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Salicylic acid increased aldose reductase activity and sorbitol accumulation in tomato plants under salt stress

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Abstract

Increased aldose reductase (ALR) activities were detected in the leaf tissues of tomato plants grown for 3 weeks in culture medium containing 10^{-7} or 10^{-4} M salicylic acid (SA), and in the roots after the 10^{-4} M SA pretreatment. The ALR activity changed in parallel with the sorbitol content in the leaves of the SA-treated plants. Salt stress elicited by 100 mM NaCl enhanced the accumulation of sorbitol in the leaves of control plants and as compared with the untreated control the sorbitol content in the SA-pretreated leaves remained elevated under salt stress. DEAE cellulose anion-exchange column purification of the protein precipitated with 80 % $(NH_4)_2SO_4$ revealed two enzyme fractions with ALR activity in both the leaf and the root tissues. The fraction of the leaf extract that was not bound to the column reacted with glucose and glucose-6-P as substrates, whereas glucose was not a substrate for the bound fraction or for root isoenzymes. The root enzyme was less sensitive to salt treatment: 50 mM NaCl caused 30 % inhibition in the leaf extract, whereas the enzyme activity of the root extract was not affected. It is suggested that increased ALR activity and sorbitol synthesis in the leaves of SA-treated tomato plants may result in an improved salt stress tolerance.

Additional key words: alterations of ALR activity *in vitro,* enzyme purification, *Lycopersicon esculentum*, NaCl.

Introduction

Polyols are non-reducing, water-soluble sugar alcohols that are generally formed through the reduction of aldoses or their phosphate esters. Mannitol, the most-widely distributed sugar alcohol, has been identified in more than 100 species (Bieleski 1982), and the occurrence of *myo*-inositol and its derivatives is also general in the plant kingdom (Loewus and Loewus 1983). Sorbitol, an important representative of polyols, is a primary photosynthate and a major translocated form of sugars in the phloem of fruit trees in the *Rosaceae* family (Moing *et al*. 1997). Aldose reductase (alditol:NAD(P)$^+$ 1-oxidoreductase, EC 1.1.1.21; ALR) is considered a key enzyme of the polyol pathway leading to the accumulation of sorbitol. ALR belongs to the superfamily of aldo-keto reductases, which catalyse the NADPH-dependent reduction of various aldehydes and reactive carbonyl metabolites. They may reduce a range of substrates, including aldoses (*e.g*. glucose or glucose-6-phosphate), aliphatic (*e.g*. methylglyoxal and 4-hydroxynon-2-enal, 4-HNE) or aromatic aldehydes (*e.g*. benzaldehyde), and they also participate in the detoxification of xenobiotic aldehydes such as acrolein (Kolb *et al.* 1994). Glutathione conjugates of reactive aldehydes (RAs) are also effectively reduced by aldo-keto reductases (Ramana *et al.* 2000).

Sorbitol is synthesized in the species of the *Rosaceae* family by sorbitol-6-phosphate dehydrogenase (S6PHD) (also called aldose-6-phosphate reductase), which converts glucose-6-phosphate to sorbitol-6-phosphate (Loesher 1987). Sorbitol-6-phosphate is then converted to sorbitol by a specific phosphatase (Zhou *et al*. 2003) and sorbitol can also be converted to fructose by NAD$^+$-dependent sorbitol dehydrogenase (SDH) (Oura *et al.* 2000). These reactions were earlier revealed in the source leaves, but not in the sink organs (Loescher 1987).

Little information is available concerning sorbitol biosynthesis and accumulation in other plant species. Sorbitol has been detected in the source leaves of tomato

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Abbreviations: ABA - abscisic acid; ALR - aldose reductase; 4-HNE - 4-hydroxynon-2-enal; RAs - reactive aldehydes; ROS - reactive oxygen species; SA - salicylic acid; S6PHD - sorbitol-6-phosphate dehydrogenase.
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(Schauer *et al.* 2005) and in the green fruits of plants overexpressing *Arabidopsis* hexokinase, *At*HXK1 (Roessner-Tunali *et al.* 2003). NAD^+-dependent SDH gene expression, enzyme activity and the biochemical characterization of the enzyme were recently reported in tomato leaves and roots by Ohta *et al.* (2005).

Polyols are compatible solutes functioning in pressure potential maintenance. The accumulation of mannitol in *Olea europaea* (Conde *et al*. 2007) and that of sorbitol in apple cultivars (Šircelj *et al*. 2007) or in *Malus hupehensis* (Meng *et al*. 2008) has been shown to facilitate osmotic adjustment during salt and drought stress, respectively. Polyols may stabilize macromolecules and increase tolerance to oxidative stress under unfavourable environmental conditions (Kanayama 2009). Transgenic tobacco plants overexpressing a bacterial mannitol-1-phosphate dehydrogenase in the chloroplast, accumulated mannitol, which protected the photosynthetic apparatus from oxidative damage generated by methyl viologen (Shen *et al.* 1997). In contrast, transgenic tobacco plants overexpressing sorbitol-6-phosphate dehydrogenase, and hence containing an exceptionally high amount of sorbitol, exhibited symptoms of oxidative damage, with necrotic lesions on their leaves (Sheveleva *et al.* 1998). Introduction of the whole metabolic apparatus of the sorbitol cycle, however, led to a protective effect without a negative influence upon plant growth and reproduction (Deguchi *et al*. 2006).

In the absence of effective scavenging systems, the other group of aldehydes, RAs, may accumulate in plant tissues under abiotic or biotic stresses. RAs may react with DNA and oxidize membrane lipids and proteins, causing cellular damage, and thereby increasing the harmful effects of environmental stresses. These toxic RAs may be derived from basic metabolic pathways, such as glycolysis (*e.g*. methylglyoxal), or may be products of lipid peroxidation (*e.g*. 4-HNE).

The accumulation of the ALR protein in rice was stress-responsive. This protein accumulated in vegetative tissues of drought-tolerant rice genotypes in response to abscisic acid (ABA), osmotic and salt stress (Sree *et al*. 2000). The ectopic expression of the *MsALR* gene from *Medicago sativa* in tobacco under the control of the CaMV 35S promoter also increased in response to ABA, osmotic stress and H_2O_2 (Oberschall *et al.* 2000), and resulted in a higher tolerance to dehydration stress in transgenic plants. The authors suggested that the enhanced stress tolerance of the transformed plants was not due to an increased synthesis of sorbitol, but could be related to decreased levels of reactive oxygen species (ROS) and RAs, in consequence of the increased expression and activity of MsALR.

Salicylic acid (SA), another derivative of the aromatic biosynthesis in plants, improved the acclimation to different abiotic stressors, including chilling (Szalai *et al.* 2000) and osmotic or salt stress (Tari *et al.* 2002). SA induced H_2O_2 accumulation and oxidative stress and activated the enzymatic or non-enzymatic antioxidative defence systems in various plant species (Szepesi *et al*. 2005, 2009, He and Zhu 2008, Mutlu *et al*. 2009). It has also been reported that SA pretreatment mitigated the salt stress injury of tomato by increasing the activity of glutathione S-transferase, an enzyme catalysing the glutathione conjugate formation of toxic metabolic intermediates. Moreover, SA treatment decreased the accumulation of thiobarbituric acid-reactive compounds such as malondialdehyde (Szepesi *et al.* 2008).

The aim of the present work was to establish whether SA treatment can modulate the activity of ALR and thereby elicit changes in sorbitol accumulation in the leaf and root tissues of tomato plants under salt stress. We characterized the biochemical properties of the enzymes prepared from the leaf and root tissues, and determined the *in vitro* effects of SA and different concentrations of NaCl on the enzyme activity. We were also interested in whether sorbitol accumulation in SA-pretreated plants can alleviate the osmotic stress caused by 100 mM NaCl.

Materials and methods

Plants: Seeds of tomato (*Lycopersicon esculentum* Mill. syn. *Solanum lycopersicum L.*) cv. Rio Fuego plants were germinated and the plants were grown in hydroponic culture as described earlier (Szepesi *et al.* 2009). Prior to being subjected to salinity stress, the plants were pretreated for 3 weeks with 10^{-7} or 10^{-4} M SA. Salt stress was imposed on the plants by adding 100 mM NaCl to the growth medium. SA was present in the culture medium throughout the whole period of the experiments. The samples were prepared from the second, fully-expanded young leaves or from roots, in 3 replicates, 7 - 10 d after the NaCl treatment. The experiments were repeated 3 - 5 times.

Extraction and purification of aldose reductase: The extraction of plant material and partial purification of ALR were carried out by the method of Sree *et al*. (2000). ALR extracts were prepared from fresh tissues, because most of the enzyme activity was lost in the frozen state. One g of plant material was homogenized at 0 ^oC with 4 cm^3 of 0.135 M potassium phosphate buffer (pH 7.4) containing 10 mM β-mercaptoethanol and 0.5 mM phenylmethylsulphonyl fluoride. The extracts were centrifuged at 10 000 *g* for 30 min at 4 ^oC (*Sorvall RC5B* centrifuge, *Ivan Sorvall Inc*., Norwalk, CT, USA). The protein content of the supernatant was determined with the Folin reagent according to Lowry *et al.* (1951), by means of a computing double-beam spectrophotometer (*CE5501*, *Cecil Instruments*, Cambridge, UK), and the extracts were used for the determination of the total extractable ALR activities of the leaf and root tissues. The supernatant was then saturated to 40 % with

$(NH_4)_2SO_4$ for 30 min at 0 °C and, after centrifugation (10 000 *g*, 30 min, 4 °C), to 80 % for 30 min. After a second centrifugation, the precipitated proteins were dissolved in the extraction buffer and dialysed with the same buffer for 3 h at 4 °C. The buffer was changed every hour. The protein extract was also desalted on a *Sephadex G-50* column. The different fractions of ALRs were separated on an 8 cm^3 DEAE cellulose anion-exchange column (*Reanal,* Budapest, Hungary) and eluted with 0.135 M potassium phosphate buffer (pH 7.4) with a linear saline gradient of KCl (0 - 1 M). Fractions of 1.5 cm^3 were collected at a flow rate of 0.4 $cm^3\ min^{-1}$. The protein contents and ALR activities of the fractions were determined.

Determination of ALR activity: The ALR activity was determined in a total volume of 1 cm^3. The reaction mixture contained 0.05 cm^3 of substrate (2 mM 2-naphthaldehyde or 20 mM D,L-glyceraldehyde, *Sigma*, St. Louis, MO), 0.05 cm^3 of 2 mM NADPH (*Reanal*) and 0.025 cm^3 of enzyme extract in 0.875 cm^3 of 0.135 M potassium phosphate buffer (pH 7.4). The enzyme activity was followed by the oxidation of NADPH at 340 nm. In some cases, 2 mM D-glucose or 2 mM D-glucose-6-phosphate was used as substrate. One unit of enzyme activity (U) was defined as the amount of enzyme catalysing the oxidation of 1 nmol of NADPH per min (Mundree *et al.* 2000).

Measurement of sorbitol content: From 0.5 g of plant material frozen in liquid N_2, sorbitol was extracted with 5 cm^3 of 2 mM HEPES buffer (pH 7.5) in 80 % ethanol. Samples were incubated at 80 °C for 1 h, and then centrifuged at 4 °C (15 000 *g*, 30 min). The pellet was extracted twice with the same solution and the combined supernatant was evaporated to dryness. The dry residue was dissolved in distilled water, and the sorbitol content was determined enzymatically with the use of a *Boehringer-Mannheim/R-Biopharm* (Darmstadt, Germany) sorbitol/xylitol kit. D-Sorbitol is oxidized by NAD^+-dependent sorbitol dehydrogenase to D-fructose, and the reduced NADH is consumed in a diaphorase reaction for the reduction of iodonitrotetrazolium. The absorbance of formazan is measured at 492 nm.

Statistics: All data presented are means ± SE. After *ANOVA*, Duncan's multiple comparisons were performed. Differences were considered significant if $P \leq 0.05$.

Results

The crude extracts of the tomato leaves and roots displayed ALR activity (Fig. 1), which could be increased by using a three-step protocol that included $(NH_4)_2SO_4$ precipitation followed by dialysis or desalting on a *Sephadex G-50* column (Table 1). Desalting of the dissolved 80 % $(NH_4)_2SO_4$ precipitate resulted in a further 7.17-fold purification.

In an attempt to purify the protein, the extract was applied to a DEAE cellulose anion-exchange column. In the leaf extracts, two fractions were observed. Most of the enzyme activity was not retained on the column, and was purified 26-fold. The second, smaller peak, which was retained on the column, could be eluted with increasing concentrations of KCl. This fraction exhibited a lower specific activity and was purified 10-fold after elution (Fig. 1*A*). The root extract also contained two main ALR fractions. The protein fraction which was not retained on the column did not exhibit ALR activity. The proteins which were eluted with an increasing linear gradient of 0 - 1 M KCl included an active fraction with two well-separated peaks (Fig. 1*B*). The degree of purification was 8- and 6-fold, respectively. This suggests that the root extracts may contain at least two ALR isoenzymes.

K_m values were determined from the initial velocities by varying the substrate concentrations. It was found that the enzyme extracts from the leaf and root tissues exhibited different affinities for the substrates D,L-glyceraldehyde and 2-naphthaldehyde. The root ALR had a higher affinity than the leaf enzyme for D,L-glyceraldehyde, but a ~6-fold lower affinity for 2-naphthaldehyde (Table 2). The V_{max} values of the reactions did not differ significantly between the root and

Table 1. Partial purification of NADPH-dependent aldose reductase from leaf and root tissues of 8-week-old tomato plants. The enzyme activities were measured with 20 mM D,L-glyceraldehyde as substrate in the leaf extract and with 2 mM 2-naphthaldehyde in the root extract in the presence of 2 mM NADPH.

		Total protein [mg]	Total activity [ΣU mg^{-1}(f.m.)]	Specific activity [U mg^{-1}(protein)]	Purification
Leaf	crude extract	37.21	32.82	0.88	
	40 % $(NH_4)_2SO_4$ precipitate	15.90	28.50	1.79	2.03
	80 % $(NH_4)_2SO_4$ precipitate	12.02	25.88	2.15	2.44
Root	crude extract	2.92	394.20	19.93	
	40 % $(NH_4)_2SO_4$ precipitate	2.44	199.68	18.19	0.91
	80 % $(NH_4)_2SO_4$ precipitate	3.03	147.59	48.71	2.44

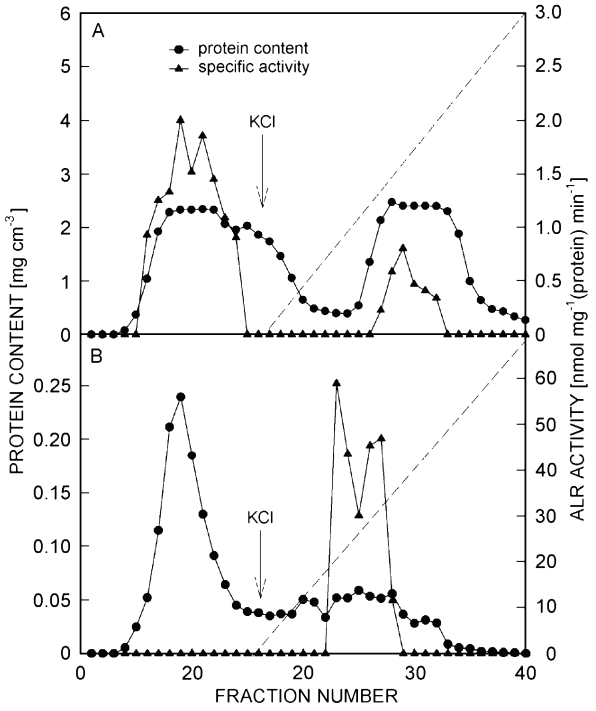

Fig. 1. DEAE cellulose chromatography of aldose reductase extracted from tomato leaf (*A*) and root (*B*) tissues. The protein content of the extracts was precipitated in 80 % $(NH_4)_2SO_4$, and, after desalting, the dissolved fraction was applied to the column equilibrated with 0.135 M K-phosphate buffer (pH 7.4). Proteins were eluted with increasing concentrations of KCl (0 - 1 M). The specific activities of the fractions were determined with 2 mM naphthaldehyde as substrate.

leaf enzymes with the same substrates. It can be concluded that 2-naphthaldehyde is a better artificial substrate for both the leaf and the root ALR.

The first combined fraction of the leaf extract, which was not bound to the column, demonstrated activity with 2 mM glucose-6-phosphate and glucose [specific activities 0.49 and 1.23 nmol mg^{-1}(protein) min^{-1}, respectively], whereas the second fraction, eluted with KCl, accepted only glucose-6-phosphate as substrate [specific activity 0.57 nmol mg^{-1}(protein) min^{-1}]. A high specific activity of the root ALR was observed with 0.2 mM glucose-6-phosphate as substrate [specific activity 13.86 nmol mg^{-1}(protein) min^{-1}]. No enzyme activity with glucose was found in the root tissues.

Table 2. K_m [mM] and V_{max} [nmol mg^{-1}(protein) min^{-1}] values of NADPH-dependent ALR extracted from leaf or root tissues for different substrates.

Substrate	K_m		V_{max}	
	leaf	root	leaf	root
D,L-glyceraldehyde	3.643	1.198	1.756	1.690
2-naphthaldehyde	0.033	0.207	23.923	24.870
NADPH	17.990	1.842	27.250	35.710

Pretreatment with 10^{-7} or 10^{-4} M SA led to significant increases in ALR activity in the leaves and pretreatment with 10^{-4} M SA also did so in the root tissues (Fig. 2). There was a small rise in ALR activity with respect to the basal activity in the leaves of the salt-stressed plants, and the activity remained significantly higher than in the untreated control in the leaves of the SA-pretreated plants under salt stress. A contrasting trend was observed in the root tissues under salt stress. 100 mM NaCl reduced the ALR activity, especially at 10^{-4} M SA. The extractable ALR activity decreased significantly with increasing plant age (unpublished results).

Sorbitol contents of the leaf tissues displayed very similar trends, increasing significantly above the untreated control level after SA pretreatments and in the leaves of the salt-stressed plants. In the root tissues, however, the sorbitol contents were not affected by the treatments (Fig. 2). The content of sorbitol remained below the osmotically relevant levels in both the leaf and the root tissues.

Table 3. *In vitro* effects of increasing NaCl and SA concentrations on the activity of NADPH-dependent ALR partially purified *via* 80 % $(NH_4)_2SO_4$ precipitation from the leaf or root tissues of 8-week-old tomato plants. ALR activity was determined with 2 mM 2-naphthaldehyde as substrate in the presence of 2 mM NADPH at 25 °C and expressed as a percentage of the control. The activity of the leaf ALR was 3.01 ± 0.2 and that of the root enzyme was 169.4 ± 9.0 nmol mg^{-1}(protein) min^{-1}. Means ± SE, n = 3.

NaCl [mM]	ALR [%] root	leaf	SA [mM]	ALR [%] root
0	100	100	0	100
50	106.3 ± 1.4	70.5 ± 4.0	0.5	121 ± 6.6
100	79.3 ± 2.7	49.2 ± 5.7	1.0	172 ± 7.5
200	65.6 ± 4.5	30.9 ± 4.0	1.5	172 ± 1.7
250	49.2 ± 2.3	28.9 ± 5.7	5.0	106 ± 10.5

To gain an insight into the putative regulatory role of cytoplasmic NaCl and SA concentrations on ALR activities, the *in vitro* effects of these compounds on the enzyme activities were determined (Table 3). It was found that NaCl resulted in a considerable inhibition of the activity. The root ALR was much less sensitive to Na^+, because at a concentration of 50 mM, which can occur in the cytoplasm under salt stress (Fricke 2004), the ALR activity was not influenced in the root tissues. However, the same concentration of NaCl resulted in a 30 % decrease of the ALR activity extracted from the leaf tissues. The effects of SA on the ALR activity of the root tissues fitted an optimum curve. Up to 0.5 mM, there were no changes in ALR activity, whereas subsequent SA treatment, up to 1.5 mM, led to activation (~70 %).

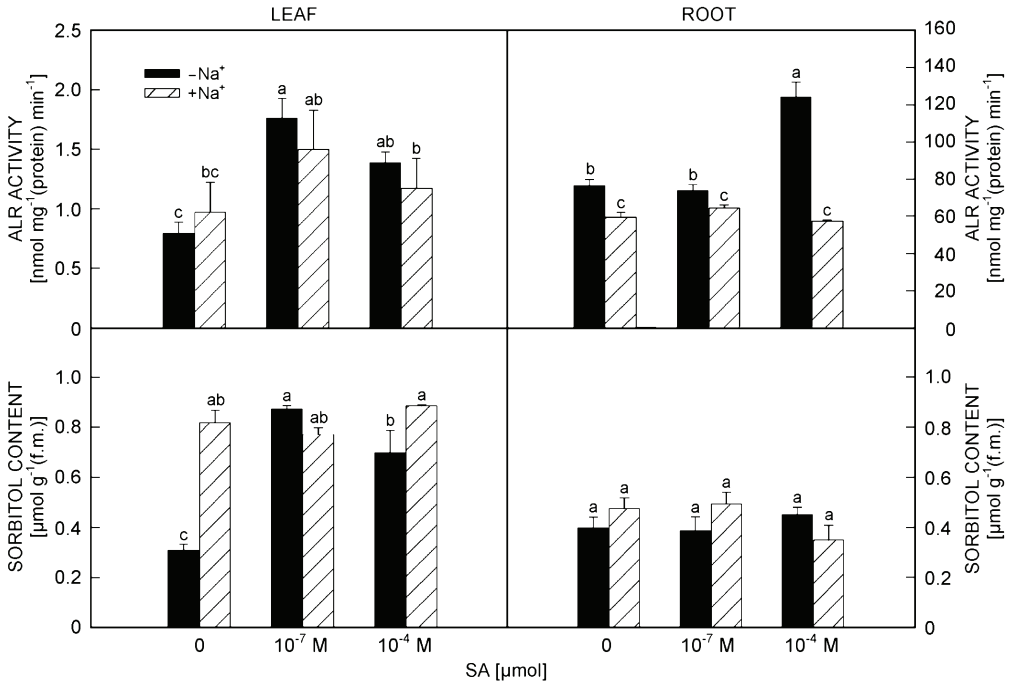

Fig. 2. Activity of aldose reductase (ALR) in the leaf and root tissues with 2 mM 2-naphthaldehyde as substrate, and accumulation of sorbitol in the leaves and roots after 10^{-7} M or 10^{-4} M salicylic acid (SA) pretreatment in 8-week-old tomato plants exposed for 7 d to 100 mM NaCl. Means denoted by different letters are significantly different at $P \leq 0.05$, as determined by Duncan's multiple range test.

Discussion

ALR could be isolated from the leaf and root tissue of untreated tomato plants. In the leaf extract, the unbound protein fraction purified in a single chromatographic step on a DEAE cellulose anion-exchange column exhibited ALR activity with 2-naphthaldehyde and several other substrates, such as D,L-glyceraldehyde, glucose-6-phosphate and glucose. The second fraction, eluted from the gel bed with a linear gradient of KCl, showed activity with the same substrates except glucose. Thus, the leaf extract may catalyse the formation of sorbitol, both from glucose-6-phosphate as NADPH-dependent aldose-6-phosphate reductase and from glucose. If sorbitol accumulates, sorbitol-6-phosphate must be hydrolysed by sorbitol phosphatase.

SA pretreatments increased the activity of ALR in tomato leaves. As compared with the untreated control, NaCl treatment enhanced the activity of the leaf enzyme to a small extent in the crude tissue extract, and the activity remained high in SA-pretreated leaves under salt stress. In the leaf tissues of the SA-treated plants, the sorbitol content changed in parallel with the ALR activity and it increased significantly in the SA-pretreated leaves and after NaCl exposure in the control plants. The sorbitol concentrations also remained above the levels of the control in the SA-pretreated leaves under high salinity. This suggests that tomato leaves can accumulate sorbitol under salt stress, and SA pretreatment caused the accumulation of this compound in the leaves before salt exposure. Similarly, it was found that drought stress increased the sorbitol content (Šircelj *et al.* 2007) and the conversion of glucose to sorbitol in mature apple leaves (Wang *et al.* 1996).

The root extract contained at least two protein fractions with ALR activity, exhibiting lower affinity for 2-naphthaldehyde and higher affinity for D,L-glyceraldehyde, than those of the leaf extract. The roots contained a very low concentration of sorbitol that did not change significantly in response to various treatments. This suggests that the primary function of ALR in tomato root tissues is different from that in leaf tissues and ALR may be involved in other reactions.

The *in vitro* effect of NaCl indicated that the root enzyme was less sensitive to salt treatment, and a physiologically relevant intracellular concentration of NaCl (50 mM) inhibited only the leaf ALR. In contrast with expectations, SA at low concentrations did not inhibit the ALR activity *in vitro*, and higher concentrations activated the enzyme. *In vivo*, however, 10^{-4} M SA increased the ALR activity in the roots. This may be due to a secondary effect of SA through its influence on H_2O_2 accumulation, since the ALR gene expression can be enhanced by H_2O_2 (Oberschall *et al.* 2000, Horváth *et al.* 2007).

In summary, concentration-dependent, SA-induced increases in the *in vivo* ALR activities in both the leaf and the root tissues may contribute to the enhanced salt stress

tolerance of tomato plants. The efficiency of glucose-6-phosphate and glucose as substrates in the enzyme assay of the leaf extract, and the increased content of sorbitol, provide support for the existence of a metabolic pathway leading to the accumulation of sorbitol in the leaves of SA-treated tomato plants. ALR-like activity can also be involved in the detoxification of RAs, inasmuch as lower contents of thiobarbituric acid reactive-substances have been found in the same tissues of tomato (Szepesi *et al.* 2008). Sorbitol, a compatible solute, may play an important role in the osmotic balance of cell compartments. The highest concentrations of sorbitol has been found in the chloroplast stroma of common plantain, sea plantain and peach plants (Nadwodnik and Lohaus 2008), suggesting that sorbitol may contribute to cell structure protection and osmotic adaptation of this compartment. It can be concluded, however, that on a whole cell basis, sorbitol is present in osmotically irrelevant amounts in the tissues of salt-stressed tomato plants.

In the leaves of SA-pretreated tomato, we have measured high fructose contents (Poór *et al.* 2010). Thus, besides the putative antioxidant function, sorbitol synthesis may ensure a balance between hexoses, glucose and fructose, and hence may modify the sugar-induced changes in gene expression under abiotic stress.

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