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RESEARCH PAPER

ABA- and ethylene-mediated responses in osmotically stressed tomato are regulated by the *TSS2* and *TOS1* loci

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Abstract

The study of mutants impaired in the sensitivity or synthesis of abscisic acid (ABA) has become a powerful tool to analyse the interactions occurring between the ABA and ethylene signalling pathways, with potential to change the traditional view of the role of ABA as just being involved in growth inhibition. The *tss2* tomato mutant, which is hypersensitive to NaCl and osmotic stress, shows enhanced growth inhibition in the presence of exogenous ABA. The *tos1* tomato mutant is also hypersensitive to osmotic stress, but in contrast to *tss2*, shows decreased sensitivity to ABA. Surprisingly, blocking ethylene signalling suppresses the growth defect of *tss2* seedlings on ABA, NaCl, and osmotic stress, but not the osmotic hypersensitivity of *tos1*. The ethylene production of *tss2* seedlings is increased compared with that of control seedlings under osmotic stress. In addition, the *tss2* plants are hypersensitive to root growth inhibition by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). This suggests that, in addition to ABA regulation, *TSS2* acts as a negative regulator of endogenous ethylene accumulation. As previously shown in *Arabidopsis*, it is shown here that extensive cross-talk occurs between the ABA and ethylene signalling pathways in tomato and that the *TSS2* and *TOS1* loci appear as regulators of this cross-talk.

Key words: Abscisic acid, ethylene production, osmotic stress, root growth, tomato, *tos1*, *tss2*.

Introduction

The hormonal regulation of plant growth and development is a complex trait. Interactions among hormones are widespread and counteracting effects of different hormones on a given developmental process are common. These interactions appear to occur at many levels, including both positive and negative reciprocal effects on synthesis (Riov *et al*., 1990; Ghassemian *et al*., 2000; Hansen and Grossman, 2000; Hussain *et al*., 2000; Sharp *et al*., 2000; Spollen *et al*., 2000), and both positive and negative interactions between signalling pathways (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000; Gazzarrini and McCourt, 2001; Federoff, 2002; León and Sheen, 2003).

Recently, phenotypic analyses have determined that several response mutants have altered sensitivities to more than one hormone, indicating that a single signalling component can act in two or more different hormone responses. The *axr2* mutants of *Arabidopsis* have increased sensitivity to abscisic acid (ABA), ethylene, and auxins (Wilson *et al*., 1990), mutations in the brassinosteroid biosynthetic gene *SAX1* confer increased ABA sensitivity to the seed (Ephritikhine *et al*., 1999), and mutants defective in their response to ethylene also shows altered ABA sensitivity (Beadouin *et al*., 2000; Ghassemian *et al*., 2000). Moreover, the complexity of these relationships is increased by the fact that the hormonal effects can differ in stressed and non-stressed conditions and will also depend on the developmental history of the tissue.

In *Arabidopsis*, the ethylene pathway regulates seed dormancy negatively by inhibiting ABA signalling, while

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Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; 1-MCP, 1-methylcyclopropene; AVG, L-α-(2-aminoethoxyvinyl)-glycine.

these two pathways act synergistically in inhibiting root growth (Beadouin *et al*., 2000; Ghassemian *et al*., 2000). *ERA3*, a gene involved in ABA sensitivity, has recently been cloned and found to be allelic to the *ETHYLENE INSENSITIVE2* (*EIN2*) gene demonstrating a clear interaction between the ABA and ethylene signalling pathways (Ghassemian *et al*., 2000). This is further supported by the identification of the constitutive ethylene response mutant (*ctr1*) and *ein2* as enhancer and suppressor mutations, respectively, of the *abi1-1* mutant (Beaudoin *et al*., 2000).

Although it is known that ABA is an essential mediator in triggering the plant response to dehydration (Bray, 1997; Leung and Giraudat, 1998; Borsani *et al*., 2003; Botella *et al*., 2005) the hormonal relationships that determine the plant responses to water stress are not well understood. In this context, ABA has generally been regarded as an inhibitor of shoot growth (Trewavas and Jones, 1991; Davies, 1995; Munns and Cramer, 1996). This view was based on observations that (i) ABA accumulates to high concentrations in plants experiencing water deficits or other adverse conditions, often correlating with growth inhibition, and (ii) applications of ABA usually result in growth inhibition. However, the interpretation of these results is difficult due to the uncertainty as to whether the effects of applied ABA are predictive of the role of endogenous ABA (Trewavas and Jones, 1991; Sharp *et al*., 1994). Paradoxically, it has been observed for over 30 years that ABA-deficient mutants are often shorter and have smaller leaves than the corresponding wild types and that leaf and stem growth can be substantially restored by applying ABA (Imber and Tal, 1970; Bradford, 1983; Quarrie, 1987). The inhibited shoot growth of ABA-deficient mutants of tomato and *Arabidopsis* has been attributed to shoot water deficits, and the growth-promoting effect of applied ABA has been assumed to result from improvement in the plant water balance (Bradford, 1983; Neill *et al*., 1986; Nagel *et al*., 1994; Léon-Kloosterziel *et al*., 1996).

Although it was reported that ethylene production was greater in ABA-deficient mutants of tomato (Tal *et al*., 1979) and *Arabidopsis* (Rakitina *et al*., 1994), and that these tomato mutants exhibited morphological symptoms characteristic of excess ethylene, such as leaf epinasty and adventitious rooting (Tal, 1966; Nagel *et al*., 1994), the possibility that ethylene is a cause of shoot growth inhibition in ABA-deficient mutants was not considered until recently (Wright, 1980; Bradford and Hsiao, 1982; Sharp *et al*., 2000; Sharp, 2002; LeNoble *et al*., 2004). Recent studies have revealed that an important role of endogenous ABA is to limit ethylene production and that this reduction is required for the maintenance of root elongation at low water potentials (Spollen *et al*., 2000; Sharp *et al*., 2004). Consistent with the finding that ABA restricts ethylene production, it was reported that under well-watered conditions ethylene production was greater in shoots of the *flacca* (*flc*) tomato mutant (Tal *et al*., 1979) in water-stressed maize seedlings (Sharp, 2002), and in whole plants of the *aba1 Arabidopsis* mutant. The occurrence of the findings in maize, tomato, and *Arabidopsis* suggests that the restriction of ethylene production may be a widespread function of ABA, and that endogenous ABA may often function to maintain rather than inhibit plant growth (Sharp, 2002).

Identification of the *tss2* and *tos1* tomato (*Solanum lycopersicum* cv. Moneymaker) mutants indicates that both increased and decreased sensitivity to ABA may lead to a decreased tolerance to osmotic stress. Therefore, an appropriate ABA perception and/or signalling are required for osmotic tolerance (Borsani *et al*., 2002). In this report, it is shown that *tss2* under osmotic stress or exogenous ABA shows increased ethylene production compared with control plants, while *tos1* already has elevated ethylene production in control growth conditions. It is also shown that in contrast to *tos1*, *tss2* hypersensitivity to both osmotic stress and ABA is prevented by blocking ethylene signalling. The expression of genes induced by ABA and ethylene is also altered in these mutants. These results suggest that the *TSS2* and *TOS1* loci are required for maintaining low ethylene production under both osmotic stress and increased ABA levels.

Materials and methods

Plant materials and growth conditions

Wild-type, *tss2* and *tos1* (*Solanum lycopersicum* cv. Moneymaker) seeds used in this study were obtained as described previously (Borsani *et al*., 2001, 2002). Seed were sterilized with 40% (v/v) commercial bleach for 30 min and washed several times with sterile water. The seeds were first germinated until radicle emergence in sterile water in order to improve germination uniformity. The basal agar medium contained Murashige and Skoog (MS) salts, (Murashige and Skoog, 1962) with 3% (w/v) sucrose, and 0.7% (w/v) agar. The MS medium consists of the following: 1690 mg l^{-1} NH_4NO_3, 1900 mg l^{-1} KNO_3, 370 mg l^{-1} $MgSO_4.7H_2O$, 170 mg l^{-1} $KHPO_4$, 378 mg l^{-1} $CaCl_2.2H_2O$, 27.8 mg l^{-1} $FeSO_4.7H_2O$, 37.2 mg l^{-1} disodium EDTA, 0.7495 mg l^{-1} NaI, 6.3 mg l^{-1} H_3BO_4, 16.9 mg l^{-1} $MnSO_4.H_2O$, 8.6 mg l^{-1} $ZnSO_4.7H_2O$, 0.25 mg l^{-1} $Na_2MO_4.2H_2O$, 0.025 mg l^{-1} $CuSO_4.5H_2O$, and 0.025 mg l^{-1} $CoSO_4.6H_2O$. The various agar plates used in this work were made by adding the appropriate amount of mannitol, ABA, Ag^+, and AVG to the molten basal medium. Light provided by cool-white fluorescent bulbs was at 50 μE m^{-2} s^{-1} with 16 h of light at 22 °C, 8 h of dark at 18 °C, and 70% relative humidity. Varying levels of Ag^+ and AVG in the media were achieved by adding appropriate amounts of $AgNO_3$ and AVG. The $KMnO_4$ treatment was performed by including 15 g of solid $KMnO_4$ into a sterile filter paper bag taped to the lid of the Petri dish. The appropriate amount of 1-MCP gas (Ethylbloc™, Floralife, Walterboro, SC) was generated according to the manufacturer instructions and injected into a Petri dish with a sealed rubber septum in the lid.

Growth measurements

For growth measurements, 10 seedlings were used per treatment, and three replicates were made for each treatment. Three-day-old seedlings with 2 cm long roots were transferred from vertical agar plates

containing MS medium onto a second agar medium that was supplemented with different treatments. Increases in root length were measured after 2 d of treatment.

Ethylene measurements

Seeds were germinated and six homogenous seedlings were grown in 150 ml glass tubes filled with 50 ml of liquid MS medium and capped with cotton to permit gas exchange. The seedlings were grown for 7 d and were thereafter subjected to different treatments by changing the growth medium for MS (control treatment), MS+200 mM mannitol and MS+20 μM ABA. After the medium change the tubes were capped with a rubber cap used to take gas samples and the seedlings remained in the same conditions for 3 d. Ethylene accumulated in this time was measured in a gas chromatograph provided with a flame ionization detector.

Real-time quantitative RT-PCR (QRT-PCR)

The protocols used for RT-PCR were essentially as described by Benitez *et al*. (2005). The primers used for *LapA* amplifications were *LapAF* 5′-ACAGCTTGATTCCGAATTGAAT-3′ and *LapAR* 5′-TGGCAGAGGCAGAGTTAATCTT-3′. The primers used for *GluB* amplifications were *GluBF* 5′-ACGTTGATTGGCAATTCTTATC-3′ and *GluBR* 5′-TTCCTATATTGACGCGATCCAT-3′.

Statistical analysis

Analyses of variance was performed with data from three independent experiments and means from ethylene blocker experiments were compared using Duncan's test at the P=0.05 level. Percentages analysis was performed using previous angular transformation.

Results

Blocking ethylene signalling abolishes the ABA hypersensitivity of tss2

The *tss2* and *tos1* tomato mutants were identified as hypersensitive to NaCl and mannitol, respectively (Borsani *et al*., 2001, 2002). The *tss2* mutant is hypersensitive to Na^+, Li^+, as well as to general osmotic stresses created by mannitol, sorbitol, and choline chloride (Borsani *et al*., 2001). The *tss2* mutant is hypersensitive to ABA (Borsani *et al*., 2001), while the *tos1* mutant exhibits reduced sensitivity to ABA (Borsani *et al*., 2002). Extensive interactions between ABA and ethylene signalling pathways in *Arabidopsis* and tomato have been shown (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000; Le Noble *et al*., 2004). As shown in Fig. 1A, root growth of *tss2* and *tos1* is hypersensitive and hyposensitive, respectively, to all ABA concentrations analysed. It was determined whether blocking ethylene perception could affect the ABA root growth sensitivity exhibited by *tss2* and *tos1*. For this purpose, the ABA responsiveness in wild-type, *tos1*, and *tss2* seedlings was measured in the presence of Ag^+, which blocks the perception of ethylene (Tanimoto *et al*., 1995; Morgan and Drew, 1997). As shown in Fig. 1B, the hypersensitivity of *tss2* to ABA was abolished by adding Ag^+ to the growth medium. In fact, no significant differences in ABA response were found between wild type, *tos1*, and *tss2* when the medium was supplemented with Ag^+.

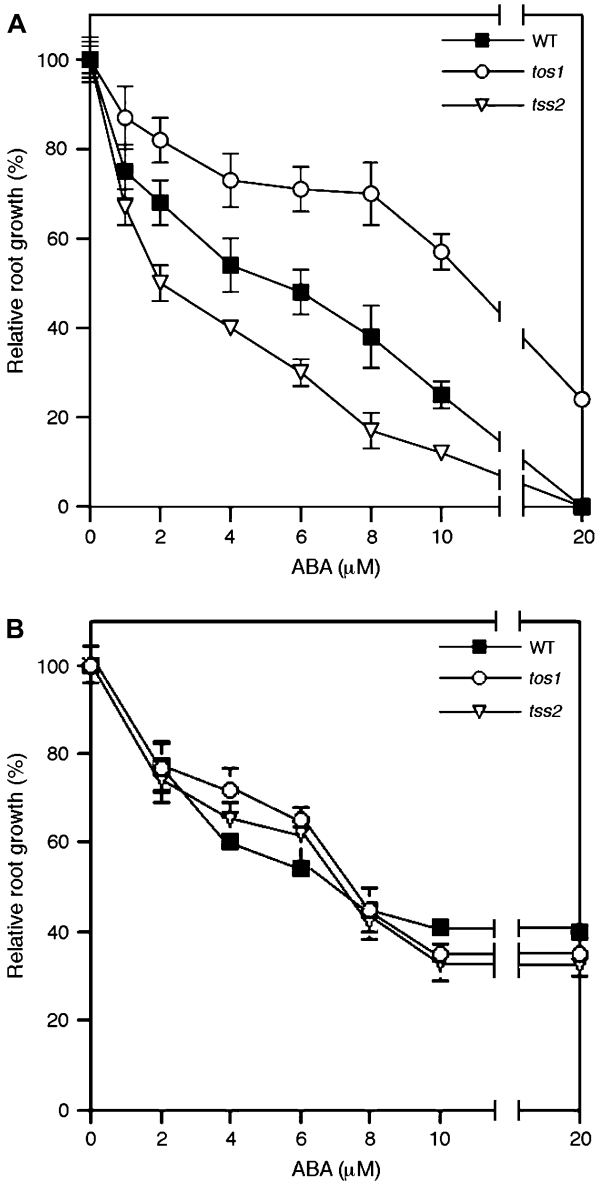

Fig. 1. Blocking ethylene signalling abolishes the ABA hypersensitivity of *tss2* and the ABA hyposensitivity of *tos1*. (A) ABA dose–response for root growth inhibition. Seed of wild type, *tos1*, and *tss2* were germinated and grown for 3 d on MS medium. The resulting seedlings were incubated vertically on MS medium supplemented with the indicated concentrations of ABA, and the root length was scored after 2 d. Root growth of ABA-treated seedlings was expressed as a percentage relative to controls incubated on MS medium. Results are the means of three independent experiments and the bars indicate SD. (B) ABA dose–response for root growth inhibition in the presence of 15 μM Ag^+. Root growth of ABA-treated seedlings was expressed as a percentage relative to controls incubated on MS medium plus Ag^+. Results are the means of three independent experiments and the bars indicate SD.

To confirm that the effect of Ag^+ was specifically due to ethylene perception, the ABA sensitivity of *tss2* and *tos1* was analysed after the application of 1-methylcyclopropene (1-MCP), L-α-(2-aminoethoxyvinyl)-glycine (AVG), and solid $KMnO_4$. 1-MCP is an ethylene antagonist used for blocking ethylene perception (Sisler and Serek, 1999).

AVG is an inhibitor of ACC synthase and has been used for blocking ethylene biosynthesis (Ghassemian *et al.*, 2000). $KMnO_4$ is a potent oxidant that has been used to remove ethylene produced by seedlings from the air (Tieman *et al.*, 2000). As shown in Fig. 2, Ag^+, 1-MCP, and AVG treatments suppressed the growth defect of *tss2* in ABA. Only when the seedlings were grown in the presence of $KMnO_4$ did the *tss2* mutant remain slightly hypersensitive to ABA. However, in this treatment wild-type and *tos1* growth was also reduced compared with that of the other treatments, perhaps because $KMnO_4$ did not adequately oxidize all of the endogenous ethylene produced or the ethylene effect was faster than the capacity of $KMnO_4$ to remove this compound.

Blocking ethylene signalling abolishes the osmotic and NaCl hypersensitivity of tss2 *but has no effect on* tos1

The hypersensitivity of *tss2* to ABA could be overcome by blocking ethylene perception. It was therefore determined whether *tss2* and *tos1* mutants were affected in their normal responses to NaCl and mannitol in the presence of Ag^+. As previously reported, *tss2* but not *tos1* is hypersensitive to NaCl (Fig. 3; Borsani *et al.*, 2002). The *tss2* hypersensitivity to NaCl was rescued by blocking ethylene by the use of Ag^+ in the growth medium. In the presence of mannitol, both *tos1* and *tss2* were hypersensitive (Fig. 3). Blocking ethylene perception by including Ag^+ in the medium abolished the osmotic hypersensitivity of *tss2*. By contrast, *tos1* remained hypersensitive to osmotic stress.

tss2 *but not* tos1 *is hypersensitive to ACC*

Because both the insensitivity of *tos1* and the hypersensitivity of *tss2* to ABA could be overcome by blocking ethylene perception, it was determined whether these mutants were affected in their response to ethylene. As shown in Fig. 4, root growth of the wild type, *tos1*, and *tss2* was measured in the presence of different concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) that is rapidly converted to ethylene (Beaudoin *et al.*, 2000). It was found that the *tss2* root growth did not differ from that of the wild type at 0.5 μM ACC. However, it was hypersensitive when grown on media containing 1, 5 or 10 μM ACC (Fig. 4). This result suggests that *TSS2* is a negative regulator of both ethylene and ABA signalling pathways. While *tos1* growth was diminished relative to wild type on control medium, its growth was similar to that of the wild type when the medium was supplemented with up to 10 μM ACC, resulting in some insensitivity to ACC in terms of relative root growth (Fig. 4).

Studies of several species indicate an important role of endogenous ABA in limiting ethylene production and maintaining primary root growth during periods of low water potential (Sharp, 2002). It was therefore determined whether ethylene content was affected in *tss2*, *tos1*, and the

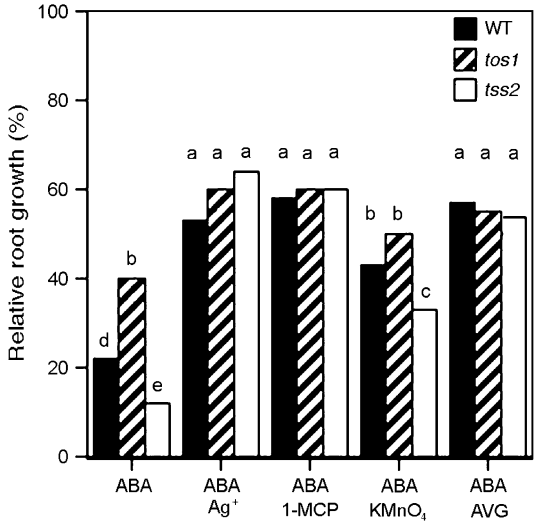

Fig. 2. Effect of blocking ethylene signalling on ABA response for root growth. Effect of Ag^+, 1-MCP, $KMnO_4$, and AVG on ABA inhibition of *tos1* and *tss2* root growth. Seed of wild type, *tos1*, and *tss2* were germinated and grown for 3 d on MS medium. The resulting seedlings were incubated vertically on MS medium containing 20 μM ABA (ABA) or MS medium containing 20 μM ABA and 15 μM Ag^+ (ABA Ag^+), ~1 ppm 1-MCP (ABA 1-MCP), 15 g of solid $KMnO_4$ (ABA $KMnO_4$) and 0.015 μM AVG (ABA AVG). The root length was scored after 2 d. Root growth was expressed as a percentage relative to controls incubated on MS medium or MS medium supplemented with Ag^+, 1-MCP, $KMnO_4$, and AVG. Results are the means of three independent experiments and values followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's multiple range test.

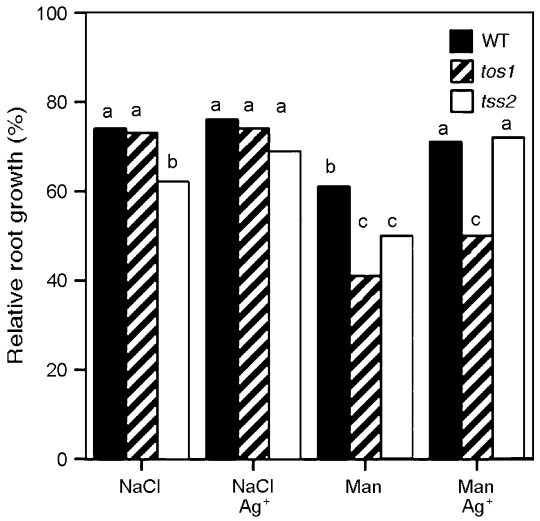

Fig. 3. Effect of blocking ethylene signalling on NaCl and mannitol response. Effect of Ag^+ on NaCl and mannitol inhibition of *tos1* and *tss2* root growth. Root elongation of wild type (WT), *tos1*, and *tss2* seedlings was measured to quantify their sensitivities to 100 mM and 210 mM mannitol inhibition in either the absence or 15 mM Ag^+. Root growth was expressed as a percentage relative to controls incubated on MS medium or MS medium supplemented with Ag^+. The experiment was performed similarly as described in the legend of Fig. 1. Results are the means of three independent experiments and values followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's multiple range test.

corresponding wild-type genotype Moneymaker in control conditions and after exogenous ABA application as well as after osmotic stress. The ethylene production in the ABA-deficient mutant *flacca* and the corresponding control genotype Ailsa Craig was also analysed.

As shown in Table 1, wild type and *tss2* showed similar levels of ethylene production, while the *tos1* and the *flacca* mutant both showed increased levels of ethylene production when growing in control MS medium. This result may explain why *tos1* always exhibits reduced growth in control medium compared with the wild type (Borsani *et al*., 2002). When ABA was added to the growth medium, wild-type seedlings reduced the production of ethylene while *tos1* did not show any difference in the rate of ethylene after ABA treatment, probably due to the insensitivity showed by this mutant to this hormone. Interestingly, *tss2* showed ~2 fold increase in the production of ethylene in contrast to the wild type, which reduced its production of ethylene. This increased ethylene production after exogenous ABA application may explain the hypersensitivity of *tss2* root growth in medium containing ABA. Similar responses were found in Ailsa Craig and the *flacca* mutant, where exogenous ABA reduced the production of ethylene. It has previously been shown that mannitol treatment increases the endogenous ABA content in wild type, *tss2* and *tos1* (Borsani *et al*., 2002). Despite this ABA increase, osmotic stress generated by mannitol produced a small increase in ethylene production in the wild type. This increase in ethylene production was much higher in *tss2* and *tos1* indicating the incapacity of these mutants to regulate ethylene production under osmotic stress.

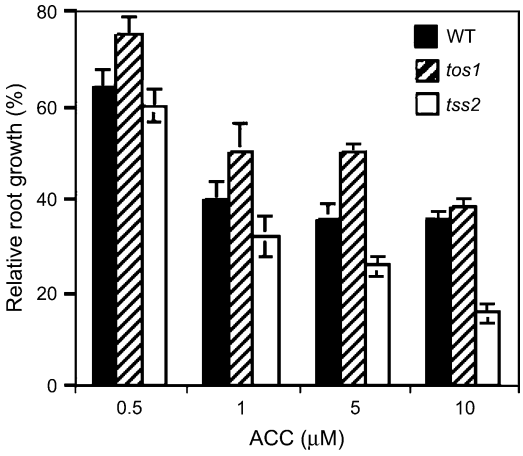

Fig. 4. *tss2* is hypersensitive to ACC. ACC dose–response for root growth inhibition in *tos1* and *tss2*. Seed of wild type, *tos1*, and *tss2* were germinated and grown for 3 d on MS medium. Resulting seedlings were incubated vertically on MS medium supplemented with the indicated concentrations of ACC, and their root length was scored after 2 d. Results are the means of three independent experiments and the bars indicate SD.

Table 1. *Ethylene production in tomato seedlings grown in control medium, medium containing 200 mM mannitol, or medium containing 10 μM ABA*

MoneyMaker and Ailsa Craig are the genetic backgrounds of *tss2* or *tos1* and *flacca* mutants, respectively. Seedlings were grown for 4 d in capped tubes, and the accumulated ethylene was measured as described in the Materials and methods. Values shown are means of three independent experiments ±SD of ethylene produced.

Genotype	Ethylene production (nmol g^{-1} FW d^{-1})		
	Control	ABA (10 μM)	Mannitol (200 mM)
MoneyMaker	17.1±0.2	4.6±0.3	27.2±1.2
tss2	14.1±0.3	31.0±1.4	46.3±1.6
tos1	24.5±1.7	22.2±0.8	61.5±1.8
Ailsa Craig	10.2±0.3	1.2±0.1	21.1±1.2
flacca	21.2±0.7	5.3±0.7	23.1±1.7

Genes regulated by ABA and ethylene in tomato show altered expression in tss2 *and* tos1 *mutants*

Because *TSS2* and *TOS1* are genes involved in both ABA sensitivity and the regulation of ethylene production after exogenous ABA application and mannitol stress, there was interest in determining the role of these loci in regulating gene expression under ABA and after mannitol treatment. For this purpose, the *LapA* and *GluB* genes were selected and their expression patterns were studied in wild-type, *tss2*, and *tos1* seedlings after ABA and mannitol treatment by real-time quantitative RT-PCR (Fig. 5). No difference in the expression was detected under control conditions in either of the genes in all the genotypes analysed (data not shown). The *LapA* gene encodes a leucine aminopeptidase, whose transcripts are up-regulated by ABA, salinity and water deficit (Chao *et al*., 1999). *GluB* encodes a basic β-1,3-glucanase whose transcripts are induced by ethylene (Chao *et al*., 1999; Wu and Bradford, 2003).

Expression of *LapA* was induced by ABA and mannitol stress in the wild-type, *tss2*, and *tos1* seedlings (Fig. 5). However, the induction of *LapA* expression was higher in the *tss2* than in the *tos1* and the wild type, which showed similar induction after mannitol treatment. The expression of *GluB* followed a similar pattern to *LapA* with the exception that the gene was not induced after ABA treatment (Fig. 5). The data suggest that the *TSS2* and the *TOS1* loci play a negative role in the expression of *LapA* and *GluB* after osmotic stress.

Discussion

Cross-talk between ABA and ethylene

There are many reports in the literature of interactions between ethylene and ABA. Antagonistic interactions between ABA and ethylene mediate the rate of grain filling in rice, whereby a high ratio of ABA to ethylene enhances grain-filling rate (Yang *et al*., 2004). Cross-talk studies between ethylene and ABA signal transduction in *Arabidopsis*

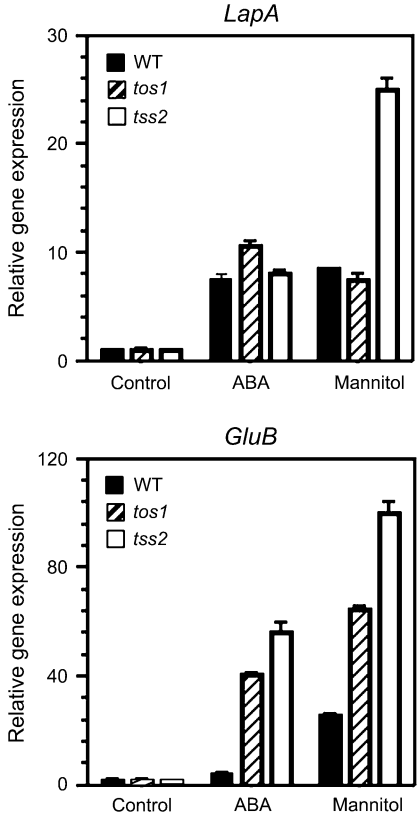

Fig. 5. Expression of stress-regulated genes is altered in *tos1* and *tss2*. Expression of *LapA* and *GluB* by quantitative real-time PCR (QRT-PCR) in wild type, *tos1*, and *tss2* seedlings after exogenous ABA application and mannitol stress. The *y*-axis represents fold differences in gene expression relative to that of the corresponding genotype in control medium. Seed of wild type, *tos1*, and *tss2* were germinated and grown for 7 d on MS medium and then transferred to MS medium (control) or supplemented with 100 μM ABA or 150 mM mannitol for 2 d.

using an ethylene-overproducing mutant and ethylene-insensitive mutants have shown inhibitory effects of ethylene on ABA-induced stomatal closure (Tanaka *et al*., 2005). There are additional genetic analyses showing the existence of substantial interactions between ABA and ethylene signalling cascades in *Arabidopsis* (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000). However, the cross-talk between ABA and ethylene signalling pathways appears to be rather complex and dependent upon the tissue analysed.

Various types of stresses have been reported to promote ethylene production in different tissues in a number of plant species (Narayana *et al*. 1991; Morgan and Drew, 1997). An overproduction of ethylene induced by drought has frequently been related to fruit abortion in cotton (Guinn, 1976) and reduction in grain weight in wheat (Xu *et al*., 1995; Beltrano *et al*., 1999). By contrast, applications of ethylene inhibitors increase grain weight in wheat (Beltrano *et al*., 1994, 1999) and maize (Cheng and Lur, 1996). In addition, it improves dry matter partitioning and grain-filling of basal rice kernels (Mohapatra *et al*., 2000; Naik and Mohapatra, 2000), whereas the application of ethephon, an ethylene-generating growth regulator, produces an opposite effect.

Maintenance of root growth at low water potential is an adaptive trait of osmotic stress tolerance. ABA is likely to play a role in this process by regulating the activity of the putative wall-loosening enzymes such as xyloglucan endotransglycosylase (Wu *et al*., 1994) or by inducing the accumulation of proline (Ober and Sharp, 1994). Other studies demonstrate an important role for endogenous ABA in restricting ethylene production at low water potentials in tomato (Sharp *et al*., 2000; Spollen *et al*., 2000; LeNoble *et al*., 2004). Consistent with this, it was found that ethylene production is enhanced in the ABA-deficient tomato mutant *flacca* (Tal *et al*., 1979; Table 1) and the ABA-hyposensitive tomato mutant *tos1* (Borsani *et al*., 2002; Table 1). This enhanced ethylene production could not be fully restored to normal levels with the application of exogenous ABA and higher ethylene production remained in the *flacca* mutant compared with that of the wild type (Tal *et al*., 1979; this study). However, ABA treatment did not have any effect on ethylene production in the *tos1* mutant, which maintained a high ethylene production constitutively. This role of ABA in controlling ethylene production is supported by further genetic and biochemical data, i.e. a reduction of root elongation at low water potential in the maize ABA-deficient *vp5* mutant and after application of fluridone, a chemical inhibitor of ABA biosynthesis (Spollen *et al*., 2000).

TSS2 *and* TOS1 *loci regulate ethylene production under osmotic stress*

The *tss2* mutation increases root growth sensitivity to both ABA and to the ethylene precursor ACC. Blocking ethylene perception abolished ABA hypersensitivity of *tss2*, suggesting that *TSS2* could be a negative regulator of both signalling pathways. It was proposed that the ethylene signal transduction pathway might have ethylene-independent functions with other hormones (Gamble *et al*., 1998). Therefore, an alternative possibility is that ABA can stimulate the ethylene signal transduction pathway independent of ethylene. A study in *Arabidopsis* has shown that different genes of ACC synthase are up-regulated by ABA treatment (Wang *et al*., 2005). Similar effects of ABA in tomato could explain the increase of ethylene production under ABA and osmotic treatment.

The results suggest that interactions between ABA and ethylene signalling cascades in tomato are synergistic for inhibiting root growth. This conclusion is supported by the fact that *tos1*, an ABA hyposensitive mutant, exhibits some insensitivity to ACC. Therefore, root growth was improved and *tss2* ABA hypersensitivity was abolished whether the ethylene synthesis was reduced by AVG or the ethylene sensitivity was reduced by Ag^{+}. This is in apparent

contradiction to previous results obtained in *Arabidopsis*, where AVG increased the sensitivity of the roots to ABA (Ghassemian *et al*, 2000). However, it is important to note that AVG could produce different effects in tomato and *Arabidopsis*, because tomato roots are far more sensitive to AVG than *Arabidopsis* roots. *Arabidopsis* roots can grow on medium containing 2 μM AVG without apparent inhibition (Ghassemian *et al*., 2000). In these experimental conditions the 2 μM AVG in the medium completely inhibited tomato root growth. Furthermore, a 20-fold reduction in the concentration of AVG in the medium (0.1 μM) was enough to reduce tomato root growth by ~50% (data not shown).

As shown in Fig. 2, blocking ethylene perception or action encourages *tos1* root growth to similar levels as in a medium supplemented with ABA, but only reaches ~60% growth of plants in the control medium, with the remaining 40% of the root growth independent of the ethylene–ABA interaction pathway. This result confirms that ABA inhibition of root growth does not appear to be exclusively mediated by this common ABA–ethylene signal transduction pathway.

The increase of endogenous ABA in *tss2* and wild-type seedlings was similar when grown in MS medium and after mannitol stress (Borsani *et al*., 2002). Therefore, the increase in ethylene production in *tss2* after osmotic stress cannot be explained by a reduced ABA concentration, as occurred in mutants defective in ABA biosynthesis, such as *flacca* (Table 1). In fact, when wild-type plants, such as Moneymaker or Ailsa Craig, or the ABA-deficient *flacca* were treated with exogenous ABA the amount of ethylene production was reduced compared with *tss2* and *tos1*. The increased ethylene production of *tss2* when either ABA or mannitol was applied suggests that *TSS2* is required to restrict ethylene production under osmotic stress through ABA action. The ethylene production in the *tos1* mutant is higher than in the wild type in the control medium, probably due to the insensitivity that this mutant shows to ABA (Borsani *et al*., 2002). This insensitivity explains why exogenous ABA produced similar amounts of ethylene in *tos1* as observed when grown in the control medium. Mannitol treatment, however, increased the ethylene production in *tos1*, indicating that as for *TSS2*, *TOS1* is required for the regulation of ethylene production under osmotic stress.

Altered gene regulation in tss2 *and* tos1

The difference in ABA sensitivity as well as ethylene production of *tss2* compared with the wild type could explain the differences in gene expression for *LapA* and *GluB*. The *LapA* gene is induced by ABA in tomato, but shows little response to ethylene (Chao *et al*., 1999), which explains the absence of difference in induction between *tss2* and the wild type. However, the induction is higher in *tos1* indicating a de-regulation *LapA* expression in this mutant. After mannitol treatment, expression of *LapA* in *tss2* was significantly higher than in the wild type, suggesting that *tss2* is a negative regulator of *LapA* expression under osmotic stress conditions. The expression of *GluB* in *tss2* and *tos1* also showed differences compared with the wild type, which partially reflects the differences in ethylene production of the genotypes.

Use of mutant analysis shows that the network involving ABA, salt, and osmotic signalling is rather complex and includes a multiplicity of intersecting signalling pathways (Ishitani *et al*., 1997; Foster and Chua, 1999; Borsani *et al*., 2005). Mutational analysis is especially suited for making inroads into complex systems such ABA and ethylene because mutations in individual components can reveal their effects on the entire system. It is reported here that both *TSS2* and *TOS1* seem to be involved in ABA responses. However, while all the *tss2* phenotypes can be explained by an uncontrolled ethylene production after osmotic stress, the *tos1* phenotypes are more complex and suggest additional roles for *TOS1* in ABA responses, in addition to ethylene regulation. Initial mapping work in *tos1* showed that the gene mutated is localized in chromosome III (data not shown). Future identification and cloning of *TOS1* and *TSS2* will provide more answers in order to complete the complex puzzle of ethylene–ABA interaction.

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