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Gradual Long-Term Water Stress Results in Abscisic Acid Accumulation in the Guard-Cell Symplast and Guard-Cell Apoplast of Intact *Vicia faba* L. Plants

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ABSTRACT

As signals that integrate plant water status, abscisic acid (ABA) made within leaves and that imported from roots contribute to stomatal control. Guard cells exhibit multifaceted responses that are initiated through ABA's interaction with outward-facing plasmamembrane receptors or internal receptors. These responses include short-term regulation of ion transport that alters stomatal conductance as well as long-term regulation of gene expression. These molecular effects have been studied intensively, but the *in planta* guard-cell ABA contents must also be known if regulation of gas exchange by ABA is to be understood. Water was withheld from *Vicia faba* L. plants for up to 6 days. Over this period, Ψ_{Leaf} dropped from −0.33 ± 0.03 to −0.87 ± 0.04 MPa and leaf conductance fell from 0.14 ± 0.01 to 0.02 ± 0.00 $mol \cdot m^{-2} \cdot s^{-1}$. Concomitantly, leaf ABA concentration increased from 298 ± 43 to 6317 ± 500 $nmol \cdot kg^{-1}$ (fresh mass) and leaf-apoplast ABA concentration increased from 273 ± 37 to 4609 ± 333 nM. The main purpose of this study was to determine the ABA content in guard cells dissected from surrounding tissue. Initially, the ABA content was 3.2 ± 0.1 fg guard-cell $pair^{-1}$, virtually all of which was symplastic. Six days after withholding of water from the plants, the guard-cell symplast ABA content increased to 10.0 ± 1.1 fg ABA·guard-cell $pair^{-1}$, whereas the guard-cell apoplast ABA content increased by more than 10 fold to 1.5 ± 0.7 fg ABA·guard-cell $pair^{-1}$ (~1.5 μM). These results contrasted with ABA compartmentation in guard cells dissected from plants that were water stressed by a different protocol. The relationship between guard-cell ABA pools and other ABA pools and the importance of compartmentation of ABA by guard cells are discussed.

Key words: Abscisic acid; Apoplast; Conductance; Guard cell; Stoma; Symplast; Water stress

INTRODUCTION

Abscisic acid (ABA) is found throughout higher plants where it accumulates in response to water deficiency and other environmental and developmental factors. Both the root and the shoot system,

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including guard cells (Cornish and Zeevaart 1986), synthesize and accumulate ABA (Zeevaart 1999). The effects of ABA on guard cells have been the focus of many studies because of the importance of gas exchange to the plant's physiology and because these cells respond in myriad ways. In brief, ABA regulates guard-cell ion transport through Ca^{2+}- (McAinsh and others 1997) and H_2O_2-mediated (Pei and others 2000) processes that rely on either Ca^{2+} uptake (Hamilton and others 2000; Schroeder and Hagiwara 1990) or on release of Ca^{2+} from internal stores (Blatt and others 1990; Gilroy and others 1990; Leckie and others 1998). In turn, Ca^{2+} inhibits the hyperpolarizing ATPase (Kinoshita and others 1995) and the K^+_{in} channel (Luan and others 1993), which act cooperatively to accumulate K^+, a primary osmoticum that causes stomatal opening. In addition, ABA activates depolarizing anion channels (Schmidt and others 1995) and K^+_{out} channels (Blatt 1992) causing K^+ release and stomatal closure. Concomitantly, ABA modulates ion traffic via several transporters across the tonoplast, effects also mediated by Ca^{2+} (Allen and Sanders 1996; Allen and others 2000). The preceding outline emphasizes the role of Ca^{2+} in ABA responses (see also Blatt 1999), but it is important to note that ABA regulation of guard-cell ion transport processes may occur in a Ca^{2+}-independent fashion also (Allan and others 1994; Allen and others 1998; Romano and others 2000). Finally, ABA regulates gene expression in guard cells (Müller-Röber and others 1998) as it does in other cells (Leung and Giraudat 1998), including genes that are not involved in osmolyte accumulation and dissipation (Aghoram and others 2000). In summary, ABA is well established as a potent regulator of stomatal guard cells. ABA is synthesized by guard cells, as mentioned, and ABA moves to guard cells from other leaf cells (Popova and others 2000) when the leaf is stressed. In addition, ABA is exported from water-stressed roots (Davies and Zhang 1991; Jackson 1993, 1997) and accumulates in guard cells (Zhang and others 2001), providing a mechanism for avoiding water stress (Tardieu 1996). Guard cells have internal (Allan and others 1994; Schwartz and others 1994; Wang and others 1998) and external (Anderson and others 1994) loci for ABA perception, implying that the exact cellular site of ABA accumulation will determine its effect. Interesting in this regard is that ABA accumulates in the guard-cell symplast when detached leaves are water-stressed (Harris and others 1988), but imported ABA accumulates in the guard-cell apoplast (Zhang and Outlaw 2001b), at least over the short term. However, no information is available on ABA accumulation in guard cells of intact plants subjected to gradual water stress. Water was therefore withheld from potted plants and the ABA contents of guard cells were determined. Under these physiological conditions, both the guard-cell symplast and the guard-cell apoplast were identified as sites of ABA accumulation.

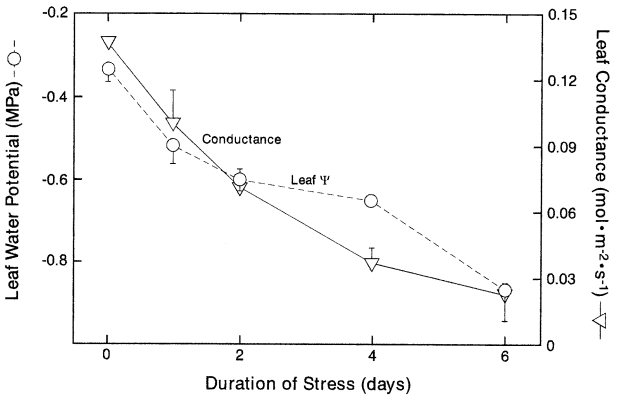

Figure 1. Effect of withholding of water on leaf water potential (○) and leaf conductance (▽). Intact plants were cultured in pots in a growth cabinet; water stress was imposed by discontinuing the daily watering regime. Results are expressed as x ± SE (n = 3 independent experiments with one leaf sampled once for each determination at each time point).

Materials and Methods

Plant Material and Stress Imposition

Soil culture. Broad bean (*Vicia faba* L. cv Longpod) plants were cultured in Metro-Mix 220 in 1-L pots in a growth cabinet set on a 16-h day beginning at 0600 hours, a day/night temperature of 25/20°C, a relative humidity of 60%, and a PFD (400–700 nm) of 600 µmol photons·m^{-2}·s^{-1} that was provided by a combination of incandescent and fluorescent lamps. Soluble nutrients were included at watering each day at 1700 hours; complete details are given in Ewert and others (2000). The third youngest fully expanded bifoliate was used in all experiments, which began at 1000 hours.

Water stress was imposed in one of two ways. Usually, water was withheld for up to 6 days (Figures 1–3 and, as indicated, in Figure 4). In these long-term experiments, stress imposition was staggered so that all plants were the same age at harvest, which was done at once. (According to this protocol (Figures 1–3), the initial values are also control values for all stress treatments.) In one experiment (Figure 4, as indicated), 200 mL 30% (w/v) PEG 8000 ($\Psi \approx -1.1$ MPa, Michel and others 1983) was

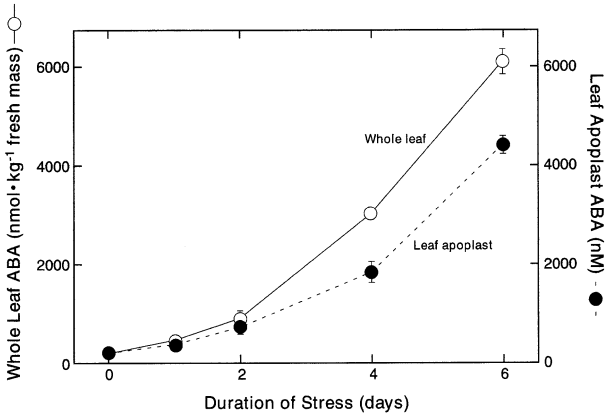

Figure 2. Effect of withholding of water on the ABA concentration in whole leaf (○) and in leaf apoplastic sap obtained by a pressure chamber (●). The analytical errors in the triplicate analyses of ABA for each sample were negligible. Other details are as in Figure 1.

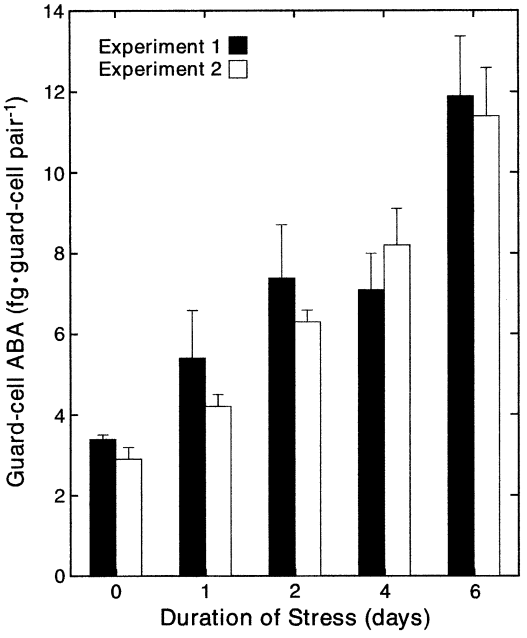

Figure 3. Effect of withholding of water on the ABA content of guard cells. The results of two experiments are shown; other data are in the text. Each column corresponds to one assay conducted in triplicate (x ± SE) on an extract of pooled guard cells.

poured into the pot and samples were taken 60 min later.

Hydroponic culture. Surface-sterilized seeds, as above, were allowed to imbibe aerated water (1 day) germinated in darkness (1 week), and cultured hydroponically (quarter-strength Hoagland's solution, 2 weeks) in a growth cabinet set to the same condi-

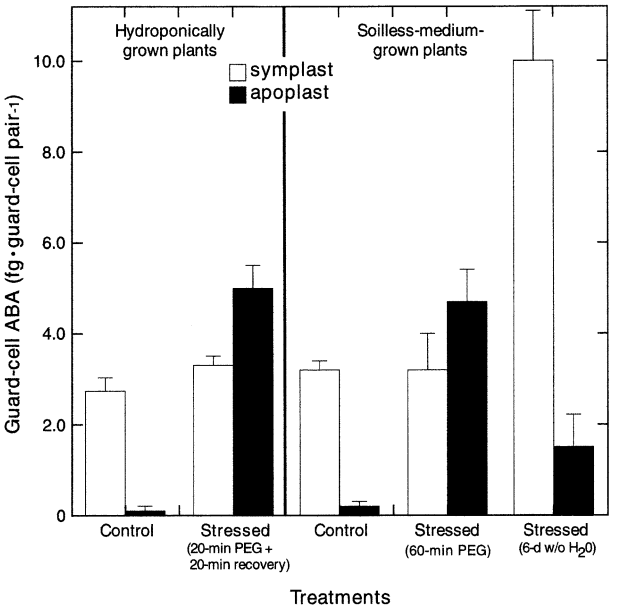

Figure 4. ABA content of the guard-cell symplast and the guard-cell apoplast under water-sufficient and water-stress conditions. For the guard-cell apoplast, 1 fg ABA·guard-cell pair^{-1} is equivalent to approximately 1 µM; the guard-cell symplast volume is dependent on stomatal aperture size, preventing a simple conversion of guard-cell symplast content to concentration. The values for long-term stress (last pair of columns) are x ± SE (n = 4 independent determinations of guard-cell ABA content of 4 plants of different growth lots). All other values are single novel experiments (x ± SE of analysis, Figure 3) that correspond to datum sets published elsewhere (Harris and others 1988; Zhang and Outlaw 2001a).

tions as used for the soil culture. For 1 week before experiments, plants were cultured individually in 1-L nutrient chambers. The nutrient solution was aerated, replenished daily, and replaced on alternate days. Plants were selected 24 h before experiments for uniformity.

Water stress was imposed by addition of PEG (0.2 g PEG 8000 added per mL of nutrient solution). After 20 min, the roots were rinsed and then transferred to fresh nutrient solution for 20 min prior to sampling. The effect of PEG on water potential (Ψ = −0.5 MPa) was calculated as above. The use of PEG for this type of experiment is discussed by Zhang and others (2001).

Leaf Conductance

A LI-1600 steady-state porometer (LI-COR, Inc., Lincoln, NE, USA) was used to measure leaf conductance as detailed elsewhere (Zhang and others 2001).

Water Potential

A pressure chamber (PMS Instrument Co., Corvallis, OR, USA) was used to measure water potential. Because of the irregularly shaped succulent petiolule of *Vicia,* modifications to the pressure-chamber sealing grommet were necessary (Ewert and others 2000).

Tissue Collection and Extraction

Leaf samples. A 1.5-cm^2 sample of leaflet blade devoid of mid-rib was excised, frozen in liquid-N_2 slurry, broken into fragments (~4-mm^2), and stored at −80°C until extraction. Leaf fragments, approximately 4 mg, were homogenized 3× in 80 µL 80% (v/v) aqueous methanol that contained 0.001% (w/v) 2,6-di-t-butyl-p-cresol. The complete homogenate (240 µL) was incubated for 12 h in a covered, silanized borosilicate 6 × 50 mm tube in darkness at 4°C. The supernatant of a low-speed centrifugation was dried under N_2 and then redissolved in ~300 µL of methanolic tris-buffered saline (10% (v/v) methanol in 50 mM tris, pH 8.1, 1 mM $MgCl_2$, 150 mM NaCl). Aliquots, 0.6 µL, were used for ABA analysis in triplicate.

Apoplast sap. Apoplast sap was collected by use of the pressure chamber and application of 0.2 MPa pressure in excess of the water-potential-balancing pressure. The first sap extruded, 2–3 µL, was discarded. The next 2–3 µL of extruded sap was diluted with three volumes of tris-buffered saline (see above). The pressure required to extrude sap is less than would damage the petiolule (Ewert and others 2000). Data on ABA concentrations in sap expressed by the pressure bomb must not be overinterpreted (Jokhan and others 1996, 1999).

Guard cells. Fresh-frozen leaflet fragments obtained concomitantly with whole-leaflet samples (see above) were freeze-dried and guard-cell pairs were dissected from the abaxial epidermis. These guard cells contained both the symplastic and apoplastic ABA pools (Zhang and Outlaw 2001a). In parallel, abaxial epidermal peels were harvested from fresh leaflet and rinsed to remove apoplast contents (Weyers and Hillman 1979). These peels were then frozen in N_2 slurry and freeze-dried. Guard cells subsequently dissected from these peels contained only the symplastic compartment. The apoplastic ABA pool was calculated as the difference in guard-cell ABA between the two guard-cell preparations. Previous work (Harris and others 1988; Zhang and Outlaw 2001a, b) demonstrated that these methods accurately measure the *in planta* apoplastic and symplastic pools of guard-cell ABA under stress and non-stress conditions. Further histochemical details are provided elsewhere (Passonneau and Lowry 1993) and the precision of dissection, less than 2 µm, is documented by Outlaw and Zhang (2001).

For each assay, 50–100 guard-cell pairs were placed in a cluster 1 cm inside a horizontal 6 × 50-mm silanized borosilicate tube as described before (Zhang and Outlaw 20001b). Then, 0.2 µL 80% (v/v) aqueous methanol that contained 0.001% (w/v) 2,6-di-t-butyl-p-cresol was delivered on top of the guard cells, and the tube was quickly sealed with parafilm. Extraction was overnight, in darkness, at 4°C. After addition of 2 µL of methanolic tris-buffered saline (see above), 0.6 µL-aliquots were analyzed in triplicate for ABA.

Abscisic Acid Analysis

The micro-scale ELISA of Harris and others (1988) with modifications (Zhang and others 1991) was used to measure ABA in the 0.1- to 12-fmol range. This assay has been validated in many ways (comparison with HPLC, internal standardization, nonreactivity with ABA metabolites).

Results

Physiological Effects of Withholding of Water

In three independent experiments, the water potential of leaflets on control plants was −0.33 ± 0.03 MPa (Figure 1). After withholding of water for 1 day, the water potential dropped to −0.52 ± 0.04 MPa ($p < 0.03$, paired *t*-test). The water potential continued to decline for the course of the experiment and reached −0.87 ± 0.04 MPa 6 days after withholding of water. Leaf conductance was initially 0.14 ± 0.01 $mol \cdot m^{-2} \cdot s^{-1}$ (Figure 1) and dropped to 0.10 ± 0.02 $mol \cdot m^{-2} \cdot s^{-1}$ ($p = 0.05$, unpaired *t*-test) 1 day after withholding of water. Six days after withholding of water, leaf conductance was only 0.02 ± 0.00 $mol \cdot m^{-2} \cdot s^{-1}$ ($p < 0.001$, paired *t*-test, compared to the control value).

Whole-Leaf and Leaf-Apoplast ABA Concentration Changes During Water Stress

The ABA concentration in whole-leaflet samples prior to withholding of water was 298 ± 43 $nmol \cdot kg^{-1}$ (fresh mass) (Figure 2). After water had been withheld for 1 day, the leaf ABA concentration increased to 563 ± 38 $nmol \cdot kg^{-1}$ (fresh mass) ($p < 0.01$, paired *t*-test). The rate of increase of whole-leaf ABA concentration was greater after 2 days of withholding of water (~1300 $nmol \cdot kg^{-1}$ (fresh

mass)·d^{-1}). At the end of the experiment, the whole-leaf ABA concentration had reached 6317 ± 500 nmol·kg^{-1} (fresh mass), that is, greater than 20 times control value. The initial leaf-apoplast ABA concentration was 273 ± 37 nM. After water had been withheld for 1 day, this value appeared to increase (p = 0.12, paired t-test); 2 days after withholding of water, the leaf-apoplast ABA concentration, 866 ± 220 nM, had increased significantly (p = 0.05, paired t-test). Mirroring the whole-leaf ABA concentration, the leaf-apoplast ABA concentration increased rapidly (~930 nM·d^{-1}) after 2 days of withholding water.

Guard-Cell ABA Content Changes During Water Stress

In two of the three experiments, guard cells were dissected from intact freeze-dried leaflet. These isolated cells contain both the symplastic and apoplastic ABA contents (see Materials and Methods). Guard cells of well-watered plants contained 3.2 ± 0.1 fg ABA·guard-cell pair^{-1} (n = 4; two shown in Figure 3 and two replicates not shown). During the whole-plant stress imposed by withholding of water, the guard-cell ABA content increased by 1.4 fg ABA·guard-cell pair^{-1}·day^{-1} (R^2 = 0.99) and after 6 d, reached a final value of more than 11 fg ABA·guard-cell pair^{-1}, that is, 3.7 times the control value.

Localization of ABA in the Symplastic and Apoplastic Compartments of Guard Cells of Water-Stressed Plants

Whether ABA accumulates in the symplastic compartment (Harris and others 1988) or in the apoplastic compartment of guard cells (Zhang and others 2001; Zhang and Outlaw 2001a, b) depends on the means by which stress is imposed. The ABA contents of these compartments were therefore determined for potted plants (Figure 4) and, as a reference, for plants grown in hydroponic culture. In unstressed plants, the guard-cell symplast ABA content was 2.4 and 3.2 fg ABA·guard-cell pair^{-1} for hydroponic and potted plants, respectively. In contrast, less than 10% of the guard-cell ABA was contained in the apoplastic compartment of unstressed plants, independent of the method of culture (Figure 4). A 20-min pulse of water stress imposed by addition of PEG to the roots of hydroponically cultured plants followed by a 20-min recovery period resulted in an increase in the guard-cell symplast ABA content and a much larger increase, approximately 30 times, in the guard-cell apoplast ABA content (Zhang and Outlaw 2001a). In these experiments (left panel, Figure 4), those results were corroborated: the guard-cell apoplast ABA content of a control plant in hydroponic culture was 0.1 ± 0.1 fg ABA·guard-cell pair^{-1} compared with a content of 5.0 ± 0.5 fg ABA·guard-cell pair^{-1} after stress.

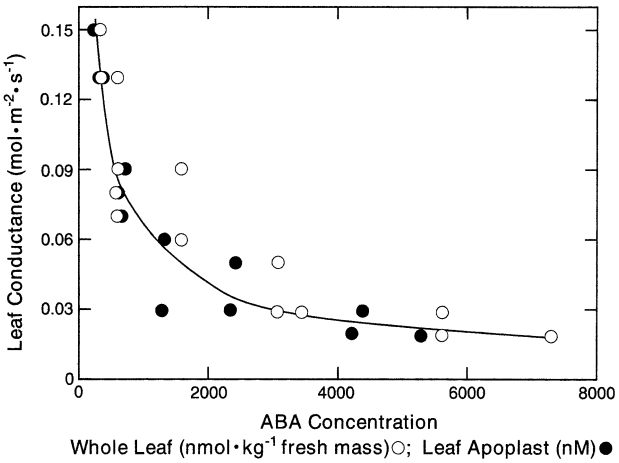

Figure 5. The relationship between whole-leaf ABA concentration (○) and leaf-apoplast ABA concentration (●) and leaf conductance. These data are plotted from the experiments shown in Figures 1 and 2.

The effect of short-term stress to potted plants on the ABA content of the guard-cell compartments was assessed by addition of PEG to the roots (right panel, Figure 4). The dominant effect was an elevation of the guard-cell apoplast ABA content, increasing from 0.2 fg ABA-guard-cell pair^{-1} to 4.7 fg ABA·guard-cell pair^{-1}. The effect of long-term stress was assessed by withholding of water (Figures 1–3). After 6 days of water stress, the guard-cell symplast ABA content increased by approximately 3 fold to 10.0 ± 1.1 fg ABA guard-cell pair^{-1} whereas the guard-cell apoplast ABA content increased by greater than 10 fold, to 1.5 ± 0.7 fg ABA·guard-cell pair^{-1} (n = 4; right panel, Figure 4).

Relationship between Leaf ABA Concentration and Leaf Conductance

On a logarithmic scale, both whole-leaf ABA concentration and leaf-apoplast ABA concentration were strongly correlated (R^2 > 0.85) with leaf conductance (Figure 5). Half saturation of the leaf-apoplast ABA effect on leaf conductance was approximately 0.5 μM.

Relationship Between Guard-Cell ABA Content and Leaf Conductance

The guard-cell ABA content and leaf conductance were correlated (Figure 6; R^2 = 0.91); half saturation was observed at 5 fg ABA·guard-cell pair^{-1}.

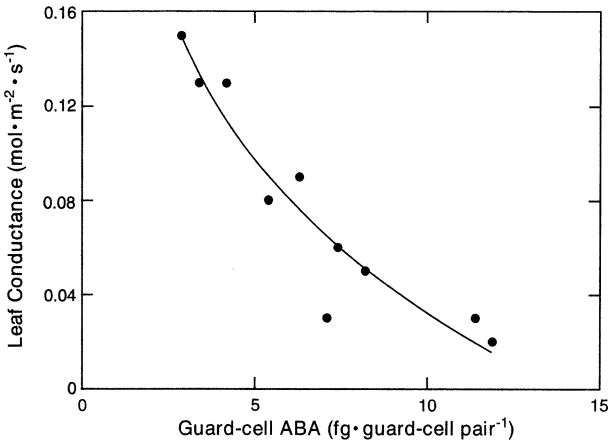

Figure 6. The relationship between guard-cell ABA content and leaf conductance. The individual whole-cell ABA contents (Figure 3) are plotted against the cognate individual conductances (Figure 1).

Discussion

The most prominent response to water stress is an elevation in the ABA concentration. In turn, guard cells must be key targets for ABA action, but only two previous studies (Zhang and others 2001; Zhang and Outlaw 2001a) have addressed water stress-induced changes in guard-cell ABA contents in intact plants. Those two reports focussed on root-source ABA. In the first report (Zhang and others 2001), one-half of the root system of hydroponic plants was stressed by PEG, resulting in little effect (less than or equal to 0.1 MPa) on Ψ_{Leaf}. In the second report (Zhang and Outlaw 2001a), a pulse of stress was imposed on the whole root system of hydroponic plants by submersion in PEG for 20 min. This treatment resulted in a large effect on Ψ_{Leaf} (Δ nearly equal to 0.6 MPa), but the effect was temporary, lasting less than 1 h. In both of those reports, the changes in guard-cell ABA content were correlated with leaf conductance or stomatal aperture size. The present results (Figure 6) extend this observation to long-term studies on intact plants that were gradually water stressed. Altogether, the simplest interpretation of these various studies is that guard-cell ABA is the major determinant of stomatal aperture size, but additional work is required to understand *in planta* interactions with other substances, for example Ca^{2+} (Wang and others 1998), and factors that influence ABA localization within the leaf, for instance pH (Hartung and others 1998).

Initiation of an ABA response requires co-localization of ABA and the receptor(s). Studies have implicated both internal and external loci for ABA perception by guard cells, as discussed above, so important goals are to understand the molecular consequences of internal and external perception and to define the circumstances under which ABA accumulates in the guard-cell apoplast and symplast. Progress toward the latter goal is underway. First, guard cells of detached water-stressed leaves accumulate ABA in the symplastic compartment (Harris and others 1988). As guard cells can synthesize ABA (Cornish and Zeevaart 1986) and because these detached leaflets were maintained under nontranspiring conditions for several hours after stress, the simplest interpretation is that guard cells accumulate internally synthesized ABA and respond to it. Under these circumstances, therefore, internal perception is implicated. At the other extreme, guard cells of leaves infused with ABA through the petiole (Zhang and Outlaw 2001b) accumulate ABA exclusively in their apoplastic compartment in the short term. These guard cells respond to ABA too, implicating external perception of root-source ABA. Similarly, when plants are given a pulse of water stress, guard-cell symplast ABA content changes only modestly, but the large changes in guard-cell apoplast ABA concentration are correlated with stomatal aperture size (Zhang and Outlaw 2001a). Therefore, those observations also implicate an external locus for ABA perception. The present results, reflecting a more physiologically relevant imposition of water stress, are intermediate. The entire plant was water stressed, and ABA accumulated in both the guard-cell apoplast and symplast. Indirectly, the results (Figures 1, 4) provide additional evidence for the role of internal ABA perception as discussed in the following. After 6 days of water stress when leaf conductance was 0.02 $mol \cdot m^{-2} \cdot s^{-1}$, the guard-cell apoplast ABA concentration was approximately 1.5 µM. However, this concentration of guard-cell apoplast ABA corresponds to a leaf conductance of greater than 0.05 $mol \cdot m^{-2} \cdot s^{-1}$ (Figure 5 of Zhang and Outlaw 2001b), consistent with an additive role of guard-cell symplast ABA (Figure 4).

The relationship between the guard-cell apoplast ABA concentration and the leaf-apoplast ABA concentration is of particular interest. In an earlier study (Zhang and Outlaw 2001a), roots of hydroponically grown plants were given a 20-min pulse of water stress as discussed above. The leaf-apoplast ABA concentration increased and reached a maximum, approximately185 nM, at 40 min from stress initiation. In contrast, the maximum guard-cell apoplast ABA concentration, also at 40 min after stress initiation, was about 2.5 µM, or approximately 13.5 times the leaf-apoplast ABA concentration. In a second study (Zhang and Outlaw 2001b), 1 µM ABA

was infused into the petioles of intact plants to simulate import of root-source ABA. In that study, the maximum guard-cell apoplast ABA concentration, more than 3 μM, was greater than 6 times the leaf-apoplast ABA concentration. The accumulation of ABA in the guard-cell apoplast was hypothesized to result from evaporation of the apoplast solution from the guard-cell wall. This proposed mechanism would cause stomata to respond to apoplast ABA concentration in concert with the transpiration rate. Consistently, the somewhat smaller amplification in the second study was correlated with a smaller stomatal aperture size, and therefore lessened transpiration.

In contrast to the previous studies (Zhang and Outlaw 2001a, b), here, the leaf-apoplast ABA concentration at the end of the stress period exceeded 4 μM (Figure 2), when the guard-cell apoplast ABA concentration was less, about 1.5 μM (Figure 4; conversion factors in Ewert and others 2000). In the present study, leaf conductance (Figure 1) was very low, equivalent to a stomatal aperture size of less than 2 μm, for 2 days before the guard-cell apoplast ABA concentration (Figure 4) was determined. The simplest conclusion is that the ratio (leaf-apoplast ABA concentration)/(guard-cell apoplast) is controlled in part by transpiration rate, which provides support for the amplification hypothesis. In other words, the leaf-apoplast ABA concentration required for half saturation of the effect on stomatal conductance will vary, depending on the transpiration rate. This interpretation, however, is qualified, as the ABA concentrations in the present long-term study were much higher than those in previous short-term studies (Zhang and Outlaw 2001a, b).

In summary, these results, along with previous ones (Harris and others 1988; Popova and others 2000; Zhang and others 2001; Zhang and Outlaw 2001a, b), show that guard-cell ABA content changes with water stress, that the changes are correlated with stomatal aperture, that guard cells respond to both internal and external ABA, and that the water-stress protocol determines whether ABA accumulates in the guard-cell symplast or the guard-cell apoplast.

ACKNOWLEDGMENTS

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