



RESEARCH PAPER

# Wounding increases salt tolerance in tomato plants: evidence on the participation of calmodulin-like activities in cross-tolerance signalling

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Received 15 November 2005; Accepted 22 March 2006

## Abstract

**Cross-tolerance is the phenomenon by which a plant resistance to a stress results in resistance to another form of stress. It has previously been shown that salt stress causes the accumulation of proteinase inhibitors and the activation of other wound-related genes in tomato plants (*Solanum lycopersicum*). However, very little is known about how different stresses interact with one another, and which are the signalling components that interrelate the responses triggered by different stress types. In the present work, it is shown that mechanical wounding increases salt-stress tolerance in tomato plants through a mechanism that involves the signalling peptide systemin and the synthesis of JA. Data are also provided indicating that calmodulin-like activities are necessary for the downstream signalling events that lead to cross-tolerance between wounding and salt stress. Finally, evidence was gathered supporting the hypothesis that LeCDPK1, a Ca<sup>2+</sup>-dependent protein kinase from tomato previously described in our laboratory, could participate in this cross-tolerance mechanism interrelating the signalling responses to wounding and salt stress.**

Key words: Calmodulin-like activities, cross-tolerance, salt stress, tomato, wounding.

## Introduction

Cross-tolerance is the phenomenon by which a plant resistance to a stress results in resistance to another form

of stress (Genoud and Metraux, 1999). For example, it has been shown that tobacco plants exposed to ozone or UV irradiation exhibit virus resistance (Yalpani *et al.*, 1994). Also, endophyte-infected grasses show enhanced tolerance to abiotic stresses (Malinowski and Belesky, 2000). In tomato plants, it has been shown that salt stress causes the accumulation of proteinase inhibitors and the activation of other wound-related genes (Dombrowski, 2003). However, very little is known about how different stresses interact with one another, and which are the signalling components that interrelate the responses triggered by different stress types.

Plants have developed mechanisms to cope with different stresses. Previous studies have shown that cytosolic Ca<sup>2+</sup> levels increase in plant cells in response to multiple adverse environmental conditions, including salinity, drought, cold, pathogen attack, and mechanical wounding (for reviews see León *et al.*, 2001; Nurnberger and Scheel, 2001; Xiong *et al.*, 2002). Following the increase in intracellular Ca<sup>2+</sup> concentration, signals are likely to be mediated by phosphorylation/dephosphorylation cascades. Ca<sup>2+</sup> activates these cascades by interacting with sensors such as calmodulin (CaM), Ca<sup>2+</sup>-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs) (Luan *et al.*, 2002; Harper *et al.*, 2004; Bouche *et al.*, 2005).

In particular, a number of studies have shown that CDPKs are involved in the response to several environmental stresses, suggesting that these kinases could function as cross-talk mediators between signalling pathways leading to cross tolerance (Cheng *et al.*, 2002; Sanders *et al.*, 2002; Harper *et al.*, 2004). CDPKs are Ser/Thr

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Abbreviations: ABA, abscisic acid; AOS, allene oxide synthase; CaM, calmodulin; CBL, calcineurin B-like protein; CDPK, Ca<sup>2+</sup>-dependent protein kinase; CPZ, chlorpromazine; JA, jasmonic acid; Pin2, proteinase inhibitor 2; PS, prosystemin; RWC, relative water content.

protein kinases with a unique structure that consists of an N-terminal domain of variable length and sequence, a protein kinase catalytic domain, an autoinhibitory junction domain, and a calmodulin-like domain with conserved calcium-binding motifs.

In this work, it was determined that mechanical wounding, together with its signalling molecules systemin and jasmonic acid, enhance tolerance to salt stress in tomato plants and that calmodulin-like activities are required for cross-tolerance between wounding and high salinity. Some evidence was also gathered supporting the hypothesis that LeCDPK1, a CDPK from tomato previously described in this laboratory (Chico *et al.*, 2002), could participate in this cross-tolerance mechanism interrelating the signalling responses to wounding and salt stress.

## Materials and methods

### Plant material

**Soil-grown plants:** Wild-type tomato [*Solanum lycopersicum* L. (syn: *Lycopersicon esculentum* Miller) cv. Castlemart] and transgenic tomato plants overexpressing prosystemin cDNA in the antisense (antisense-PS, McGurl *et al.*, 1992) and sense orientation (PS-overexpressing) were cultivated in a greenhouse under a 16/8 h light/dark 25/20 °C cycle. Detached compound leaves or leaflets with their corresponding petioles were used for the experiments.

**In vitro plants:** Seeds of wild-type tomato plants, were surface-sterilized with a solution of 10% (v/v) commercial bleach (0.525% sodium hypochlorite) for 5 min and washed three times with sterile distilled water. Seeds were germinated on Murashige and Skoog (MS) medium with 0.7% (w/v) agar in glass vessels. Plants were grown for 3 weeks and then transferred to liquid MS medium for 7 d before the corresponding treatments.

### Wounding treatments

Mechanical wounding was performed according to Carrera and Prat (1998) with modifications. For soil- and *in vitro*-grown plants, the main veins of apical leaflets of compound leaves were cut with a dented forceps. For relative water content (RWC) experiments, detached leaflets were wounded in the same manner. Wounding did not cause any visible alterations within the proximities of the cut, such as necrosis or loss of turgor, that could reduce the leaf area available for transpiration and therefore affect the results obtained by RWC measurements and salt tolerance analysis on soil-grown plants.

### Determination of relative water content (RWC)

RWC was determined in detached leaflets with their corresponding petiole of 2–3-month-old tomato plants cultivated in a greenhouse. Leaflets were placed in individual containers with water for 48 h before salt treatment to allow the wound-response components induced by leaflet excision to be restored to basal levels. RWC measurements were determined after 6 h of salt stress (300 mM NaCl), according to the formula:

$$RWC(\%) = 100(FW - DW)(TW - DW)^{-1}.$$

Fresh weight (*FW*) was measured at the end of the salt stress period. Turgor weight (*TW*) was determined by subjecting leaves to rehydration in water for 24 h, after salt treatment. Dry weight (*DW*) was obtained after drying the samples at 75 °C for 24 h.

*RWC*(%) values represent the mean  $\pm$ SE of eight leaflets (obtained at random from different plants of the same age) per treatment, repeated in three independent experiments. The significance of differences was determined according to *t* test where *P* values <0.05 were considered to be significant. Controls without salt treatment (basal RWC values) were carried out in each determination.

### Salt tolerance analysis on soil-grown plants

For salt tolerance assays, 4-week-old tomato plants cultivated in a greenhouse (each one grown in 170 cm<sup>3</sup> of soil), were exposed to NaCl. To minimize experimental variation, plants of each comparison group grown in individual pots were placed on the same tray and 200 ml of 200 mM NaCl solution was added to the tray every 24 h. For wounding/salt stress experiments (Fig. 2B), 24 h prior to NaCl exposure, plants were subjected to mechanical wounding as described. Wounding was performed every 48 h (three times overall). Experiments were repeated three times. Plants from one representative experiment are shown.

### RNA isolation and northern blot hybridization

Total RNA was isolated from leaves and roots of plants subjected to abiotic stress or exposed to different defence signals. Samples (0.1–1 g) were collected and ground in liquid nitrogen, and total RNA was extracted using the TRIzol Reagent (Invitrogen). Total RNA (10–20  $\mu$ g) was separated on 1.2% (w/v) formaldehyde agarose gels and blotted onto nylon membranes (Hybond N<sup>+</sup>, Amersham). Northern blots (Alwine *et al.*, 1977) were hybridized with LeCDPK1, proteinase inhibitor 2 (Pin2), or allene oxide synthase (AOS) probes labelled with prime-a-gene DNA labeling system kit (Promega). After sequential stringent washes at 65 °C, bands were visualized by autoradiography. Equal RNA loading was checked by the EtBr staining of the electrophoresis. LeCDPK1, Pin2, and AOS mRNAs were quantified relative to the RNA loading using Scion Image software.

### Preparations of plant extracts and protein kinase activity assay

Leaves from tomato plants subjected to salt stress were harvested, ground in a mortar cooled with liquid nitrogen, and extracted with 50 mM TRIS–HCl, pH 7.5, containing 2 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 20% (v/v) glycerol, and protease inhibitors (0.5 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 2  $\mu$ g ml<sup>-1</sup> soybean trypsin inhibitor, and 25 units ml<sup>-1</sup> aprotinin). The suspensions (1 ml buffer g<sup>-1</sup> wet tissue) were centrifuged at 20 000 g for 1 h and the resulting supernatant fractions were used for Ca<sup>2+</sup>-dependent protein kinase (CDPK) activity assays.

CDPK activity was determined by using histone H1 as substrate. Fractions were incubated at 30 °C for 5 min with 0.1 mg ml<sup>-1</sup> histone H1 and 10  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (specific activity 500 cpm pmol<sup>-1</sup>) in the presence of either 1 mM EGTA or 1 mM CaCl<sub>2</sub>. The reaction mixture also contained 10 mM TRIS–HCl (pH 7.5), 10 mM MgCl<sub>2</sub> and 10 mM  $\beta$ -mercaptoethanol. Reactions were stopped with the addition of cracking buffer and analyzed on 12% (w/v) SDS–PAGE.

## Results

### Calmodulin-like activities are required for salt stress tolerance and wound-related gene expression

Cross-tolerance between different stresses may implicate the existence of shared components that interrelate the signalling cascades triggered by each type of stress. As a first approach to find mutual components of salt stress and wounding signalling, the effects of the calmodulin

antagonist chlorpromazine (CPZ) on salt stress tolerance and the expression of the wound-related gene proteinase inhibitor 2 (Pin2) were tested.

Salt tolerance was evaluated by measuring water loss during salt stress by relative water content (RWC) determination in detached leaflets preincubated with 0.5–1 mM CPZ. Treatment with CPZ decreased RWC values (Fig. 1A) in a dose-dependent manner when compared with non-treated leaflets. Controls without salt treatment (basal RWC values) are also shown. CPZ did not affect basal RWC values.

The effect of CPZ on wound-induced Pin2 expression was determined in tomato plants preincubated with 0.5 mM CPZ followed by mechanical wounding. Total RNA was isolated and northern blot hybridization with the Pin2 probe was performed. As shown in Fig. 1B, CPZ inhibited wound-induced expression of Pin2.

These results indicate that both wounding and salt-stress responses are mediated by calmodulin-like activities.

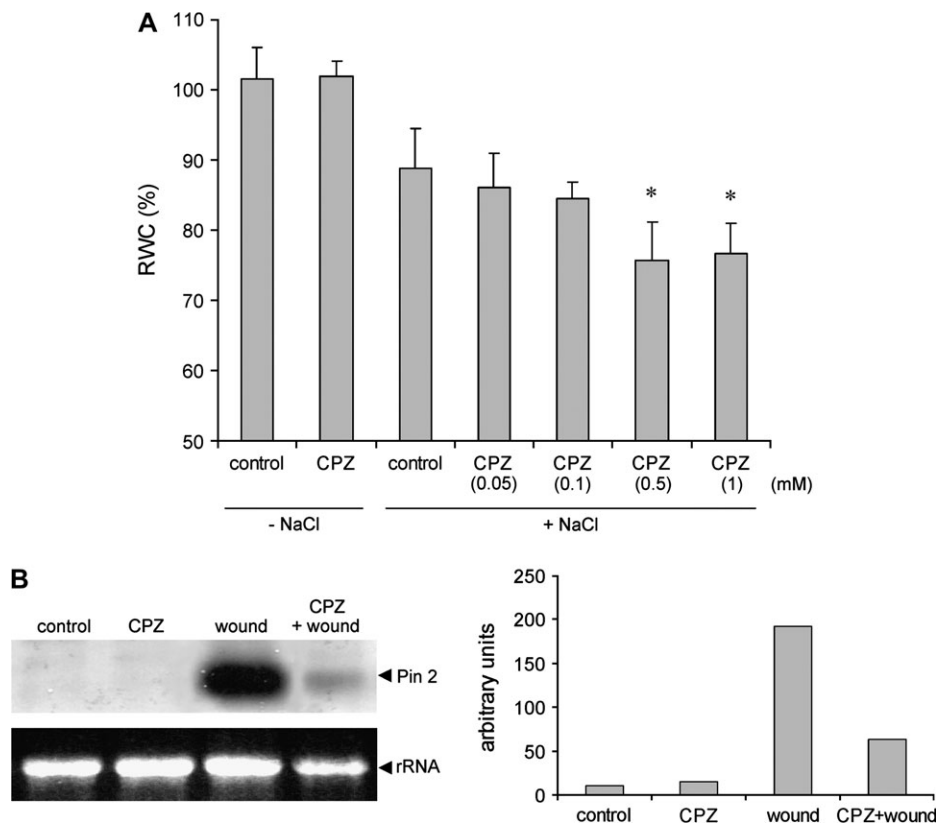
#### Wounding increases salt-stress tolerance

To assess the existence of cross-tolerance between wounding and high salinity, RWC was determined after salt stress

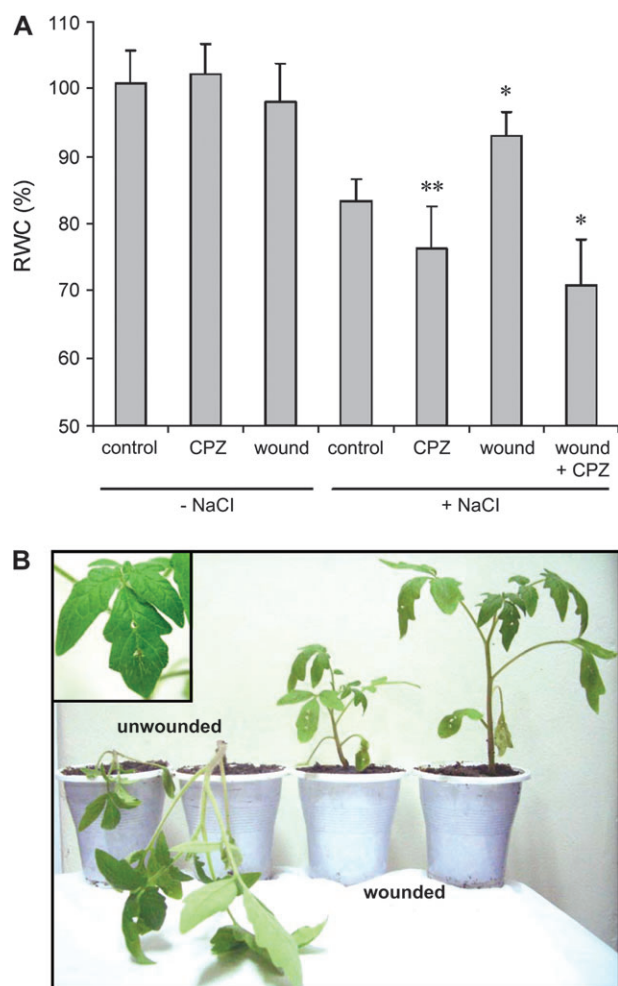
(300 mM NaCl, 6 h) in detached leaflets from wild-type tomato plants, previously subjected to mechanical wounding (3 h prior to salt treatment). As depicted in Fig. 2A, wounding increased RWC values when compared with unwounded leaflets. This effect was blocked by CPZ. Wounding did not affect basal RWC values (without salt treatment). These data indicate that mechanical wounding prevents water loss caused by salinity and that this cross-tolerance mechanism is mediated by calmodulin-like activities. These results were confirmed *in vivo* with soil-grown tomato plants cultivated in a greenhouse. As shown in Fig. 2B, tomato plants wounded with a dented forceps were shown to be more tolerant to salt stress than unwounded plants, validating the data obtained by RWC measurements.

#### Systemin and jasmonic acid increase salt stress tolerance

The 18-aa polypeptide systemin (synthesized as part of a larger 200-aa protein called prosystemin) has been shown to activate the long-distance wound signalling that regulates the expression of defensive genes in tomato leaves in



**Fig. 1.** Calmodulin-like activities are required for salt-stress tolerance and wound-related gene expression. (A) Detached leaflets of wild-type tomato plants were incubated with different doses of CPZ (0.05–1 mM) for 1 h before salt treatment (+NaCl; 300 mM NaCl for 6 h). RWC was determined as described in the Materials and methods. Controls without salt treatment (RWC basal levels) were also carried out (–NaCl). Asterisks show significant differences with a  $P < 0.001$  for CPZ+NaCl versus control+NaCl. (B) *In vitro*-grown tomato plants were incubated in the presence or absence of CPZ 0.5 mM for 2 h before subjecting them to mechanical wounding. Wounding was performed with a dented forceps, cutting the main vein of apical leaflets of compound leaves. 18 h after wounding, leaves were frozen in liquid nitrogen, total RNA was isolated and northern blot hybridization with Pin2 probe was performed (left). Relative expression levels of Pin2 are plotted on the right.



**Fig. 2.** Wounding increases salt stress tolerance. (A) Detached leaflets of wild-type tomato plants were subjected to mechanical wounding (wound) as described in the Materials and methods, 3 h prior to salt treatment. CPZ (0.5 mM) was added 1 h prior to salt treatment (CPZ). RWC was determined as described in the Materials and methods after salt stress (+NaCl; 300 mM NaCl, 6 h). Controls without salt treatment are also shown (–NaCl). \* $P < 0.001$  for wound+NaCl versus control+NaCl and wound+CPZ+NaCl versus wound+NaCl. \*\* $P < 0.05$  for CPZ+NaCl versus control+NaCl. (B) Soil-grown wild-type tomato plants cultivated in a greenhouse were subjected to mechanical wounding as described in the Materials and methods. After 24 h, unwounded and wounded plants were exposed to 200 mM NaCl solution. The photograph was taken after 5 d of NaCl treatment. Upper-left corner: wounded apical leaflet.

response to insect attack or other mechanical wounding (Pearce *et al.*, 1991). In the present work the effect of systemin on salt-stress tolerance was evaluated by determining RWC after salt stress (300 mM NaCl, 6 h) in detached leaflets from tomato wild-type plants (Fig. 3A), preincubated with 30 nM systemin for 2 h. The signalling peptide increased RWC values when compared with non-treated leaflets. Again, this effect was reversed by CPZ. Systemin did not affect basal RWC values. Similar results were obtained when the same experiment was carried out using leaflets from antisense-PS plants (data not shown). These results indicate that systemin mediates the wound-

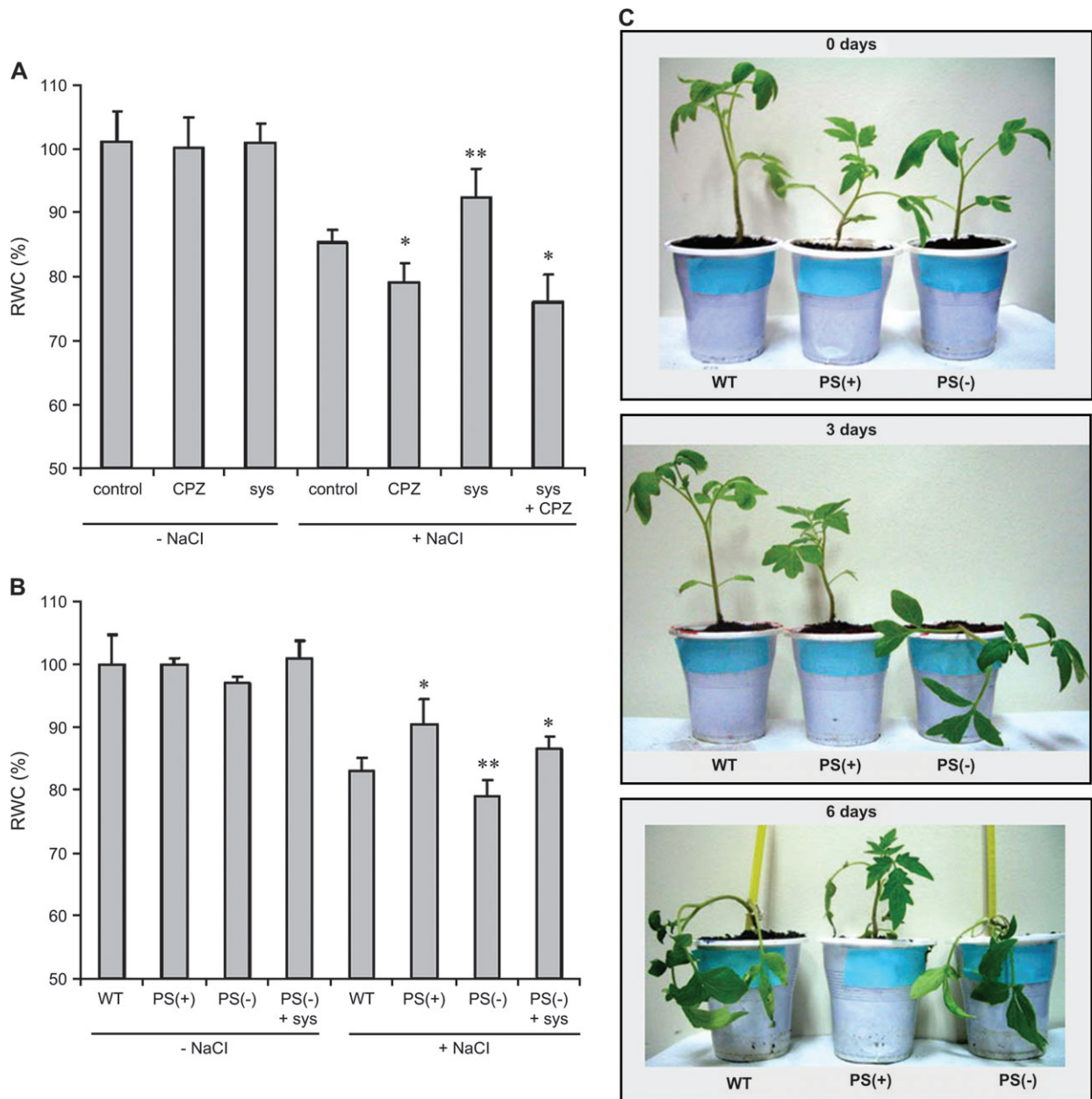
induced salt-stress tolerance through a mechanism involving calmodulin-like activities. In accordance with this concept, antisense-PS leaflets were more sensitive to high salinity than leaflets from wild-type plants, while PS-overexpressing leaflets were more tolerant than leaflets from wild-type plants (Fig. 3B). Pretreatment of antisense-PS leaflets with 30 nM systemin yielded RWC values between wild-type and PS-overexpressing scores. Similar results were obtained with soil-grown tomato plants cultivated in a greenhouse (Fig. 3C). Antisense-PS plants were shown to be more sensitive to high salinity than wild-type plants, while PS-overexpressing plants were more tolerant. Once again, *in vivo* results validated the data obtained by RWC measurements.

Jasmonic acid (JA) is synthesized from linolenic acid via the octadecanoid pathway in response to wounding, systemin or other signals generated by insect and pathogen attacks (Doares *et al.*, 1995). JA is part of the signal transduction pathway that mediates the induction of defensive genes in plants. To determine the participation of JA in wound-induced salt tolerance, RWC was measured after salt stress (300 mM NaCl, 6 h) in detached leaflets from wild-type plants, preincubated with 50  $\mu$ M JA for 1.5 h. As shown in Fig. 4, JA increased RWC values and this effect was reversed by CPZ. JA did not affect basal RWC values.

The data so far indicate that wounding increases salt tolerance in tomato plants through the generation of systemin and the synthesis of JA, both of which, in turn, activate an as yet unknown downstream signalling cascade that involves calmodulin-like activities.

#### Salt stress induces wound-related signalling genes

The induction of wound-related signalling genes by salt stress was also studied. Detached compound tomato leaves were subjected to salt stress and northern blot hybridization with prosystemin (PS) and allene oxide synthase (AOS) probes was performed. AOS is an enzyme of the octadecanoid pathway involved in the biosynthesis of JA. As shown in Fig. 5, both genes were up-regulated by salt stress. These results are similar to those found by Dombrowski (2003), who reported that salt stress induces the signalling genes cathepsin D inhibitor, PS, and lipoxygenase in tomato plants and increases protein accumulation and transcript levels of Pin2, a serine proteinase inhibitor synthesized in response to mechanical wounding or injury by chewing insects (Plunkett *et al.*, 1982). Pin2 reduces the digestibility and nutritional quality of the leaves to help defend the plant against insect predators (Johnson *et al.*, 1989; Orozco-Cardenas *et al.*, 1993). This cross-tolerance mechanism not only involves the induction of salt tolerance by wounding, but also the induction of wound-related genes by salt stress, with the possible increase in protection against mechanical wounding by herbivory.



**Fig. 3.** Systemin increases salt-stress tolerance. (A) Detached leaflets of wild-type tomato plants were treated with systemin (sys) 2 h prior to salt treatment. CPZ (0.5 mM) was added 1 h prior to salt treatment (CPZ). RWC was determined as described in the Materials and methods after salt stress (+NaCl; 300 mM NaCl, 6 h). Controls without salt treatment are also shown (-NaCl). \* $P < 0.001$  for CPZ+NaCl versus control+NaCl and sys+CPZ+NaCl versus sys+NaCl; \*\* $P < 0.01$  for sys+NaCl versus control+NaCl. (B) Detached leaflets of wild-type (WT), PS-overexpressing [PS(+)] or antisense-PS [PS(-)] tomato plants were subjected to salt stress (+NaCl, 300 mM NaCl for 6 h) followed by RWC determination as described in the Materials and methods. In addition, PS(-) leaflets were treated with systemin (sys) 2 h prior to salt stress. Controls without salt treatment are shown (-NaCl). \* $P < 0.001$  for PS(+) versus WT+NaCl and PS(-)+sys versus PS(-)+NaCl; \*\* $P < 0.01$  for PS(-) versus WT+NaCl. (C) Soil-grown wild-type (WT), PS-overexpressing [PS(+)] or antisense-PS [PS(-)] tomato plants cultivated in a greenhouse were exposed to 200 mM NaCl solution. Photographs were taken before (0 d) and after 3 d and 6 d of NaCl treatment.

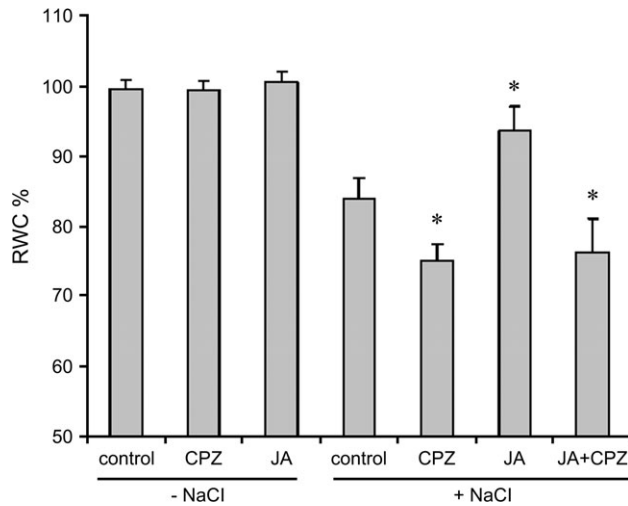
#### Wound-related signalling molecules induce *LeCDPK1* expression

Once it has been established that wound-induced salt tolerance is mediated by systemin and JA through the activation of pathways involving calmodulin-like activities, evidence was gathered supporting the hypothesis that

*LeCDPK1* could participate in this cross-tolerance mechanism interrelating the signalling responses to wounding and salt stress.

CPZ is a calmodulin antagonist that can also inhibit the calmodulin-like domain characteristic of CDPKs. At the same doses used in our experiments, CPZ has been shown

to block LeCDPK1 activity *in vitro* completely (Chico *et al.*, 2002). As has been previously shown that this kinase is induced by mechanical wounding, the effects of the wound-related signalling molecules systemin and JA on LeCDPK1 expression were studied here.



**Fig. 4.** JA increases salt-stress tolerance. Detached leaflets of wild-type tomato plants were treated with jasmonic acid (JA) 1.5 h prior to salt treatment. CPZ (0.5 mM) was added 1 h prior to salt treatment (CPZ). RWC was determined as described in the Materials and methods after salt stress (+NaCl; 300 mM NaCl, 6 h). Controls without salt treatment are also shown (-NaCl). \* $P < 0.001$  for CPZ+NaCl versus control+NaCl, JA+NaCl versus control+NaCl and JA+CPZ+NaCl versus JA+NaCl.

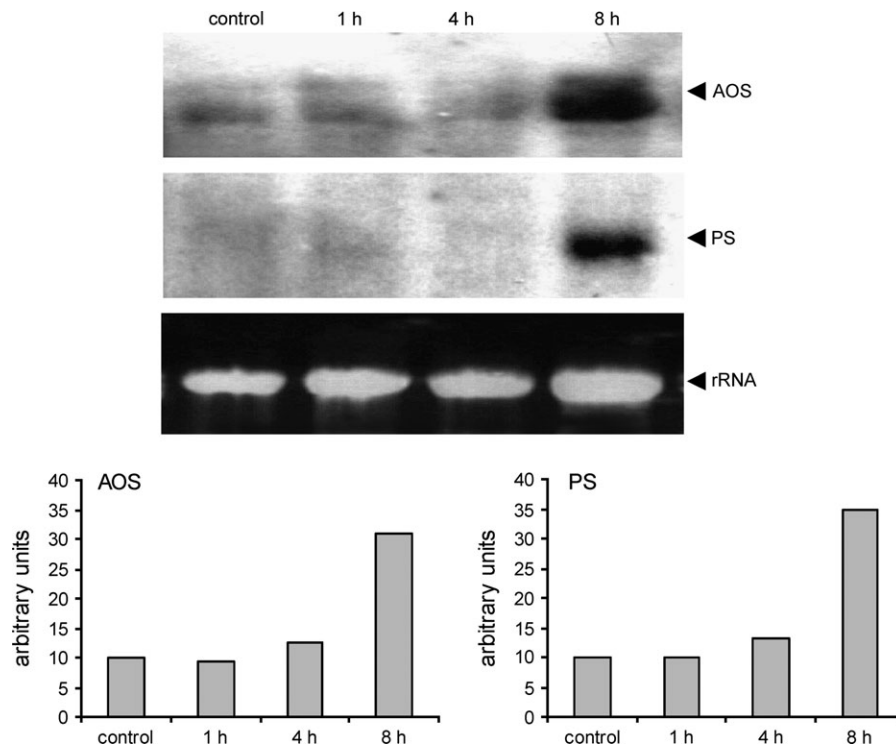
Detached compound tomato leaves were treated with 30 nM systemin for different time periods and northern blot hybridization with the LeCDPK1 probe was performed. Systemin induced LeCDPK1 expression with a maximal effect after 4 h (Fig. 6A). Similarly, treatment of *in vitro*-grown tomato plants with 50  $\mu$ M JA also induced the kinase expression (Fig. 6B), but the maximal effect was observed after 1 h, rather than after 4 h as seen with the systemin treatment.

According to these observations it is probable that LeCDPK1 is induced after wounding by the JA generated via the octadecanoid pathway triggered by systemin.

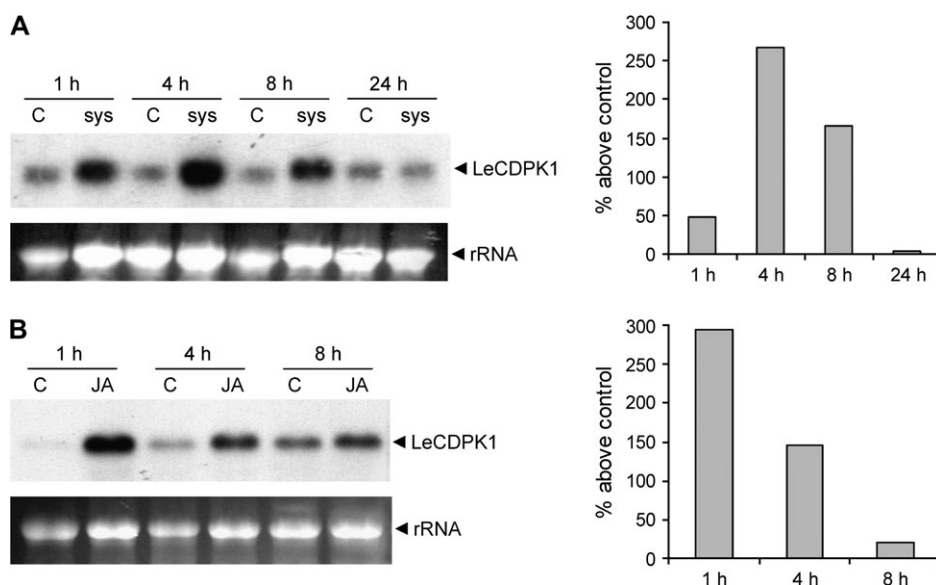
Induction of LeCDPK1 by wounding and its regulatory molecules correlates with the onset of wound-induced salt-stress tolerance. Systemin enhanced LeCDPK1 expression within 1–4 h and a similar pattern of induction was described previously in response to mechanical wounding (Chico *et al.*, 2002), while JA increased LeCDPK1 mRNA levels within 1 h. Salt-stress tolerance was increased after wounding the leaflets 3 h prior to salt addition (Fig. 2A), and after pretreatment with systemin (Fig. 3A) and JA (Fig. 4) for 2 h and 1.5 h, respectively.

#### Salt stress and abscisic acid (ABA) induce LeCDPK1

To gain further insight into the participation of LeCDPK1 in the cross-tolerance between wounding and salt stress, the expression of LeCDPK1 under high salinity was studied.



**Fig. 5.** Salt stress induces wound-related signalling genes. Detached compound leaves of wild-type tomato plants were placed in containers with water 48 h before subjecting them to salt stress (500 mM NaCl) for different time-periods. Total RNA was isolated and northern blot hybridization with prosystemin (PS) and allene oxide synthase (AOS) probes was performed. Relative expression levels of AOS and Pin2 are plotted at the bottom.



**Fig. 6.** Wound-related signalling molecules induce LeCDPK1 expression. (A) Detached compound leaves of wild-type tomato plants were placed in containers with water 48 h before systemin (30 nM, sys) treatment. After different time-periods (1–24 h), total RNA was isolated and northern blot hybridization with LeCDPK1 probe was performed. (B) *In vitro*-grown wild-type tomato plants were treated with 50  $\mu$ M jasmonic acid (JA) for different times (1–8 h). Total RNA was isolated and northern blot hybridization with LeCDPK1 probe was performed. Relative expression levels of LeCDPK1 expressed as % above controls are plotted on the right (A, B). C: control.

To determine the kinetics for salt-induced activation of the LeCDPK1 gene, detached tomato compound leaves were exposed to high salinity during different time periods (0.5–8 h). LeCDPK1 expression was determined by northern blot analysis. As observed in Fig. 7A, the maximal induction occurred after 8 h of salt treatment. Next, tomato plants grown *in vitro* were treated with different doses of NaCl (100–500 mM) for 8 h. NaCl induced LeCDPK1 expression in leaves and roots (Fig. 7B). NaCl (200 mM) was sufficient to increase LeCDPK1 mRNA levels in leaves but not in roots, where higher concentrations of salt were required. This result may be consistent with the fact that roots are more tolerant to high salinity than the aerial parts of the plants (Flowers *et al.*, 1977). Accordingly,  $Ca^{2+}$ -dependent protein kinase (CDPK) activity, determined using histone H1 as a phosphate acceptor, increased in parallel to LeCDPK1 mRNA induction in leaves (Fig. 7C), although other CDPKs might contribute to the measured CDPK activity. These results suggest that LeCDPK1 participates in the signalling pathway leading to a salt-stress response.

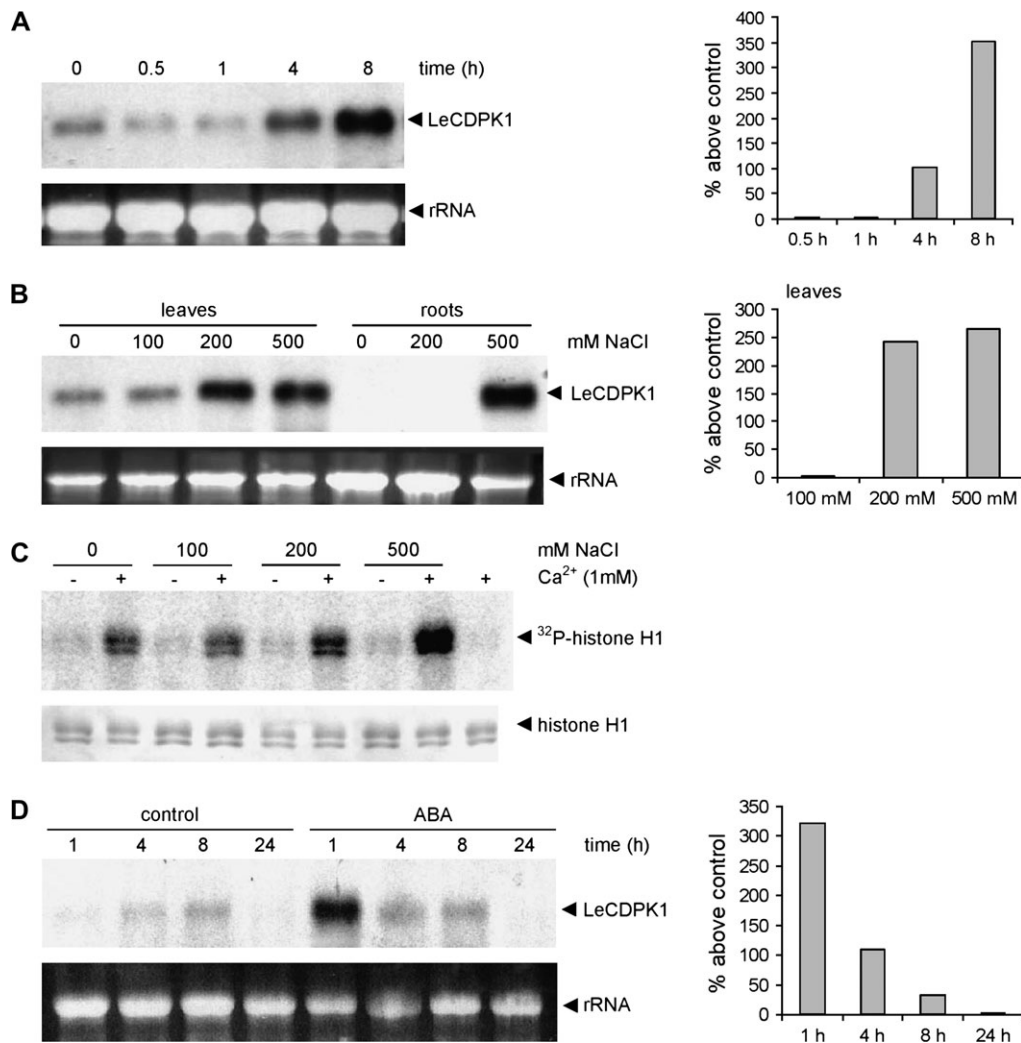
ABA regulates several aspects of plant development including seed development and desiccation tolerance of seeds, and plays a crucial role in the plant response to abiotic stresses such as salinity, drought, and cold (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). Since LeCDPK1 is induced by salt stress, the effect of ABA on the expression of this gene was examined (Fig. 7D). Expression of LeCDPK1 was induced in *in vitro*-grown tomato plants treated with 100  $\mu$ M ABA, which produced a maximal effect after 1 h. This observation confirms the concept that LeCDPK1 could participate in the salt-stress response.

## Discussion

The present work provides new data about cross-tolerance mechanisms between different stress types in plants. This study demonstrates for the first time that wounding increases salt-stress tolerance in tomato plants through a mechanism that involves the signalling peptide systemin and the subsequent synthesis of JA (Figs 2–4). Evidence is also provided indicating that calmodulin-like activities are necessary for the downstream signalling events that lead to cross-tolerance. A diagram representing the mechanisms described in this report is depicted in Fig. 8.

It has previously been shown that LeCDPK1 is induced by mechanical wounding (Chico *et al.*, 2002). In this report, new information was obtained about the signalling pathways in which LeCDPK1 may participate and about its regulation by wound and salt-stress signalling molecules. It was determined that wounding increases LeCDPK1 levels through the generation of JA acid triggered by systemin (Fig. 6). On the other hand, it was demonstrated that LeCDPK1 is induced by high salinity and ABA (Fig. 7).

Considering that wounding, systemin and JA increase salt-stress tolerance in parallel with the induction of LeCDPK1, and that high salinity induces wound-related signalling genes (Fig. 5) concomitantly with LeCDPK1, it is hypothesized that this kinase may function as a cross-talk node between both signalling pathways, activating downstream components that modulate cellular responses leading to cross-tolerance. However, the participation of other tomato CDPKs isoforms or other proteins with

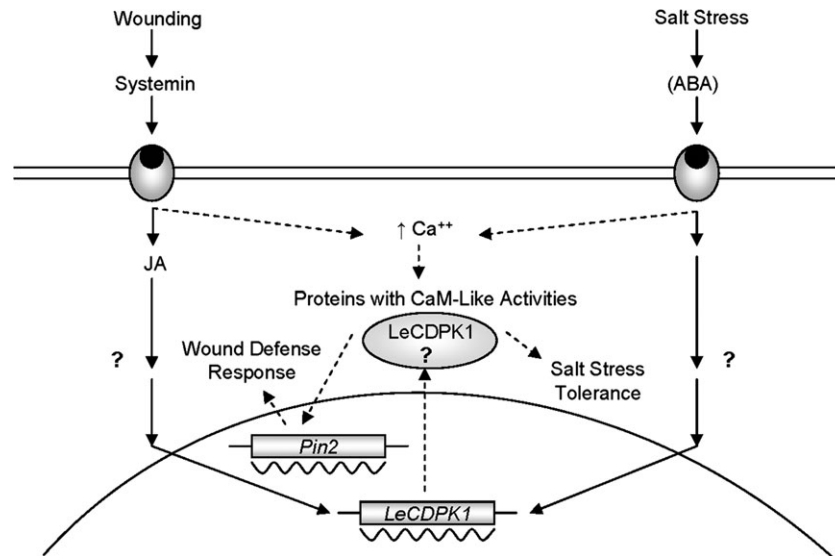


**Fig. 7.** Salt stress and ABA induce LeCDPK1. (A) Detached compound leaves of wild-type tomato plants were treated with 500 mM NaCl for different time periods. Total RNA was isolated and northern blot analysis for LeCDPK1 was performed. (B) *In vitro*-grown wild-type tomato plants were treated with different doses of NaCl for 8 h. Total RNA from leaves and roots was isolated and northern blot analysis for LeCDPK1 was performed. Relative expression levels of LeCDPK1 expressed as % above controls are plotted on the right (A, B). (C) *In vitro*-grown wild-type tomato plants were treated with different doses of NaCl for 8 h. Protein extracts were obtained from leaves and CDPK activity was determined using histone H1 as substrate. (D) *In vitro*-grown wild-type tomato plants were treated with 100  $\mu$ M abscisic acid for different times. Total RNA was isolated and northern blot analysis for LeCDPK1 was performed. Relative expression levels of LeCDPK1 expressed as % above controls are plotted on the right.

calmodulin-like domains cannot be excluded as mediators of cross-tolerance between mechanical wounding and salt stress in tomato plants. To this regard, calcineurin B-like proteins (CBLs) are calcium sensors that specifically target a family of protein kinases called CIPKs (CBL-interacting protein kinases) (Kim *et al.*, 2003). In particular, a member of the CBL family, CBL1, is induced by salt stress and wounding in *Arabidopsis* (Cheong *et al.*, 2003). Therefore, it can not be discarded that the effect of CPZ on the increase of salt-stress tolerance by wounding shown in this report is due, at least in part, to the activity of a putative tomato orthologue of CBL1. Genetic approaches such as silencing of the LeCDPK1 gene would yield the results needed to confirm the function of this kinase in the cross-tolerance mechanism.

In the field, plants are subjected to wounding by herbivores. Artificial damage caused by crushing the leaf with a forceps is not always a suitable model of biotic stress inflicted by herbivores (Karban and Myers, 1989), mainly due to the differential responses triggered by chemical compounds of insect saliva (Alborn *et al.*, 1997). However, artificial injury in tomato plants has been widely used to mimic the wounding component of herbivore attack since the study carried out by Green and Ryan (1972). It is well known that the wound response addressed in the present work, that is generation of systemin with the consequent release JA and the induction Pin2 expression, occurs after herbivorous insect attack as well as after artificial mechanical wounding (O'Donnell *et al.*, 1996; Ryan, 2000). Moreover, the LeCDPK1 gene was found to be induced in





**Fig. 8.** A simplified diagram representing the mechanisms described in this work. In tomato plants, wounding induces the production of systemin, which initiates several events including the synthesis of JA (Ryan, 2000). JA induces the expression of *LeCDPK1* in addition to other defence-related genes. On the other hand, salt stress, probably through ABA, also induces the *LeCDPK1* gene. High cytosolic calcium concentrations induced by systemin and salt stress (Ryan, 2000; Zhu *et al.*, 2002) activate proteins with calmodulin-like activities, possibly *LeCDPK1*, which are necessary for both the development of salt-stress tolerance and the expression of *Pin2*, an important component of the wound defence response.

tomato plants exposed to feeding by *Spodoptera eridania* (DA Capiati *et al.*, unpublished data). According to these observations, although the chemical compounds of insect saliva are absent in the wounding treatments performed in this study, the artificial damage inflicted with a forceps seems to be an appropriate representation of the mechanical injury caused by chewing insects or larger herbivores.

Similarly, excised-leaf RWC assays do not necessarily yield the same response as an intact plant would. Nevertheless, the results of RWC assays indicating that mechanical wounding prevents water loss caused by salinity and that systemin increase salt-stress tolerance were confirmed *in vivo* with soil-grown tomato plants cultivated in a greenhouse, therefore validating the data obtained by excised-leaf RWC measurements.

Cross-tolerance between two different stresses could be generated by signalling cross-talk by means of shared components that interrelate the signalling cascades triggered by each type of stress; therefore, any one of those stresses activates the response that leads to resistance to the other stress. Alternatively, the generation of cross-tolerance could be at the response level, that is, when two stresses require similar protective mechanisms, then the response to one will automatically generate tolerance to the second. The data from this paper tend to support the signalling cross-talk idea, providing evidence on the participation of calmodulin-like activities (possibly *LeCDPK1*) as signalling molecules that interconnect the responses triggered by wounding and salt stress. Nevertheless, the possibility can not be excluded that the product of some wound-related genes, such as the hydrophilic protein prosystemin,

could function as osmoprotectants increasing salt tolerance (McGurl *et al.*, 1992).

The implications of this kind of cross-tolerance for plant performance in the field remain unclear. The activation of the pathways that lead to salt tolerance when the plant is subjected to wounding by herbivores represents a high cost of energy and resources, therefore, it is likely that this mechanism represents an advantage for the survival of the plant in the field. Even though it is also possible that wound-mediated activation of the salt-stress tolerance response could simply be a physiological constraint due to the high functional redundancy in stress-signalling pathways.

## Acknowledgements

We thank Dr Clarence A Ryan (Washington State University, Pullman, WA, USA) for his generous gift of the systemin and the transgenic tomato plants (PS-overexpressing and antisense-PS) and Dr Arjen ten Have (Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Buenos Aires, Argentina) for providing the tomato seeds. Financial source: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica, Universidad de Buenos Aires.

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