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CSIRO Publishing
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The ascorbic acid cycle mediates signal transduction leading to stress-induced stomatal closure

Albino Maggio[A], *Michael G. McCully, Kanogwan Kerdnaimongkol*[B], *Ray A. Bressan, Paul M. Hasegawa and Robert J. Joly*[C]

Center for Plant Environmental Stress Physiology, Purdue University, 1165 Horticulture Building, West Lafayette, IN 47907–1165, USA.
[A]Current address: National Center of Energy, Environment and Innovative Technology, C.R. Trisaia, S.S. Jonica Km 419–500, 75026 Rotondella (MT), Italy.
[B]Current address: Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[C]Corresponding author; email: joly@purdue.edu

Abstract. Using a combination of pharmacological approaches, mutation analysis and a gene silencing strategy, we present evidence that treatment of tomato (*Lycopersicon esculentum* Mill.) plants with exogenous ascorbate (AsA) subsequently increases the level of cellular AsA and causes stomatal closure. Using the ABA-deficient mutants *flacca* and *sitiens*, we show that the AsA-mediated induction of stomatal closure requires the participation of ABA. In addition, ABA acts independently of its role in mediating another stress response, proline accumulation. Because cellular AsA level was not elevated during stomatal closure, we hypothesized that stomatal closure relies on the activation of the AsA cycle and possible accumulation of intermediate components, such as monodehydroascorbate, that have been reported to be involved in mediating stress-induced responses. To establish a link between H_2O_2 production, the AsA cycle and stomatal closure, we also evaluated the effect of AsA treatment on catalase-deficient transgenic plants, which have a constitutively high level of H_2O_2. Interestingly, stomata of catalase-deficient plants were much more responsive to AsA treatment, compared with wild-type control plants. Because an increase in cellular H_2O_2 upon stress has been widely documented in many organisms and has been interpreted as a signal that initiates a cascade of stress-induced responses, we suggest that stress-induced stomatal closure is mediated by H_2O_2 and activation of the AsA cycle.

Keywords: ABA, ascorbate, gas exchange, glutathione, leaf water relations, proline, tomato.

Introduction

Plants exposed to hyperosmotic stress activate biochemical pathways that lead to adaptive responses, including osmotic adjustment, ion detoxification, production of compatible solutes, protection against reactive oxygen species, regulation of leaf gas exchange, and modulation of root and leaf water fluxes (Niu *et al.* 1995; Ingram and Bartels 1996; Hasegawa *et al.* 2000; Maggio *et al.* 2001, 2002). Some of these responses, such as ion compartmentation, production of compatible solutes, and stomatal movement are functionally coordinated, and are likely to be regulated by common and/or interacting signal transduction pathways (Zhu *et al.* 1997).

Stomatal closure is among the earliest events occurring in plants exposed to hyperosmotic stress, and it is mediated by the stress hormone ABA (Tardieu and Davies 1993; Lösch and Schulze 1995). Some of the components downstream of ABA that are required for stomatal closure are known (Assmann 1993; MacRobbie 1998). Upon stress, increased ABA at the site of action causes depolarization of the guard cell plasmalemma and tonoplast membrane potentials which, in turn, leads to K^+ efflux through the activation of outward-rectifying K^+ channels. This process is associated with ion efflux (Cl^- and/or malate^{2-}), and is mediated by an increase in cytoplasmic pH and Ca^{2+} (Assmann 1993; MacRobbie 1998). Much less is known about the ABA upstream components that link perception of the stress signal to ABA synthesis and/or mobilization and, ultimately, to ABA-mediated responses like stomatal closure.

AsA is a major reducing agent in plants, and can be found in both photosynthetic and non-photosynthetic tissues in millimolar concentration (Noctor and Foyer 1998). In addition to its well-documented role as an antioxidant (Njus and Kelley 1991), electron transporter (Smirnoff 1996), and enzyme cofactor (Liso *et al.* 1985), AsA has been reported

Abbreviations used: A, net CO_2 assimilation rate; AsA, ascorbate; DHA, dehydroascorbate; GL, L-galactono-1,4-lactone; g_s, stomatal conductance; GSH, glutathione; GSSG, glutathione disulfide; MDHA, monodehydroascorbate; PPFD, photosynthetic photon flux density; ψ_w, water potential.

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to be involved in the regulation of gene expression and cellular redox potential (Noctor and Foyer 1998). These latter two functions, together with a known sensitivity of the AsA pool to water deficit (Smirnoff 1993) suggest that the AsA cycle may operate not only to scavenge free radicals but also to activate, and possibly regulate, other stress-induced responses (Smirnoff 1996). Based on this information, we hypothesized that hyperosmotic stress could be linked to activation of the AsA cycle by stress-induced production of H_2O_2 that is reduced through the AsA cycle.

Preliminary experiments in our laboratory revealed that an increase of the plant AsA pool by pretreatment of tomato root systems with a 5 mM solution of AsA caused dramatic protection against either a gradually-imposed water stress or severe osmotic shock. In further investigations, we discovered that one component of the observed AsA-mediated protective effect was the rapid induction of stomatal closure. That there was a relationship between the AsA cycle and a general stress response was not surprising (Smirnoff 1993, 1996). However, the possibility of a specific physiological link between AsA and stomatal response has not been considered before, to our knowledge.

Materials and methods

Plant material and growth conditions (salt stress experiment)

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Better Boy) were sown in June 1998 into flats containing a 2:2:1 (v/v/v) mixture of perlite, peat moss and top soil, and maintained in a greenhouse under approximately 28°C daytime maximum and 23°C night-time minimum temperature and approximately 50% relative humidity. Two weeks after germination, 12 seedlings were transferred to each of eight 30-L plastic containers filled with 0.5× Hoagland's solution (Hoagland and Arnon 1950). Plants were maintained in aerated, hydroponic culture under the greenhouse conditions described above for an additional 2 weeks, until they had produced four or five fully-expanded leaves. Plants and treatments were arranged in a completely randomized block design on the greenhouse bench, and three replicate plants were sampled for each of four treatments. At 16:00 on the day prior to imposition of salt shock, sodium ascorbate (Sigma, St Louis, MO, USA) was added to the nutrient solution of four of the eight containers, bringing the final AsA concentration to 10 mM. After 18 h, NaCl was added to the nutrient solution of two AsA-pretreated and two non-pretreated containers, bringing the final concentration to 250 mM. In summary, the four treatments were: (i) untreated control; (ii) AsA pretreated; (iii) 250 mM NaCl; (iv) AsA pretreated followed by 250 mM NaCl.

Plant materials and growth conditions (water stress experiment)

Tomato seeds were sown singly in 1.4-L (15 cm diameter) plastic pots containing a 2:2:1 (v/v/v) mixture of perlite, peat moss, and topsoil under the greenhouse conditions described above. Plants were grown for an additional 2 weeks until they had produced four or five fully-expanded leaves. At this time, 12 plants were selected for uniform size and arranged in a randomized design on a greenhouse bench. Six plants were irrigated to pot capacity daily (non-stressed control), while water was withheld from the remaining six until permanent stomatal closure was detected. Stomatal conductance (g_s) of stressed and control plants was monitored daily using a steady-state porometer (model 1600; Li-Cor, Lincoln, NE, USA).

Responses of detached leaves

Thirty tomato plants were grown in aerated hydroponic culture (0.5× Hoagland's solution) in a controlled-environment growth chamber (model E15; Conviron, Winnipeg, Manitoba, Canada). Very-high-output fluorescent lamps provided 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) to the upper leaves. Plants were exposed to a 12-h photoperiod, with day and night temperatures of 25 and 23°C, respectively. Relative humidity was approximately 80%.

After 4 weeks, the two uppermost expanded leaves of each plant were excised with a razor blade and immediately transferred to 50-mL Falcon tubes filled with the same nutrient solution as used for hydroponic culture. Leaf petioles were inserted through holes in the tube closures and sealed with Parafilm to prevent evaporation of water. An acclimation period of 30 min was used before any treatments were applied. After g_s was measured to verify that there had been no wound-induced stomatal closure, concentrated solutions of AsA, L-galactono-1,4-lactone (GL), or ABA were injected into the bathing solution to obtain the desired final concentrations. AsA and ABA were added to obtain final concentrations of 5 mM and 100 μM, respectively. GL (Sigma) was added to provide concentrations of 1, 5 or 10 mM. After addition of each specific chemical, g_s was monitored.

Catalase-deficient transgenic plants

Transgenic tomato plants expressing an antisense catalase gene were generated previously (Kerdnaimongkol and Woodson 1999), and used here to test their stomatal responsiveness to exogenous AsA. We used transgenic lines 11-12 and 11-30 (here renamed *an*-cat11-12 and *an*-cat11-30), which exhibited a 2- and 1.5-fold increase in H_2O_2, respectively, in leaf extracts relative to the wild-type control (cv. Ohio) (Kerdnaimongkol and Woodson 1999). Leaf H_2O_2 levels were confirmed using a spectrophotometric assay (Kerdnaimongkol and Woodson 1999).

Fifteen plants of each line (wild type, *an*-cat11-12 and *an*-cat11-30) were grown singly for 4 weeks in 1.4-L (15 cm diameter) pots in a controlled-environment growth room. Day and night temperatures were 25 and 23°C, respectively, during 16-h photoperiods. Relative humidity was approximately 50%, and a mixture of metal halide, high-pressure sodium, and incandescent lamps provided a PPFD of approximately 300 μmol m^{-2} s^{-1} at the top of the plant canopy.

When plants had four or five fully-expanded leaves, pots were randomized and treatments imposed. For each group of 15 plants, ten were irrigated every other day with deionized water while five were irrigated to pot capacity with a 5 mM AsA solution at the same time interval. g_s was monitored daily using the Li-Cor steady-state porometer. After three AsA applications, a 30–50% decrease in g_s was detected in the AsA-treated catalase antisense transgenic plants. No reduction was observed in the wild-type control.

At this time, water was withheld from all AsA-pretreated plants and from five of the ten plants that had been irrigated previously. The alternate-day irrigation regime was maintained for the five remaining plants. In summary, for each of the three lines studied, we established three treatments: irrigated control, soil water deficit, and AsA pretreatment, followed by water deficit. Seven days after withholding water, stressed plants exhibited a high degree of wilting — leaf water potential (ψ_w) and g_s were measured at this time. Plants were then watered to pot capacity to evaluate their ability to recover from the stress.

Leaf water relations and gas exchange measurements

For the water stress experiments described above, g_s was measured between 10:00 and 12:00 using a steady-state porometer (model 1600; Li-Cor) on the first and second uppermost expanded leaves of four

plants for each treatment. For the salt stress experiment, net CO_2 assimilation rate (A) and g_s were assessed at approximately hourly intervals following AsA treatment, and 2 h after NaCl treatment, using a portable photosynthesis system (model CIRAS-1; PP Systems, Haverhill, MA, USA) fitted with a 20-W halogen light source that provided a PPFD of approximately 900 μmol m^{-2} s^{-1} to the leaf surface. Leaf ψ_w was measured using a pressure chamber (model 600; PMS Instruments Co., Corvallis, OR, USA) on the same leaves used for g_s analysis (salt stress experiment only). Following measurement of ψ_w, a 0.5 g sample of fresh leaves was sealed and frozen in liquid nitrogen for later determination of ABA and AsA concentrations. For the water stress experiment, g_s was monitored daily until values measured in stressed plants had decreased to 20–30% that of irrigated controls. At that time, four leaflets (two each for ABA and AsA measurement) were harvested from the uppermost expanded leaf of each of the 12 plants and frozen in liquid nitrogen.

Proline, AsA, glutathione and ABA determination

Frozen leaves (0.5 g fresh weight) were ground to a fine powder with a mortar and pestle. Proline content was determined after methanol:chloroform:water extraction (12:5:1 v/v/v) by the acid ninhydrin procedure (Troll and Lindsley 1955). AsA was measured by the decrease in absorbance at 265 nm upon addition of AsA oxidase, and dehydroascorbate was measured after non-enzymic reduction using glutathione (GSH) (Foyer *et al.* 1983). Total GSH and glutathione disulfide (GSSG) were determined using GSH reductase and 2-vinylpyridine (Roxas *et al.* 1997), and GSH was calculated as the difference between total GSH and GSSG. Samples for ABA quantification were frozen in liquid nitrogen, weighed, and lyophilized. ABA was extracted from powdered tissue in methanol as described previously (Birkenmeier and Ryan 1998), and ABA levels were quantified using an indirect enzyme-linked immunosorbent assay method (Phytodetek ABA; AGDIA Inc., Elkhart, IN, USA).

Statistical analysis

Unless otherwise stated, levels of significance of differences between treatments were determined using the F-test of the ANOVA. Means were separated using the least significant difference procedure at $P<0.01$. Statistical analysis was performed using the MSTAT-C software (Crop and Soil Science Department, Michigan State University, Version 2.0).

Results

Effects of AsA pretreatment on salt- and water-stressed plants

Pretreatment with AsA for 18 h elicited a strongly protective effect in tomato plants subsequently subjected to hyperosmotic shock (Fig. 1). Two hours after exposure to 250 mM NaCl, untreated plants had lost turgor and exhibited severe wilting. In marked contrast, AsA-pretreated plants did not show any apparent symptoms of osmotic shock (Fig. 1*A*). Leaf ψ_w was significantly higher in plants pretreated with AsA, regardless of whether NaCl was later added to the nutrient solution or not (Fig. 1*B*). The effect of AsA pretreatment was greater in NaCl-shocked plants, however, and leaf ψ_w of pretreated plants was more than 0.6 MPa higher than that measured in non-pretreated plants. A and g_s in AsA-pretreated plants were 70 and 80% lower, respectively, than in non-pretreated, non-stressed controls. (Fig. 2). Two hours after NaCl treatment, A and g_s had decreased to very low values in both AsA-pretreated and non-pretreated plants.

AsA was readily taken up in treated plants (Table 1*A*), and levels of AsA in roots and leaves of AsA-treated plants were each significantly higher than in untreated controls. In contrast, dehydroascorbate (DHA), GSSG and GSH pools

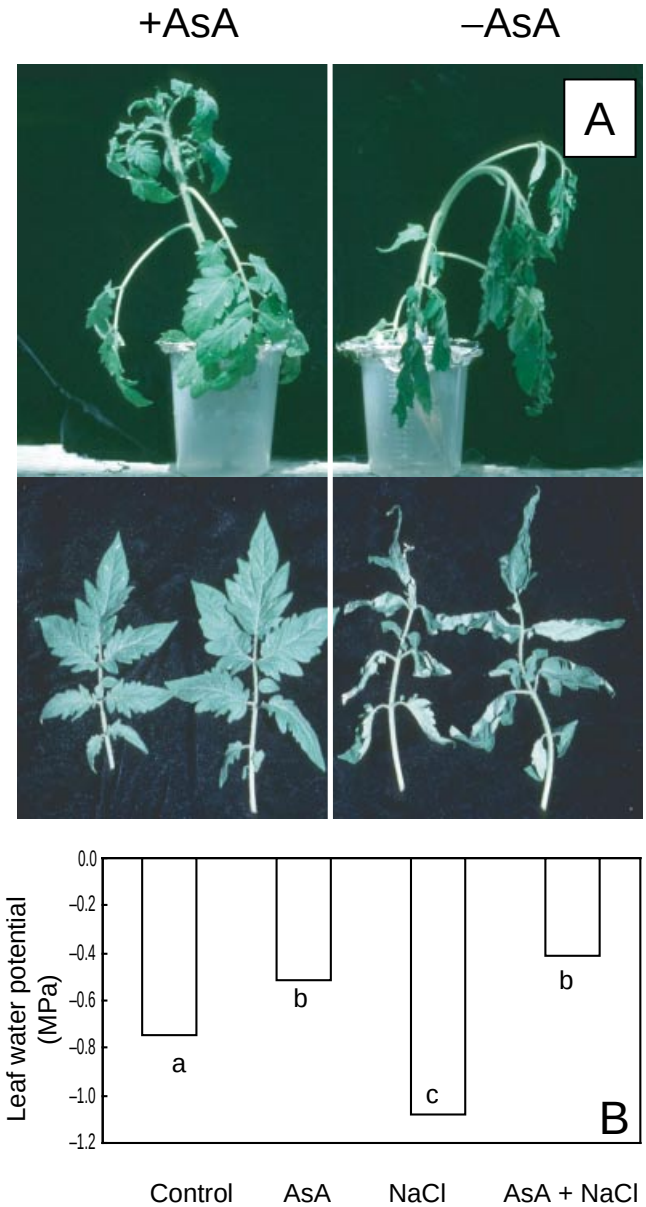

Fig. 1. Effect of AsA pretreatment on 4-week-old hydroponically-grown tomato plants subsequently subjected to osmotic shock by addition of NaCl to the nutrient media. (*A*) AsA-pretreated plants and non-pretreated controls photographed 2 h after addition of NaCl (final concentration 250 mM). AsA pretreatment was 10 mM for 18 h. (*B*) Leaf water potentials measured 2 h after addition of NaCl to the hydroponic solution for each of four treatments (untreated control; AsA-pretreated; 250 mM NaCl; AsA-pretreated followed by 250 mM NaCl). Different letters indicate significant difference at $P<0.01$ ($n = 3$).

were not significantly affected by AsA treatment, with the exception of DHA in roots of AsA-treated plants, which was higher than untreated controls where DHA was not detectable. To verify the specificity of AsA in mediating stomatal closure, we analysed the effect of GL (a precursor of AsA) taken up by detached tomato leaves. GL was rapidly converted to AsA in a nearly linear manner (Fig. 3). The appearance of AsA was inversely correlated with g_s of detached leaves. g_s decreased markedly, consistent with nearly complete stomatal closure, at concentrations of GL between 1 and 5 mM.

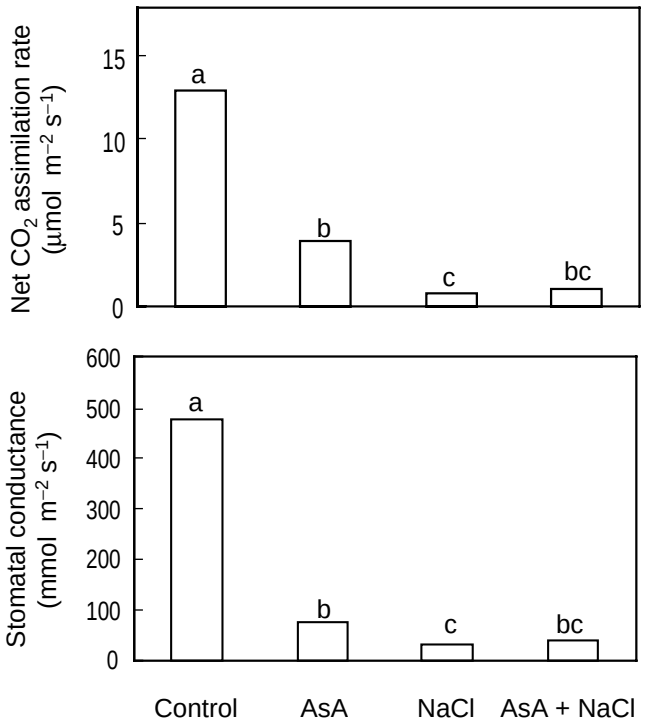

Fig. 2. Effect of 18 h AsA pretreatment on A and g_s of hydroponically-grown tomato plants subsequently subjected to 250 mM NaCl in the nutrient media. Measurements were made 2 h after addition of NaCl. Treatments were: untreated control; AsA-pretreated; 250 mM NaCl; AsA-pretreated followed by 250 mM NaCl. Different letters indicate significant difference at $P<0.01$ ($n = 3$).

AsA does not cause an increase in leaf ABA

Since stomatal closure upon stress typically occurs via ABA, we wanted to test whether or not this was the case for the AsA-mediated response. Tomato plants exposed to either water deficit or salt stress exhibited typical stress-induced increases in leaf ABA content (Table 2). However, although water stress, salt stress and AsA pretreatment each reduced g_s to approximately one-third of the levels measured in non-stressed controls, AsA did not cause an increase in *ex novo* synthesis of ABA (Table 2).

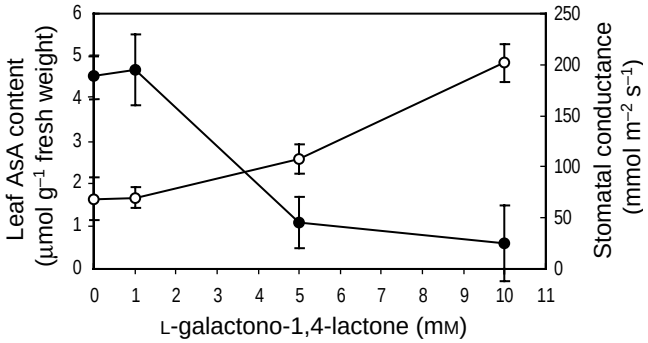

Fig. 3. Dose-response of detached tomato leaves to increasing concentration of the AsA precursor GL in the nutrient solution. Effects on leaf AsA content (○) and g_s (●) are shown. Data are means ± s.e. of three replicate plants.

Table 1. Effects of AsA, water deficit and salt stress on tissue concentrations of intermediates of the AsA–GSH cycle

Tissue concentrations of AsA, DHA, GSH and GSSG in roots and leaves of tomato plants treated with 10 mM AsA and non-treated controls. AsA and DHA concentrations are given in μmol g^{-1} fresh weight. GSH and GSSG are in nmol g^{-1} fresh weight. In the same row, different letters indicate significant difference at $P<0.01$, according to Student ($n = 8$). AsA and DHA concentrations (μmol g^{-1} fresh weight) in leaves of tomato plants subjected to water stress (by withholding irrigation water) or salt stress (by addition of NaCl to the hydroponic solution). For each experiment, different letters within a row indicate significant difference at $P<0.05$ ($n = 3$ for water stress experiment; $n = 3$ for salt stress experiment). ND, not detectable; NM, not measured

	Control		AsA-treated	
	Leaves	Roots	Leaves	Roots
AsA	2.67 ± 0.34 a	0.61 ± 0.09 b	4.70 ± 0.76 c	6.03 ± 0.39 d
DHA	0.21 ± 0.01 a	ND	0.33 ± 0.07 a	0.47 ± 0.05 b
GSSG	69.93 ± 9.81	50.04 ± 8.3	48.81 ± 11.34	50.16 ± 4.44
GSH	116.28 ± 6.99	148.53 ± 8.0	148.53 ± 8.04	127.32 ± 15.7
	Irrigated control	Water stressed	0 mM NaCl	250 mM NaCl
AsA	2.80 ± 0.18 a	1.7 ± 0.37 b	2.5 ± 0.09 a	1.8 ± 0.18 b
DHA	0.19 ± 0.017	0.15 ± 0.032	NM	NM

Response of ABA-deficient mutants flacca *and* sitiens *to AsA treatment indicates that ABA is required for AsA-induced stomatal closure*

Because ABA levels did not change upon AsA treatment (Table 2), it was essential to verify whether ABA was required for AsA-induced stomatal closure. For this purpose, we used two ABA-deficient tomato mutants, *flacca* and *sitiens*, known to contain approximately 15 and 26%, respectively, of the wild-type ABA level (Zeevaart and Creelman 1988). In a time-response experiment, we verified that AsA (5 mM) and ABA (100 μM) treatments had very similar effects on the kinetics of stomatal closure (Fig. 4*A*). Detached tomato leaves (wild type) treated with ABA or AsA exhibited a 50% reduction in g_s after 30 and 45 min, respectively (Fig. 4*A*). In *flacca* and *sitiens*, however, AsA did not have any effect within 1 h of treatment (Fig. 4*B*).

Activation of the AsA cycle causes stomatal closure

Analysis of the constitutive AsA and DHA pools upon stress showed that water and salt stress both deplete the AsA pool without affecting the DHA pool (Table 1*B*). This result suggests that activation of the AsA cycle, and possible accumulation of intermediate components of the cycle, rather than an increase of the absolute AsA pool itself, may have been the cause for the observed stomatal response to AsA treatment. To test this possibility we utilized catalase-deficient transgenic plants, which constitutively accumulate H_2O_2 (Fig. 5). Transgenic tomato plants with decreased catalase and increased H_2O_2 levels were strongly sensitized to AsA pretreatment and subsequent dehydration stress (Fig. 5). After only three irrigations with 5 mM AsA, g_s was reduced by 50 and 25% in catalase-deficient transgenic lines *an*-cat11-12 and *an*-cat11-30, respectively. Under these conditions the AsA treatment was not sufficient to affect g_s in the wild-type control (Fig. 5). This partial stomatal closure was associated with protection against subsequent dehydration stress. Withholding irrigation for 7 d caused severe dehydration, as assessed visually (Fig. 6*A*) and by leaf ψ_w (Fig. 6*B*), in wild-type plants both pretreated

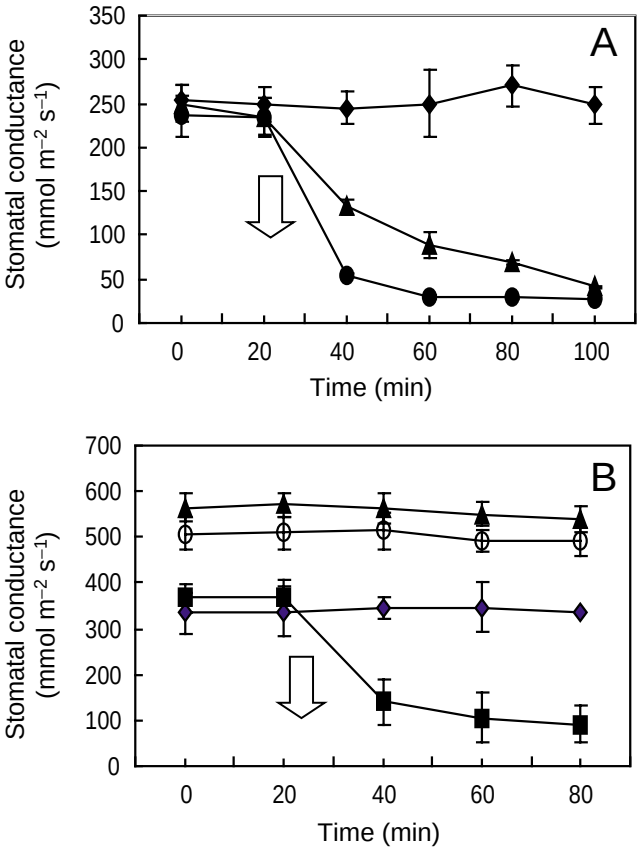

Fig. 4. Time response of g_s in detached tomato leaves following addition of ABA or AsA to the nutrient solution. (*A*) Response of g_s in wild-type tomato leaves to 5 mM AsA (▲) and 100 μM ABA (●), relative to a distilled water control (◆). Large arrow indicates time of addition of AsA or ABA. Data are means ± s.e. of three replicate plants. (*B*) Effect of 5 mM AsA treatment on g_s in detached leaves of the ABA-deficient mutants *flacca* (○) and *sitiens* (▲), relative to wild-type tomato leaves (■). A non-treated wild-type control (◆) is shown. Large arrow indicates time of addition of AsA. Data are means ± s.e. of three replicate plants.

Table 2. Effect of AsA, plant water deficit and NaCl shock on g_s, leaf proline content, and leaf ABA content in 4-week-old tomato plants

Water deficit was imposed by withholding irrigation water from pot-grown plants, while salt shock was induced by addition of NaCl to the hydroponic solution to obtain a final concentration of 250 mM. In both experiments, samples for proline and ABA content were taken when g_s had decreased to approximately one-third that measured in non-stressed controls. In the same column, different letters indicate significant difference at $P<0.01$($n = 4$)

	Leaf proline content (μmol g^{-1} fresh weight)	g_s (mmol m^{-2} s^{-1})	Leaf ABA content (ng 100 mg^{-1} dry weight)
Control	1.5 ± 0.2 a	331 ± 9.38 a	11.0 ± 0.95 a
AsA	1.3 ± 0.1 a	127 ± 5.57 b	10.1 ± 0.74 a
Salt shock	111.0 ± 7.5 b	98 ± 4.87 c	24.6 ± 2.24 b
Water stress	154.0 ± 12.0 c	115 ± 1.68 bc	29.0 ± 0.64 b

and not pretreated with AsA. Antisense transgenic plants not pretreated with AsA also showed severe wilting and low ψ_w. Withholding water caused leaf ψ_w to drop below –1.5 MPa in wild-type plants, regardless of AsA pretreatment. However, AsA-pretreated catalase-deficient plants maintained leaf ψ_w approximately 0.65 and 0.5 MPa higher, respectively, than non-pretreated controls, and the differences were highly significant in each case.

The AsA-mediated response is specific

Because ABA activates many stress-induced responses, including proline biosynthesis (Yoshiba *et al.* 1997), we wanted to test whether the AsA-mediated ABA activation also induced other ABA-dependent responses. Tomato plants exposed to either water deficit or salt stress exhibited a 100-fold increase in leaf proline content (Table 2), with stress-induced values being consistent with other reports for this species (Fujita *et al.* 1998). In contrast, AsA treatment did not affect the proline level, suggesting that AsA/ABA-mediated stomatal closure and ABA-mediated proline accumulation follow separate signal transduction pathways.

Discussion

In this study we present evidence for a novel pathway that leads to stress-induced stomatal closure. Specifically, we used a whole-plant approach that perturbed *in vivo* AsA cycle homeostasis in several different ways to study the possible relationships between the AsA antioxidant system, ABA, and stomatal response to stress.

Increased leaf AsA causes stomatal closure

AsA feeding through leaf petioles or root systems increased the plant AsA level without significantly altering the foliar AsA/DHA and GSH/GSSG ratios (Table 1), suggesting that AsA uptake may have enhanced the activity of enzymes in the AsA–GSH cycle, such as AsA peroxidase and AsA oxidase, allowing these pools to remain unchanged. The increase in total leaf AsA likely caused an increase in both apoplastic and cytoplasmic AsA pools. However, predicting which of these pools would have a predominant influence on stomatal response is not simple. Plant ozone sensitivity, for instance, has been shown to be associated with either a decrease in the apoplastic or cytoplasmic pools in common bean and tobacco, respectively (Orvar and Ellis 1997; Moldau *et al.* 1998). The partial stomatal closure induced by AsA pretreatment was likely sufficient to limit plant water loss caused by subsequent osmotic shock (Figs 1, 2). The observed AsA-mediated stomatal response was not caused by altered pH of the nutrient solution (which was stable at

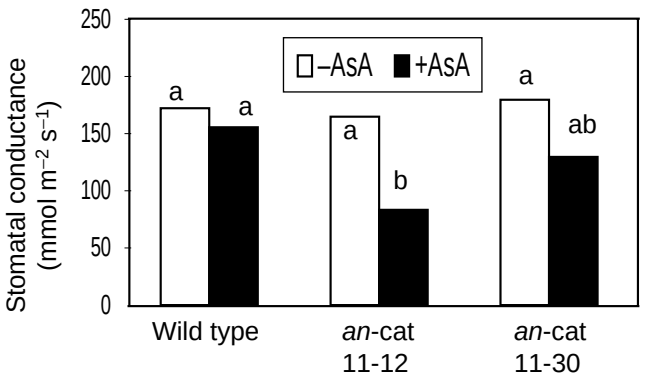

Fig. 5. Effect of AsA treatment on g_s measured in non-stressed catalase antisense transgenic tomato plants (*an*-cat11-12 and *an*-cat11-30) and wild-type control. g_s was measured following three applications of 5 mM AsA solution as a soil drench, each given on alternate days. Different letters indicate significant difference at $P<0.01$ ($n = 4$).

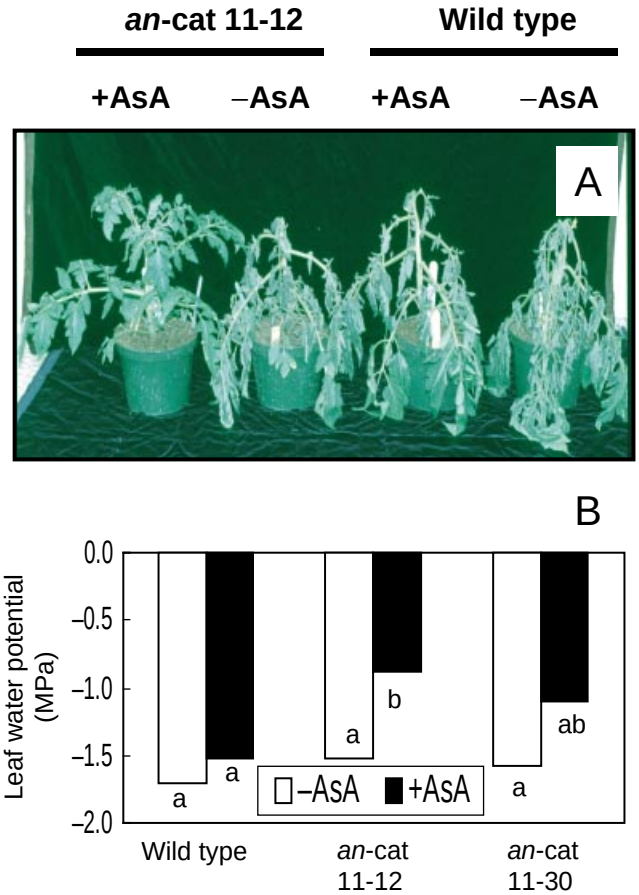

Fig. 6. Effect of AsA pretreatment (three alternate-day applications of a 5 mM solution) on subsequent response to soil water deficit in catalase antisense transgenic tomato plants (*an*-cat11-12 and *an*-cat11-30) and wild-type controls. Before imposition of water stress, leaf AsA levels were 2.5 ± 0.5 and 2.8 ± 0.31 for wild type and *an*-cat11-12 non-AsA-treated controls, respectively, and 3.4 ± 0.4 and 3.6 ± 0.6 for AsA-treated plants, respectively. (*A*) AsA-pretreated plants and non-pretreated controls photographed 7 d after withholding water. (*B*) Leaf ψ_w measured on tomato plants subjected to soil water deficit for 7 d. Recently fully-expanded leaves were sampled. Different letters indicate significant difference at $P<0.01$ ($n = 4$).

6.5 ± 0.1 upon addition of AsA) or by changes in the osmolality (0.137 ± 0.001 and 0.136 ± 0.002 MPa for the control and 10 mM AsA solutions, respectively). Furthermore, additional control treatments of either 10 mM NaCl or 10 mM sodium citrate did not initiate any measurable stomatal closure (data not shown). The specificity of AsA treatment was also confirmed upon feeding of GL, a specific precursor of AsA. GL caused a dose-dependent linear increase in leaf AsA content that was inversely correlated with g_s.

AsA-mediated stomatal closure occurs through ABA

AsA treatment did not cause stomatal closure in detached leaves of the ABA-deficient mutants *flacca* and *sitiens* (Fig. 4). These results provide unequivocal evidence that AsA-induced stomatal closure was not caused by osmotic effects. In addition, considering that the ABA level did not increase upon AsA treatment (Table 2), we conclude that AsA-mediated stomatal closure occurs via a local increase in active ABA at guard cells. Although upon water stress, ABA has been shown to be synthesized in roots and subsequently translocated to leaves via the transpiration stream (Zhang and Davies 1987; Assmann and Shimazaki 1999), a clear and conclusive sequence of events from stress perception to ABA action has not yet been established.

What is the functional relationship between AsA and stomatal closure?

The effect of environmental stresses on intracellular and extracellular AsA pools is complex. A large decrease in the AsA pool has been found to result from water deficit (Smirnoff 1993). However, AsA accumulation has been reported to increase at high light intensity, a condition often associated with high temperature and drought (Smirnoff and Pallanca 1996). In our experimental environment, both water stress and salt stress depleted the AsA pool without affecting the DHA pool (Table 1*B*). This suggests that an enzyme(s) involved in AsA homeostasis may have been activated upon stress. A plausible candidate is AsA oxidase, which is responsible for the conversion of AsA to monodehydroascorbate (MDHA). Its activation would be expected to cause an increase in the steady-state level of MDHA, as predicted from the equilibrium constant of the disproportionation reaction (Smirnoff 1996, 2000).

Osmotic stress is also known to induce the formation of H_2O_2 (Shen *et al.* 1997), which may in turn be scavenged by AsA. Because stress-induced H_2O_2 and exogenously applied AsA should both activate the AsA cycle, and both conditions (stress and added AsA) lead to stomatal closure, we hypothesized that activation of the AsA cycle may be a functional link between stress and stomatal closure. To test this possibility we used transgenic tomato plants expressing an antisense catalase gene. We predicted that a higher constitutive H_2O_2 level, caused by the impaired reactive oxygen species scavenging system of these plants, would be sufficient to cause stomatal closure and/or to hypersensitize stomata to exogenous AsA (which should also activate the AsA cycle). Inhibition of one subunit of the tomato catalase gene *pTOMCAT1* caused an increased H_2O_2 level (Kerdnaimongkol and Woodson 1999), and a clear stomatal hypersensitivity to AsA treatment (Fig. 5). Certainly, catalase-deficient plants may have other modifications of the redox pools in addition to a higher H_2O_2 level, including a large increase in the GSSG/GSH ratio accompanied by an accumulation of total GSH (Noctor and Foyer 1998). Nevertheless, our results and recently published data on the effects of increased H_2O_2 levels on guard cells (Pei *et al.* 2000; Zhang *et al.* 2001) are consistent with a direct link between cellular redox state and stomatal behaviour.

The *flacca* mutation results in cellular ABA accumulation of only 26% of wild-type plants. The effect of the reduced pool of ABA appears to have a predominating influence on stomatal behaviour over either AsA treatment or conditions that lead to H_2O_2 production (e.g. stress or catalase deficiency). In this regard, the ABA signal appears to function after AsA or H_2O_2. Evidence for the relative position of AsA and H_2O_2 in signalling stomatal closure is less clear. It does support, however, a model with H_2O_2 affecting the AsA cycle, since the AsA treatment is able to 'suppress' the lack of H_2O_2 in non-stress conditions and cause stomatal closure, but increased H_2O_2 in catalase-deficient plants is not able to 'suppress' the lack of added AsA. Of course these results could be interpreted to mean that catalase-deficient plants do not produce sufficient H_2O_2 to activate the AsA-cycle-mediated mechanism without additional AsA. This is less likely because the accepted role of AsA is to scavenge H_2O_2 rather than increase it. However the ability of AsA to potentiate the effect of increased H_2O_2 does strongly suggest that H_2O_2 may act to close stomata by a separate parallel pathway. This view is supported by the report that H_2O_2 appears to act directly on guard cell behaviour by activating a Ca^{2+} channel through the activity of the *gac2* gene product, independent, or at least partially independent, of ABA (Pei *et al.* 2000).

AsA-induced stomatal closure is mediated by a specific part of ABA signal transduction

The AsA/ABA pathway leading to stomatal closure appears to be separated from other ABA-mediated signal events, as suggested by the co-existence of low leaf proline and ABA contents upon AsA treatment (Table 2). Stress-induced ABA synthesis is currently thought to increase transcription of the *P5CS* gene (Bray 1997; Yoshiba *et al.* 1997), which encodes the enzyme that catalyses the rate-limiting step for proline biosynthesis. This, in turn, causes proline accumulation. Because AsA treatment causes partial stomatal closure before any detectable increase in ABA or proline (Table 2), it is apparent that AsA interacts with components of the

general stress-induced response that are separated from ABA synthesis activation and proline accumulation.

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