leaf ABA content. In contrast, our analyses in tomato using GC-MS reveal a much weaker relationship. The explanation could be technical. Electron capture detection of leaf ABA of flooded tomato plants may be subject to interference by substances that co-chromatograph with authentic endogenous ABA, and this requires rigorous checking. It is also possible that the radioimmunoassay used by Zhang and Davies (1987) was subject to interference by compounds other than ABA present in flooded roots. We have found such a substance(s) in xylem sap of flooded tomato plants that interferes with a similar immunoassay, thereby giving overestimates of ABA concentrations present. Indeed, this problem has contributed to two earlier reports of ours that indicated (falsely) that roots of flooded tomato plants export increased amounts of ABA to the shoots (Jackson, 1993; Jackson et al., 1993), when the converse is actually true judging from GC-MS results.

Whereas flooding decreased g_s within 4 h and sustained the effect for at least 60 h, statistically significant increases in leaf ABA, as analyzed by GC-MS, were seen only after 12 and 24 h of flooding (Figs. 2A and 4C). At earlier and later times, leaves of flooded plants contained no more total ABA than those of well-drained plants. We conclude that, if increases in leaf ABA are responsible for initiating stomatal closure induced by flooding within 4 h and maintaining it beyond 24 h, they take place in a small active pool (e.g. the guard cell apoplast) that is not fully reflected in bulk estimations. One way in which this may occur is through an alkalization of the apoplast caused by increased pH of xylem sap that occurs within 24 h of flooding (Else et al., 1995b; Jackson et al., 1996).

ABA as a Positive Message from Flooded Roots

Our present results confirm those that we reported previously (Else et al., 1995a; Jackson et al., 1996) showing that flooding does not increase delivery of ABA from roots to shoots in the transpiration stream. On the contrary, ABA

Table I. *Stomatal apertures of epidermal strips of C. communis floated on Mes buffer with or without aliquots of xylem sap collected from roots of well-drained plants or plants flooded for 48 h at rates of whole-plant transpiration*

Sap was diluted 4-fold prior to assay. ABA concentrations in xylem sap of well-drained and flooded plants as determined by GC-MS were 1.23 and 0.18 μmol m^{-3}, respectively, after dilution. Results are means ± SE of six replicates. Buffer pH was 6.15 and was not altered by the addition of sap from well-drained or flooded plants.

Time	Stomatal Apertures		
	Mes buffer	Mes buffer + flooded sap	Mes buffer + well-drained sap
h		μm	
1	18.3 ± 0.26	9.9 ± 0.28	16.2 ± 0.25
2	17.7 ± 0.37	9.9 ± 0.50	18.6 ± 0.76
3	16.0 ± 0.29	9.9 ± 0.26	16.0 ± 0.26

delivery decreases within 4 h (Fig. 2B), and this low delivery is sustained for at least 72 h. This result is compatible with the oxygen requirement of the ABA biosynthetic pathway (Creelman et al., 1987). Clearly, ABA from roots had little influence on shoot ABA content. It cannot explain the transient increase in bulk leaf ABA nor any supposed increase in ABA within the apoplastic environment of guard cells and, thus, adjacent to the presumed receptor sites for ABA (Anderson et al., 1994). If ABA from roots played a dominant role in regulating bulk leaf ABA or guard-cell apoplast ABA, concentrations in leaves of flooded plants could be expected to decrease rather than increase and/or stomata could begin to open again after 24 h. However, no such changes were seen.

ABA as an Accumulation Message

There remains the possibility that stomatal closure, the transient increase in bulk leaf ABA, and possibly masked increases in apoplastic ABA are the results of inhibited export of ABA in the phloem. This concept rests on the notion that ABA transport out of leaves in phloem sap is linked to that of Suc. Vartapetian et al. (1978) and Saglio (1985) demonstrated that oxygen-deficient roots attract less leaf assimilate. We show here that export of [^{14}C]Suc from a leaf to which it is applied is severely inhibited by 24 h of soil flooding. Thus, even if ABA biosynthesis in leaves remains unaltered, slower export would favor a buildup of ABA, which may in turn close stomata. If this rationale is sound, the effects of flooding on ^{14}C export, ABA accumulation, and stomatal closure should be reproducible in a single leaf attached to a well-drained plant by impeding phloem transport with heat girdling (Setter et al., 1980; Setter and Brun, 1981, Bradford and Hsiao, 1982, Henson, 1984; but see Reece and Riha, 1991). Our present experiments using girdled leaves confirmed this to be the case, thus supporting the possibility of an accumulation message. The results with girdling well-drained plants were reinforced by the results obtained by girdling leaves of flooded plants. Acceptance of the accumulation theory requires that the combined effect of girdling and flooding should not exceed the effect of girdling alone on a well-drained plant. We found bulk leaf ABA levels to be similar in leaves of well-drained girdled plants and those of plants that were flooded and girdled (Fig. 3C). Thus, the transient increase in leaf ABA in flooded plants can be explained in terms of an accumulation message. We found that ABA levels in girdled leaves of flooded plants were higher than those in intact flooded plants, which sustains the accumulation theory, since it is in accord with the greater effectiveness of girdling compared with flooding on inhibiting ^{14}C export from leaf 4 (99 versus 70%, respectively).

Older Leaves as a Source of ABA for Younger Leaves of Flooded Plants

The hypothesis that older leaves are a rich source of ABA for younger leaves of flooded plants was put forward by Zhang and Zhang (1994). They suggested that such leaves may be prematurely senescent and low in turgor as a result of flooding injury from unspecified causes (e.g. nitrate shortage) and thus produce ABA in increased quantities. The authors asserted that ABA from this source is exported in the phloem and recycled to younger leaves after transfer to the xylem sap in the fashion proposed by Wolf et al. (1990). However, in the present study, girdling the lower leaves did not interfere with patterns of stomatal closure and leaf hydration (Fig. 4, A and B) nor did it nullify the transient increase in leaf ABA that flooding brings about (Fig. 4C). Thus, our results do not support those of Zhang and Zhang (1994), at least from our shorter experiments.

Anti-Transpirant Activity of Sap from Flooded Plants

The absence of convincing evidence of ABA as a positive message linking soil flooding with stomatal closure or of ABA as an accumulation message that explains early closure (4 h) and prolonged closure (>24 h) encouraged us to seek other active factors. Previous reports (Munns and King, 1988; Trejo and Davies, 1991) give direct and indirect evidence for the existence of one or more such factors in xylem sap from droughted and well-watered wheat, *Phaseolus vulgaris*, and maize. Until now, direct tests that demonstrated the presence of anti-transpirants other than ABA used detached leaves and utilized their transpiration rate as an estimate of g_s. Unfortunately, such assays are subject to interference by large molecules (>0.3 kD; Munns et al., 1993) that block xylem vessels. Unless these are filtered out (Zhang and Davies, 1991) they can give a false impression of stomata-closing activity, especially in sap stored at −20°C before being assayed (Munns et al., 1993; Sinclair et al., 1995). In our experiment this problem was avoided because we used a detached epidermal strip assay (Zhang and Davies, 1991). The results show that xylem sap from tomato plants contains one or more factors other than ABA that actively close stomata. Our tests compared the activity of sap taken from well-aerated 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., 1995b). When all sap samples were diluted 4-fold, those from flooded plants reduced stomatal apertures in the *Commelina* assay within 1 h, whereas those from well-drained plants were much less active. This was the case even though GC-MS analyses of ABA showed that there was 85% less ABA in the sap from flooded plants. We have no evidence as yet concerning the chemical identity of the active component or whether larger amounts are delivered from roots of flooded plants. But we speculate that this unidentified anti-transpirant may constitute a positive message from flooded root systems that closes stomata. The precursor of ethylene ACC can be eliminated as the anti-transpirant. Although transported to the shoots in increased amounts as a xylem sap solute (Bradford and Yang, 1980; Else et al., 1995b), ACC does not close stomata in tomato plants (Bradford and Hsiao, 1982).

CONCLUSIONS

In this paper we have addressed the question of how oxygen deficiency at the roots, induced by flooding the soil, promotes stomatal closure within 4 h and sustains it for more than 48 h. No support was found for the view that increased delivery of ABA from roots or from older leaves is responsible; flooded roots exported less, not more, ABA to shoots, and lower leaves had no effect on flooding responses of younger leaves. ABA accumulation in leaves caused by inhibited export in the phloem may make a limited contribution to closing stomata on flooded plants, but the effect is small, slow to develop, and short-lived. Bioassays of xylem sap in an epidermal strip test show that sap exported from roots of flooded plants carries a factor that promotes stomatal closure and is not ABA. This unidentified substance may be responsible for much of the stomatal closure seen in flooded tomato plants.

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