Stomatal control in tomato with ABA-deficient roots: response of grafted plants to soil drying

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Received 8 May 2001; Accepted 22 February 2002

Abstract
The hypothesis that ABA produced by roots in drying soil is responsible for stomatal closure was tested with grafted plants constructed from the ABA-deficient tomato mutants, sitiens and flacca and their near-isogenic wild-type parent. Three types of experiments were conducted. In the first type, reciprocal grafts were made between the wild type and sitiens or flacca. Stomatal conductance accorded with the genotype of the shoot, not the root. Stomates closed in all of the grafted plants in response to soil drying, regardless of the root genotype, i.e. regardless of the ability of the roots to produce ABA. In the second type of experiment, wild-type shoots were grafted onto a split-root system consisting of one wild-type root grafted to one mutant (flacca or sitiens) root. Water was withheld from one root system, while the other was watered well so that the shoots did not experience any decline in water potential or loss of turgor. Stomates closed to a similar extent when water was withheld from the mutant roots or the wild-type roots. In the third type of experiment, grafted plants with wild-type shoots and either wild-type or sitiens roots were established in pots that could be placed inside a pressure chamber, and the pressure increased as the soil dried so that the shoots remained fully turgid throughout. Stomates closed as the soil dried, regardless of whether the roots were wild type or sitiens. These experiments demonstrate that stomatal closure in response to soil drying can occur in the absence of leaf water deficit, and does not require ABA production by roots. A chemical signal from roots leading to a change in apoplastic ABA levels in leaves may be responsible for the stomatal closure.

Introduction
Experiments in which leaf water status was maintained in plants as the soil dried, either by balancing pressure techniques (Gollan et al., 1986; Schurr et al., 1992) or by roots split between wet and dry soil (Gowing et al., 1990; Khalil and Grace, 1993; Stoll et al., 2000), demonstrate that stomates can close independently of the water status of the leaves. This suggests that there is a chemical signal produced by the roots that controls stomatal conductance. There is evidence that this signal is ABA; stomatal closure is often associated with an increase of ABA in xylem sap, prior to a detectable increase in leaf ABA (reviewed in Davies and Zhang, 1991), and significant stomatal closure can be induced in detached leaves by feeding synthetic ABA at concentrations similar to that occurring in the transpiration stream (Tardieu et al., 1996; Correia and Pereira, 1994). This suggests that root-produced ABA might regulate stomatal behaviour under stress. However, a number of findings are inconsistent with this hypothesis. First, there is no absolute relationship between ABA and conductance even within a given species. The relationship can vary with the time of day (Tardieu et al., 1996), the degree of stress (Correia and Pereira, 1995) or with the individual plant (Schurr et al., 1992). Possible explanations of this variable relationship include an interaction with leaf water status (Tardieu et al., 1996), or, in the absence of changes in leaf water status, changes in ion transport that might give rise to changes in xylem sap pH (Gollan et al., 1992; Wilkinson and Davies, 1997; Wilkinson et al., 1998). Second, ABA arriving in the xylem is rapidly metabolized by...
leaves; ABA fed through the xylem of detached leaves had a half-life less than 1 h (Gowing et al., 1993), although in intact leaves the half-life was a little longer (Jia and Zhang, 1997). Trejo et al. showed that the mesophyll tissue rapidly metabolized ABA fed through the xylem, so it is possible that the fine control of stomatal conductance is exerted by leaf metabolic activity rather than by the ABA arriving in the xylem (Trejo et al., 1993). Third, the ABA appearing in the xylem may not have been produced by the roots; it may have been synthesized in the leaves, translocated in the phloem to the roots, and recirculated in the xylem (reviewed by Munns and Cramer, 1996). Fourth, experiments in which normal shoots have been grafted onto either normal roots or ABA-deficient roots have found little or no effect of the root genotype on stomatal behaviour (Fambrini et al., 1995; Jones et al., 1987). Thus, the role of ABA in the xylem versus that made in the leaf in controlling stomatal conductance is not clear.

In order to test the hypothesis that root-produced ABA controls stomatal conductance in drying soil, plants with ABA-deficient roots and wild-type shoots were constructed. If ABA produced by roots is required for root-to-shoot signalling, then the response to drying soil should depend upon the root genotype. The ABA-deficient tomato mutants sitiens and flacca were used. Previous work had shown that roots of sitiens plants do not synthesize increased amounts of ABA under stress (Cornish and Zeevaart, 1988; Dunlap and Binzel, 1996), and that sitiens does not export significant amounts of ABA in the xylem even in dry soil (R Munns and VR Shashidhar, unpublished data). Three types of experiments were conducted. The first type examined patterns of water use and stomatal conductance in reciprocal grafts of both sitiens and flacca. The second type of experiment examined patterns of water use when only part of the root system was dried. These ‘split-root’ experiments used plants formed from 3-way grafts such that the shoot had a wild-type phenotype, while the root system had both a wild-type and an ABA-deficient part. The split-root design allowed the effect of root genotype on patterns of water use in response to soil drying to be examined without the possibility of dehydration-induced ABA synthesis in leaves. The third type of experiment examined changes in stomatal conductance in response to soil drying of plants whose xylem pressures were held constant using root pressurization. Reciprocal grafts formed from wild-type shoots and sitiens or wild-type roots were established in pots that could fit within a Scholander-type pressure chamber. The chambers were pressurized as the soil dried to maintain the xylem sap at atmospheric pressure and hence also the shoot water status at its maximum.

Materials and methods

Plant material and growth conditions

Seeds of the ABA-deficient mutants flacca and sitiens and their near-isogenic parental line, Rheinlands Ruhm, were obtained from the Tomato Genetic Resources Centre, University of California, Davis. Seeds were germinated on wet filter paper and grown without the addition of ABA. Experiments involving the mutant sitiens used a sandy loam (bulk density of 1.28 g cm$^{-3}$; maximum water capacity of about 0.25 g cm$^{-3}$) as the rooting medium. Experiments involving the mutant flacca were conducted using an organic-based potting medium (bulk density of 0.54 g cm$^{-3}$; maximum water capacity of about 0.4 g cm$^{-3}$). The plants were grown and experimental measurements carried out in growth chambers providing 30/20 °C day/night temperature, 60% daytime relative humidity, light intensity of 650 μmol m$^{-2}$ s$^{-1}$ and a 12 h photoperiod.

Reciprocal graft experiments

The effect of the root genotype (ABA-deficient versus wild type) on patterns of water use and stomatal conductance in response to drying soil was investigated using reciprocal grafts. Grafts were made by cutting the scion and stock plants diagonally, removing the leaves, securing the union with parafilm, and growing under a mist until new leaf growth was observed (approximately 2–3 weeks). At this stage there was no indication of reversion of the siti/wt (scion/stock) to the shoot morphology of the wild type, which occurs with time (Cornish and Zeevaart, 1988). In the experiments using the mutant sitiens, five individuals of all four graft combinations, wt/sitiens, wt/wt, sitiens/wt, and sitiens/sitiens were grown in 600 cm$^3$ pots. There was no difference in leaf area between wt/wt, wt/sitiens, and sitiens/wt plants (average of 378 cm$^2$), however, sitiens/wt plants had somewhat less total leaf area (average of 302 cm$^2$). In the experiments using the mutant flacca, six individuals each of wt/flacca and wt/wt were grown in 5.0 l pots. There was no difference in mean leaf area/plant between the two graft constructs (average of 2926 cm$^2$). Both soil volume and leaf area per plant were smaller in the sitiens experiments than in the flacca experiments, however, the relationship between leaf area (cm$^2$) and soil volume (cm$^3$) was similar (0.55 cm$^{-1}$ flacca versus 0.60 cm$^{-1}$ sitiens). The plants were watered regularly throughout their development. At the onset of the experiment, the pots were covered with plastic to prevent evaporation from the soil and watering ceased.

In the sitiens experiment, plants were allowed to dry the soil at their own rate (i.e. no water was added to the pots during the period of soil drying). The time to visible wilting varied between graft types, but was approximately 3 d for plants with a wild-type scion and 2 d for plants with a sitiens scion. Plants were weighed at hourly intervals between 11.00 h and 17.00 h, and the transpiration rate calculated. Stomatal conductance was measured at 1 h intervals during the same period using a porometer (AP4, Delta-T Devices, Burwell UK). The experiment was terminated when the plants wilted, at which point the plants were harvested and total leaf area and soil dry weight determined. Soil water content was calculated assuming a constant plant water content over the course of the experiment (3 d). Leaves were sampled for ABA content from three individuals of each graft type at the beginning and the end of the experiment (method described below). Leaflets for ABA measurement were taken from the same leaf in which stomatal conductance was measured.

In the flacca experiment, each plant was weighed at the beginning and end of the light period, allowing an average
water loss rate for the light and dark periods to be calculated. Differences in the rate of soil drying between plants were minimized by adding water to the pots such that the maximum rate of water loss did not exceed 0.05 kg d\(^{-1}\). At this rate, approximately 7 d were needed before the plants began to wilt. Measurements of water loss, stomatal conductance, and leaf water potential were made until the plants began to wilt, at which point the plants were harvested. Stomatal conductance was measured on five leaves per plant between midday and 14.00 h, using a porometer (Li-1600, Li-Cor, Lincoln USA). Measured leaves were selected from at least three different branches per plant and only the youngest fully expanded leaves were used. Leaf water potential was determined using thermocouple psychrometers (JRD Merrill Specialty Equipment Co., Model 84-13C). Leaf discs (0.4 cm diameter) were rapidly sealed into the psychrometer chambers in a temperature-controlled box (30 °C), and measurements made with a Wescor HR33T dew point microvoltmeter.

**Split-root experiments**

A split-root experiment was used to examine the role of root-produced ABA on patterns of water use in response to partial drying of the root system. Split-root plants were constructed from a three-way graft joining together material from three separate plants. A V-shaped cut was made at the base of a wild-type shoot and simultaneously grafted to the root system of two other plants, growing in separate pots, from which the shoots had been excised (Fig. 1). The success rate for these grafts was quite low and large numbers of plants could not be generated. Six *sitiens* split-root plants were generated, with wild-type shoots and both wild-type and *sitiens* root systems, growing in different pots. Two *flacca* split-root plants were similar to this, i.e. had wild-type shoots and both wild-type and *flacca* root systems, and in another two the root systems were the same (either wild type or *flacca*). In all cases the pots were tall cylinders (5 x 40 cm).

Time-domain reflectometry (TDR) was used to determine the soil water content of each pot (Fig. 1). Wave-guides, consisting of three stainless steel rods, were inserted at a spacing of 2.5 cm. Measurements using a Tektronix 1502c time-domain reflectometer were taken three times each day, and the relationship between TDR reading and volumetric soil water content determined empirically by growing tomato plants in pots identical to one used for the split-root plants.

The basic experiment consisted of monitoring rates of whole plant water use and leaf water status as one pot dried while the other was watered. In the *sitiens* experiment, the three treatments (wild-type side dried, *sitiens* side dried, or neither side dried) were applied sequentially to each plant. The order in which each plant experienced these treatments was varied. Each plant (i.e. pair of pots) was weighed three times per day, and TDR measurements taken for each pot. The amount of water needed to bring the plant to its initial weight was calculated, and the appropriate pot was watered with this amount. Stomatal conductance was measured between midday and 14.00 h on five leaves per plant using a porometer (Delta-T). Leaf relative water content was determined by rapidly weighing excised leaflets (three per plant), re-weighing them after allowing them to hydrate fully by floating them for 3 h on deionized water, and then drying the leaves to a constant weight at 65 °C.

In the *flacca* experiment, water was withheld from one side of the root system of each plant for a period of 7 d. The plants were then freely watered for several days, following which water was withheld from the other side of the root system. Stomatal conductance was measured at midday on the abaxial surface of five young, but fully expanded leaves, per plant using a porometer (Li-1600). Simultaneously, leaf discs were collected from two leaves per plant for measurements of leaf water potential using thermocouple psychrometers.

**Root pressurization experiments**

Root pressurization was used to determine whether the ability of roots to produce ABA influenced patterns of stomatal closure in response to soil drying when xylem water potentials were maintained at a constant level. Two types of grafts were made having wild-type shoots, but either wild-type or *sitiens* roots (i.e. wt/wt and wt/*sit*), and plants were established in pots designed to fit within a pressure chamber (9 x 19.5 cm). When the plants had 7–8 unfolded leaves pots were placed in a pressure chamber and the stems were sealed according to methods outlined previously (Stirzaker and Passioura, 1996). Plants were brought to balancing pressure by removing a leaflet from the most recent fully expanded leaf, and raising the pressure in the chamber until the xylem sap was on the...
point of bleeding, as judged from moisture appearing at the end of the cut petioloile. No excess bleeding was observed in other parts of the plant. The method followed that described earlier (Stirzaker and Passioura, 1996), except that the plants were in walk-in controlled environment chambers, and that the pressure was monitored and adjusted manually.

Experiments were carried out on three replicate control plants (wt/wt) and six replicate mutant grafts (wt/sit). Three different treatments were carried out sequentially on these plants. First, the soil was allowed to dry while the plant was maintained at balancing pressure. Stomatal conductance was measured until the balancing pressure reached 2 MPa. Pots were weighed at the beginning and end of the period, and the rate of water loss calculated. Second, after allowing plants to recover for 3 d, the soil was allowed to dry without balancing pressure, and conductance and pot weights measured. Third, after 9 d the soil was allowed to dry while plants were pressurized, and leaf tissue as well as xylem sap were collected for measurement of ABA.

Stomatal conductance was measured with a porometer (Delta-T) as the soil dried, on each of 6–8 leaves, starting from the youngest leaf whose laminae had expanded sufficiently to measure, and finishing with the oldest that was not starting to senesce. Conductance was measured every hour until the rate of change increased when it was measured every 20–30 min. The soil took about 2 d to dry to the extent that conductance was reduced by 50–70%. Conductance was maximum between 11.00 h and 17.00 h, and between the period of 10.00 h and 18.00 h, i.e. not less than 2 h into the photoperiod and 1 h before the end. However, as conductance was maximum between 11.00 h and 17.00 h, and decreased outside this time in well-watered plants, only data between these times are used. When the rate of drying was such that conductance started to fall outside these times, the experiment was repeated.

To measure changes in ABA during the drying period, a leaflet was detached from the most recently fully expanded leaf at the start of the drying period, and sap was collected from its petioloile while balancing pressure was monitored on the next oldest leaf, for periods of 1–2 h, while the soil was wet, (balancing pressures about 0.5 MPa), and at two times while it was drying (balancing pressures about 1.0 and 1.4 MPa). Another leaflet was sampled for ABA at the end of the drying period.

**ABA measurements in xylem sap and leaves**

Xylem sap was collected from a number of different mutant and grafted plants, growing under normal light levels in pots designed to fit within a pressure bomb, as described above. Plants were pressurized to bring the water potential of the shoots to near atmospheric, so that the most recently fully expanded leaf was on the point of bleeding. One leaflet was then cut from this leaf, and sap was collected from its petioloile, while balancing pressure was monitored on the next oldest leaf. Thus the sap was flowing at normal rates, through a virtually-intact plant. This avoids the problems of collecting sap exuding from stumps of plants with shoots removed, when the low flow rate of the sap results in artificially high concentrations of solutes (Sagi et al., 1999). Leaf material was collected as described earlier. Sap and leaflets were frozen in liquid nitrogen and stored at −80 °C. ABA was assayed by an indirect ELISA method (Walker-Simmons, 1987) using a monoclonal antibody (Idetek). All assays were made in triplicate and possible interference with cross-reactive substances was checked by analysing a range of dilutions. In some plants (wild type, *sitiens*, and *sit/wt*), some of the sap was digested by alkaline hydrolysis (pH 11 for 1 h at 60 °C) (following the method of Bano et al., 1993).

Leaf tissue was analysed for ABA by the ELISA method after extraction with hot water (Loveys and van Dijk, 1988) and passage through a C18 SepPak column. As a further check on the specificity of the assay, and possible interference with cross-reactive substances, replicate leaf samples were taken for analysis by GCMS. Leaves were extracted in 80% methanol with deuterated internal standard added, purified by filtration and ether partitioning, then analysed by GCMS (Green et al., 1997). This validated the results of the ELISA assay.

**Results**

**ABA in xylem sap**

The ABA concentration of the xylem sap from well-watered *sitiens* plants was very low, and sometimes it was undetectable (less than 0.1 nM; Table 1). Sap was hydrolysed with alkali to see if there were conjugated (esterified) forms of ABA present, but no significant amount of ABA was released (data not shown). The level of ABA increased with soil drying about 10-fold in both wild type and *sitiens*, but the level in the mutant was still low compared to the unstressed wild type (Table 1). These data show that roots of *sitiens* do not export a significant amount of ABA to the shoot.

Xylem sap was collected from reciprocal grafts of wild-type and mutant plants, both *sitiens* and *flacca*, grown under well-watered conditions. Self-grafted plants (both mutant and wild type) were examined in case the graft union interfered with the transport of ABA up or down the stem, or produced ABA of its own. Self-grafted *sitiens* (sit/sit) and *flacca* (flc/flc) transported very little ABA in xylem sap, and self-grafted wild-type (wt/wt) had ABA levels similar to those found in intact wild-type plants (compare Fig. 2 with Table 1). Reciprocal grafts between wild type and mutant (either *sitiens* or *flacca*), had xylem sap ABA concentrations that were several times higher than the self-grafted mutants although much less than self-grafted wild type (Fig. 2). The low amount of ABA in the sap of plants with mutant shoots and wild-type roots (*sit/wt and flc/wt*) had presumably arisen...

| Table 1. ABA concentrations in xylem sap collected from intact transpiring plants of wild type and *sitiens* before and after the soil was allowed to dry for 3 d |
|-----------------|-------------------------------|
|                  | Xylem ABA (nM)               |
|                  | Well-watered | Water-stressed |
| Wild type *sitiens* | 38 ± 15 | 242 ± 40 |
| *flacca*         | 0.4 ± 0.2 | 6 ± 2  |
in the roots. However, the low amount of ABA in the sap of plants with wild-type shoots and mutant roots (wt/sit and wt/flc) had presumably originated in the shoots, as the roots of the sit/sit and flc/flc plants exported little ABA (Fig. 2). This ABA could have been produced by the stem or leaves below the site of collection, and transferred to the xylem either via the roots, or by direct phloem–xylem transfer in the stem above the graft.

Reciprocal graft experiments

The stomatal behaviour of reciprocal grafts of sitiens demonstrated that the plants responded to soil drying according to their shoot genotype. In wet soil, there was no statistically significant difference between the plants with wild-type shoots (wt/wt and wt/sit) in transpiration rate (Fig. 3A) or stomatal conductance (Fig. 3B). Neither was there any difference between the plants with mutant shoots (sit/sit and sit/wt). However, there was the expected difference between shoot genotype. In wet soil, plants with sitiens shoots had almost twice the transpiration rate and stomatal conductance of plants with wild-type shoots, regardless of their root genotype (Fig. 3). Stomatal closure began at about the same soil moisture level (about 0.07 g cm\(^{-3}\)) in all grafted plants (Fig. 3A, B). At this stage, the plants with sitiens shoots were already severely wilted, almost desiccated (as first noted by Neill and Horgan, 1985). Prior to this stage, the conductance actually increased; when the soil water content fell below 0.15 g cm\(^{-3}\), the conductance increase to about 1200 mmol m\(^{-2}\) s\(^{-1}\), and remained elevated as the soil water fell to about 0.06 g cm\(^{-3}\) (Fig. 3B). This occurrence was most pronounced in the sit/sit plants.

The ABA concentration in the leaves of all the grafted plants increased to different extents as the soil dried, with the increase being greatest in the self-grafted wild-type plants (Fig. 3C). Thus, there was little correlation between bulk ABA in the leaves and stomatal closure. Figure 3C also shows that although the ABA concentration in the leaves reflects most strongly the shoot genotype, there was some influence of the roots, as the level of ABA in the wt/wt plants in well-watered soil at the beginning of the experiment was greater than in the wt/sit plants, and the initial level of sit/wt plants was greater than in sit/sit plants. As the soil dried, this pattern became more pronounced. No water relations measurements were made in this experiment.

The stomatal response of reciprocal grafts with flacca produced similar results to those with sitiens. There were no differences in transpiration rates between wt/wt and wt/flc plants, both when the soil was well watered and as the soil dried (Fig. 4A). Stomatal conductance was also independent of root genotype (data not shown), and
declined in parallel with transpiration rates. There were no differences in leaf water potential between the two types of grafts (Fig. 4B), and little decrease as the soil dried (Fig. 4B). The 4-fold decrease in transpiration as the soil water content fell from 0.20 to 0.12 g cm$^{-3}$ was accompanied by a relatively small fall in leaf water potential, about 0.2 MPa (Fig. 4B), indicating that the stomatal closure was sufficient to prevent a significant fall in leaf water status. This makes it unlikely that stomates closed because of a leaf water deficit and raises the possibility of regulatory signals coming from the roots.

**Split-root experiments**

In the split-root experiment using the sitiens mutant, water use per plant increased over the 6 d during which each treatment was applied, as a result of the increase in leaf area that occurred during the same interval. Total plant water use increased to a lesser degree when water was withheld from one portion of the root system, compared with when the entire root system remained well watered, but there was no difference if the sitiens roots or the wild-type roots were not watered (Fig. 5A). Stomatal conductance declined to about 80% of their initial values, again regardless of whether water was withheld from the sitiens or the wild-type roots (Fig. 5B). There was no significant difference in leaf water content when water was withheld from the wild-type or the sitiens root system. Average leaf relative water content (±SE) for the three treatments (wild type dried, sitiens dried, and both sides watered) were 91.6 ± 0.6, 91.4 ± 0.4, and 92.7 ± 0.1, respectively. After 4 d of drying, there was little water uptake from the dry side (Fig. 5C), so water was taken up almost exclusively from the wet side.

The sitiens roots were not able to extract water at the same rate as the wild-type roots (Fig. 5C), nor to the same final extent. For example, the amount of water used by the wild-type root system during the first day after cessation of watering was always about 40% greater than that used by the sitiens root system. After 4–5 d, the soil
water content of the wild-type side reached 0.053 g cm\(^{-3}\), while the *sitiens* side reached a minimum of only 0.065 g cm\(^{-3}\) (Fig. 5C). Yet the *sitiens* root systems were much bigger than the wild-type root systems; the ratio of wild type to *sitiens* being 0.4 : 0.6 (Table 2). This meant that the rate of water uptake per mass of root was much greater for the wild-type than the *sitiens* roots. For example, during the first day after cessation of watering, the amount of water taken up by wild-type root systems was about double that from the *sitiens* root systems.

The split-root experiment performed with *flacca* gave qualitatively similar results. Partial drying of the root system resulted in a decrease in stomatal conductance that was independent of the genotype of the roots in the drying soil (Fig. 6). There was no difference in the response of stomatal conductance when water was withheld from the *sitiens* roots or the wild-type roots (Fig. 6A). There were no differences in midday leaf water potential between plants (average 0.70 ± 0.04 MPa) and no decreases when water was withheld from one side of the split-root system (data not shown). As with the *sitiens* split-root plants, wild-type roots were better able to extract water from the soil compared with the *flacca* roots. In the plants with different roots, the minimum soil water content in the side with the wild-type roots was 0.13 g cm\(^{-3}\), compared with 0.17 g cm\(^{-3}\) in the side with *flacca* roots (Fig. 6A). However, without competition with wild-type roots, *flacca* roots were able to extract more water; in the plants with all roots of the same genotype, the minimum soil water content resulting from uptake by *flacca* roots was 0.13 g cm\(^{-3}\), almost as low as that from wild-type roots (Fig. 6B).

### Table 2. Biomass and leaf area of split-root grafted plants, with wild-type shoots grafted onto two root systems of wild type and sitiens

Plants were harvested at end of the series of experimental treatments shown in Fig. 5, in which either one or both systems were watered. Values are means ± SE (n = 6).

<table>
<thead>
<tr>
<th>Leaf area (cm(^2))</th>
<th>1539 ± 55</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW of leaves (g)</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>DW of wild-type root system (g)</td>
<td>1.69 ± 0.10</td>
</tr>
<tr>
<td>DW of sitiens root system (g)</td>
<td>2.47 ± 0.05</td>
</tr>
</tbody>
</table>

**Fig. 6.** Stomatal conductance of *flacca* split-root plants in response to water being withheld from one side of the root system. (A) Split-root plants with different root systems; water was withheld from wild-type roots (closed symbols) or *flacca* roots (open symbols). (B) Split-root plants with the same root system, either wild type (closed symbols) or *flacca* (open symbols). Each point represents the mean ± SE of all leaves measured on a single plant.

### Root pressurization experiments

Root pressurization, which was used to maintain a high shoot water status during soil drying, did not alter stomatal apertures when the soil was moist. When well-watered plants were first brought to balancing pressure (i.e. the pressure was increased such that xylem sap was visible at the end of a recently cut petiolule) there was no measurable change in stomatal aperture. Stomatal closure in response to soil drying occurred in all plants, irrespective of root genotype or application of pressure to the roots. In wt/wt plants, stomata began to close when the soil water content dropped below 0.095 g cm\(^{-3}\), and the balancing pressure was 0.8–1.0 MPa (Fig. 7A). Stomatal closure of 50% occurred at 1.8 MPa, at which point the experiment was terminated as the pressure was approaching the pressure limit of the chamber (2 MPa). The plant was re-watered, and some days later was allowed to dry again, this time without the application of balancing pressure. The relationship between conductance and soil moisture was similar to when it was pressurized, i.e. closure started when the soil moisture fell to the same level (Fig. 7A). Two other replicate plants had the same response (data not shown).

Similar patterns of stomatal closure in response to soil drying were observed with wt/sit plants. Stomatal closure started at balancing pressures of 0.8–1.0 MPa and a soil moisture content of about 0.08 g cm\(^{-3}\), with closure of about 50% occurring at 1.8 MPa (Fig. 8A). When the plant was allowed to dry without balancing pressure, stomatal closure began when soil moisture content fell to the same extent (Fig. 8B). The same result was found with three other replicate (wt/sit) plants (data not shown).

ABA content of xylem sap could only be determined for plants maintained at balancing pressure as positive xylem pressures are needed to produce exudation from
transpiring plants. At the start of the drying period, xylem sap ABA concentration in wt/sit plants was a little lower than that of wt/wt plants (Fig. 9). With soil drying, there was a large increase in the ABA concentration of xylem sap of the wt/wt plants, but very little increase in the wt/sit plants (Fig. 9). ABA content of leaf tissues was also lower in the wt/sit than the wt/wt plants at the start of the drying period, being 3.20 ± 0.24 nmol g⁻¹ FW for the wt/wt plants and 2.32 ± 0.08 nmol g⁻¹ FW for the wt/sit plants. However, the ABA concentration in the leaves increased as the soil dried in both types of plants to a similar extent, the increase being 0.52 ± 0.30 and 0.60 ± 0.06 nmol g⁻¹ FW respectively (n = 3).

Discussion
Stomatal closure in drying soil does not require root-sourced ABA

The experiments with reciprocal grafts between ABA-deficient and wild-type shoots and roots showed that the stomatal behaviour depended on the genotype of shoot, not the root. Stomatal conductance was higher when the shoot genotype was either sitiens or flacca, regardless of the root genotype, and decreased as the soil dried regardless of the root genotype. These results are similar to those of Fambrini et al. with reciprocal grafts from a wild-type sunflower and a wilty ABA-deficient mutant (Fambrini et al., 1995); they reported that the stomatal conductance during water stress was determined by the shoot rather than the root genotype. They are not inconsistent with those of Jones et al. with reciprocal grafts of wild-type tomato and sitiens or flacca, measured in well-watered conditions (Jones et al., 1987), who concluded that there was only a small and generally...
insignificant effect of the root genotype after grafting either wild-type or mutant shoots onto alternate rootstocks. They are different from those of Borel et al. with tobacco (Borel et al., 2001), who reported similar stomatal responses of ABA-deficient and wild-type shoots on a common wild-type rootstock, but in that case there may have been ABA circulating from the wild-type shoot to the mutant shoot, causing a reversion of the phenotype. Phenotype reversion of ABA-deficient shoots on wild-type roots was reported with grafts of tomato (Cornish and Zeevaart, 1988) and of sunflower (Fambrini et al., 1995).

Leaf ABA was not closely linked to stomatal behaviour. Although leaf ABA was lower when the shoot genotype was sitiens rather than wild type, and thus correlated with stomatal conductance in unstressed plants, it increased much more with soil drying in the wt/wt than the wt/sitiens plants, yet stomates of these plants closed to the same extent (Fig. 3). As the roots of the mutants did not export significant amounts of ABA in the xylem, something other than root-produced ABA was causing stomatal closure. In the experiments with reciprocal grafts, shoot water potential was not controlled, so it was possible that reduced leaf water status influenced stomatal conductance, either directly, or by inducing ABA accumulation. The succeeding two types of experiments, split-root and root pressurization, were designed to examine the effects of soil drying without changes in shoot water status. In both types of experiment, the plants had wild-type shoots and varied only in root genotype. This avoided the morphological abnormalities in leaf and stomatal architecture due to a mutant shoot phenotype (as well as the problem of reversion of shoot morphology of sitiens/wt grafts).

In the split-root experiments, patterns of stomatal conductance (sitiens and flacca experiments) or transpiration (sitiens experiment) were the same when the ABA-deficient or the wild-type root system was allowed to dry, that is, there was a small degree of stomatal closure, independent of the root genotype. Compared to some other split-root experiments (Jones et al., 1987; Gowling et al., 1990; Khalil and Grace, 1993; Stoll et al., 2000), this closure was small, but it was discernible, unlike the split-root experiment of Saab and Sharp with maize, which showed no closure at all, just a reduction in leaf expansion (Saab and Sharp, 1989). In the present study, while the soil dried around one root system, there was increasing uptake from the watered roots until, by the end of the experiment, all of the water transpired was withdrawn from the watered root system. To maintain the watered pot at its target soil water content, water was added three times per day. Perhaps the different degrees of stomatal closure in the various split-root experiments reported in the literature reflect the frequency with which the wet pot was watered.

It was surprising that the root system of sitiens was larger than the wild-type root system in the split-root grafted plants (Table 2), as roots of ABA-deficient maize seedlings grow more slowly than those of the wild type under water stress (Saab et al., 1990; Sharp et al., 1994). Furthermore, addition of ABA can largely restore root and shoot growth in flacca in unstressed conditions (Sharp et al., 2000), and presumably would also in stressed conditions. The greater growth of sitiens roots in the grafted plants described here was possibly due to signals coming from the wild-type shoots. However, the hydraulic conductance of the sitiens root system appeared to be impaired, the maximum rate of water transport per unit mass of root being about half that of wild-type roots. The inability to produce ABA was therefore still having an effect on architecture, which is consistent with suggestions that ABA enhances hydraulic conductance in roots (Zhang et al., 1995). Nevertheless, despite the greater root system, the actual rate of water uptake from the pot with the sitiens roots was less than from the wild type. The greater mass of the sitiens root system did not compensate for the lower hydraulic conductance.

In the experiments run under balancing pressure, stomatal conductance decreased as the soil dried regardless of the root genotype (stomatal behaviour was the same in wt/sitiens and wt/wt). This showed there was a signal coming from the roots that influenced stomatal conductance independently of leaf water status and, at least in the case of wt/sitiens, that this signal was not ABA produced by the roots. What is the signal?

**Signals that could be produced by ABA-deficient roots**

It is possible that roots of the ABA-deficient mutants are able to export precursors of ABA (such as the ABA-adduct identified by Netting et al., 1997) or other precursors that would presumably accumulate in mutants blocked, like sitiens and flacca, at the final step of ABA synthesis (as found in aba3; Schwartz et al., 1997). These would not have been detected by the assay system used here for ABA. Other products of the ABA biosynthetic pathway may accumulate in ABA-deficient mutants. For instance, t-xanthoxin accumulates in unstressed sitiens, and increased in water-stressed flacca to a similar extent as ABA in the wild type (Parry et al., 1988). Whether t-xanthoxin carries biological activity is unknown.

Other types of hormonal stress signals are also possible. Cytokinin concentrations are often lower in xylem sap from droughted plants (Bano et al., 1993, 1994; Shashidhar et al., 1996; Stoll et al., 2000), and can influence stomatal opening (Bradford, 1983). Spraying grapevines exposed to partial root water deficit with cytokinin restored stomatal conductance to that of fully
irrigated controls (Stoll et al., 2000). Other compounds in xylem sap may influence stomatal conductance, for example, an unidentified inhibitor of transpiration was detected in xylem sap of wheat seedlings (Munns et al., 1993).

Changes in pH in xylem sap occur in droughted plants, and there is some evidence for these being part of a root signalling system. Changes in pH could be caused by changes in ion transport induced by the lower water status of the roots as the soil dries. A decrease in cell volume or turgor of root cells could activate mechanosensitive Ca$^{2+}$ channels which could have profound effects on ion transport systems such as K$^+$ channels and indicate that stomates could be responding to ABA (Netting and Davies, 1997). Evidence that this closure was not related to xylem ABA concentration, nor to the ability of roots to produce ABA, or related to pH changes that have been observed in xylem sap (Kuboki et al., 1997). Alternatively, the ABA in the xylem from the wt/sit plants may have not arrived via the roots. The sap was collected from the most recently expanded leaf, inserted into the stem some 5 cm above the graft. The ABA may have been made within the stem above the graft, or made by the older leaves and transferred into the xylem within the stem internodal tissue.

The fate of the ABA in the xylem, whatever its origins, is unclear. Calculations of the amount of ABA in the xylem sap of the wt/wt plants that was delivered to leaves throughout the drying period showed that this exceeded the actual increase in the leaf by about 6-fold (values derived from the data for the last experiment presented above, given in the text and in Fig. 9, and assuming a transpiration rate of 6 g g$^{-1}$ FW of leaves d$^{-1}$). This indicates that there was considerable metabolism of the incoming ABA. Earlier studies showed that the mesophyll tissue rapidly metabolized ABA fed through the xylem, limiting the supply to the epidermis (Trejo et al., 1993). ABA fed through the xylem of detached leaves had a half-life less than 1 h (Gowing et al., 1993; Zhang et al., 1997a), although in intact leaves the half-life was a little longer, 2.2 h in well-watered maize (Zhang et al., 1997b) and increased under water stress to 3.6 h (Jia and Zhang, 1997). It is possible that the control of stomatal conductance by ABA is exerted by the metabolic activity in the leaves, not by the amount arriving in the xylem.

### Conclusion

Overall, these experiments show that, in tomato, as in other species, root signals operate to control stomatal conductance in drying soil. However, these data show that this closure was not related to xylem ABA concentrations, nor to the ability of roots to produce ABA, and indicate that stomates could be responding to ABA that is made in situ rather than transported from the roots via the xylem. The signal from the roots that causes this increase in ABA, or otherwise influences stomatal conductance, remains unknown, but may be a precursor, or related to pH changes that have been observed in xylem sap.

### Acknowledgements

We thank Andrew Poole for verification of leaf ABA by GCMS, and Andy Netting, Ian Dodd, John Passioura, Rod King, and Brian Loveys for helpful comments on the manuscript.
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