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The Effect of $CaCl_2$ and NaCl Salt Acclimation in Stress Tolerance and its Potential Role in ABA and Scion/Rootstock-Mediated Salt Stress Responses

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ABSTRACT

Both NaCl and $CaCl_2$ salt acclimation pre-treatment responses were examined in contrasting salt stress resistant potato genotypes, ABA-, ABA+, 9506, 'Norland' and reciprocally grafted sensitive and resistant potato genotypes. Plants were grown in a hydroponic nutrient sand culture under greenhouse conditions and were subjected to: (a) no pre-treatment; (b) NaCl pre-treatment; (c) $CaCl_2$ pre-treatment; and (d) combined $CaCl_2$ + NaCl pre-treatment and stressed with higher levels of NaCl salt for two weeks. Pre-treatment with NaCl-based acclimation generally enhanced salt stress resistance to a greater extent than $CaCl_2$. This was associated with a specific Na^+ ion effect rather than a non-specific EC-dependent response. That the ABA(-) genotype was unable to exclude Na^+ from the shoot relative to the ABA(+) and other genotypes. That the $CaCl_2$ pre-treatment also enhanced shoot K^+ except in the ABA(-) genotype suggests that both ABA and $CaCl_2$ are requirements for this mode of salt stress defence. The salt resistant rootstock positively influenced water content in the salt stressed sensitive scions as reflected through stomatal conductivity, osmotic potential, shoot growth and water content of the scions. Grafting salt stress resistant scions onto the ABA(-) rootstock increased in root growth. Grafting of salt- resistant scions under salt acclimation treatments may be an alternative approach to increasing stress resistance in commercially important potato cultivars. While the $CaCl_2$ acclimation pre-treatment was not as effective as NaCl pre-treatment, the importance of calcium in stress acclimation cannot be excluded, particularly since low levels of Ca^{2+} existed in the background nutrient and NaCl salt stress solutions. Calcium and ABA appear to be involved in increasing the ratio of K^+/ Na^+ in the shoots, associated with elevated salt stress resistance.

Keywords: ABA, calcium, electrical conductivity, graft, mutant, NaCl, potato, salt acclimation

Abbreviations: **ABA**, abscisic acid; **EC**, electrical conductivity; **ABA(-)**, ABA-deficient mutant; **ABA(+)**, ABA normal sibling; **9506**, resistant line; **LD_{50}**, lethal dose for 50% of plants; **Ψs**, osmotic potential

INTRODUCTION

The potato (*Solanum tuberosum*) is a key part of the global sustainable food system – producing more food energy on less land than corn, wheat or rice (*Oryza sativa*; www.cipotato.org). More than half of global potato production is in developing countries, rendering it an important source of food and income to millions of farmers. However, potato is moderately susceptible to salinity, with both growth and yield being adversely affected when soil electrical conductivity (EC) exceeds 2.16 to 3.38 dS m^{-1} (Katerji *et al*. 2003). Salinization has the potential to negatively impact potato production in irrigated soils, with the most deleterious effects occurring in arid and semi-arid regions where high evapotranspiration rates are coupled with increasing demands on limited supplies of high quality water sources (Rengasamy *et al*. 2006). NaCl, the most detrimental salt in saline soils, has been shown to negatively impact growth of both cultivated and wild species of potato (Bilski *et al*. 1988a, 1988b; Li *et al*. 2006), and its impact is anticipated to increase as a function of global climate change (Hijmans 2001).

The process whereby pre-exposure to lower levels of stress improves the ability of a plant to adapt to various environmental stresses is generally known as acclimation (Conroy *et al*. 1988; Guy 1990). Salt acclimation is characterized by the ability to subsequently grow at salt concentrations which otherwise would be lethal to non-acclimated plants (Amzallag *et al*. 1990b). For acclimation to occur, the cell, tissues, organ and organism must be competent to undergo the change (Amzallag and Lerner 1995). Pre-exposure to low levels of NaCl prior to NaCl salt stress increased subsequent salinity resistance in maize (*Zea mays* L.) seedlings (Rodríguez *et al*. 1997), soybean (*Glycine max*) (Umezawa *et al*. 2000), cowpea (*Vigna unguiculata*) (Silveira *et al*. 2001), rice (*Oryza sativa*) (Hassanein 2000; Djanaguiraman *et al*. 2006) and potato (Etehadnia *et al*. 2008).

There is evidence that ABA is involved in salt acclimation (Amzallag *et al*. 1990a; Noaman *et al*. 2002). NaCl pre-treatment increases ABA levels in bush bean (*Phaseolus vulgaris*) resulting in lower ion concentrations in the treated plants (Montero *et al*. 1997). In *Phaseolus vulgaris*, a Na^+ excluder, ABA mediated responses to Na^+ toxicity and the signaling of salt-induced water deficit (Sibole *et al*. 1998, 2000). Exogenous ABA resulted in an increase in salt stress resistance and dry weight accumulation in corn (*Zea mays* L.) seedlings under different salinity levels (Zhao *et al*. 1995). These changes were the result of enhanced osmotic adjustment activity, exclusion of Na^+ from the shoot and accumulation in the root. The role of ABA in coordinating growth of roots and shoots of plants and regulating tolerance responses to a number of stresses including water and salt has been reported (Sharpe and LeNoble 2002).

Whereas ABA is widely demonstrated to be involved in many physiological responses, De Silva *et al*. (1985) proposed that calcium was the secondary messenger in ABA-mediated signalling of stomatal closure. Calcium is considered to be a ubiquitous secondary messenger in plant signalling (McAinsh *et al*. 1997). The requirement of Ca^{2+} for plants has long been known to increase as NaCl soil salinity

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increases (Gerard 1971). Calcium appears to elevate plant salt stress tolerance through several mechanisms including stabilization of cell membranes and cell walls (see Palta 1996 for a review) as well as re-adjustment of carbon metabolism and Na^+ regulation (Bolat *et al.* 2006). Calcium elevates plant salt stress tolerance through mechanisms involving both re-adjustment of carbon metabolism and Na^+ regulation (Bolat *et al.* 2006). The calcium binding protein, calreticulin, was shown to be associated with salt stress tolerance and its expression in the scion was regulated by the rootstocks (Shaterian *et al.* 2005a).

Grafting is an integrative reciprocal process that contributes in transferring desirable properties in the scion/rootstock communication, allowing the examination of both scion and rootstock influence on salt tolerance (Pardo *et al.* 1998; Holbrook *et al.* 2002). Our previous studies on diploid potato indicate that rootstocks play an important role in salt stress resistance not only through regulation of calreticulin expression (Shaterian *et al.* 2005a), but also through Na^+ exclusion from the shoots and Na^+ accumulation in the roots (Shaterian *et al.* 2005b). We further demonstrated that the response of potato plants to salt stress was influenced by both the scion and the rootstock, and that this response was modulated by NaCl salt acclimation and ABA (Etehadnia *et al.* 2008). However, an examination of the effect of $CaCl_2$ salt acclimation in stress tolerance and its potential role in ABA and scion/rootstock-mediated salt stress responses has not been performed.

Our specific objectives were to: (a) examine the effects of $CaCl_2$ salt acclimation in stress tolerance by comparing potato plant responses between $CaCl_2$, NaCl and combined NaCl + $CaCl_2$ salt acclimation pre-treatments; (b) determine if salt acclimation pre-treatment responses were related to a specific ion effect or a non-specific EC effect; (c) examine the effect of $CaCl_2$ salt acclimation in ABA and scion/rootstock-mediated salt stress responses.

MATERIALS AND METHODS

Four potato lines of varying salt tolerance (Shaterian *et al.* 2005b) were used in this study; the diploid clones: 9506-resistant (a salt resistant line); 9120-05 (ABA(-), an ABA-deficient mutant, a salt sensitive line), and 9120-18 (ABA(+), an ABA-normal sibling of 9120-05, a moderately salt sensitive line). These lines were kindly provided by Dr. H. De Jong of Agriculture and Agri Food Canada, Fredericton. A tetraploid commercial cultivar, 'Norland' (moderately salt resistant), was used as a check. Plants were propagated vegetatively under greenhouse conditions by stem tip cuttings in Ottawa sand (1-2 mm diameter, 75.5% very coarse sand; 0.5-1 mm, 24.4% course sand; and < 0.5 mm, < 0.1%) under a mist system.

After three weeks, rooted plants were transferred to 1 L standard plastic pots containing the same Ottawa sand medium. Ottawa sand has minimal ion-binding capacity, which reduces interference by ion absorption in salinity or fertility trials. The pots were placed into trays (20 × 40 × 60 cm) which were automatically irrigated three times a day with fertilized water containing 2 g L-1 20-20-20 N-P-K including micronutrients (Plant Products Co. Ltd., 314 Orinda Road, Brampton, Ontario, LGT-1J1). The EC and pH of the nutrient solution were checked weekly. The EC and pH of the 0-180 mM NaCl treatment solutions ranged between 1.22-22.16 dS m^{-1} and 6.52-6.96, respectively. Six pots per tray with a single plant per pot of salt-resistant ('Norland', 9506) or relatively salt-sensitive [(ABA(-), ABA(+)] genotypes were placed in each tray. The entire pot was flooded from the bottom with nutrient solution for 5 min and drained over a 2-min period. Plants were grown under natural light supplemented with 500-600 $\mu Ms^{-1}m^{-2}$ light intensity, 14 h photoperiod, and 25/20°C day/night temperature. To prevent tangling of stems, all plants were tied to bamboo stakes during the experiment.

Physiological responses to salt stress and acclimation abilities of the four potato genotypes were evaluated in a preliminary experiment. Based on observed relative physiological response and LD50, different levels of salt stress were selected for the salt-sensitive and salt-resistant genotypes. The relatively salt stress sensitive ABA(-) and its ABA(+) sibling were acclimated by increasing the concentration of NaCl in the nutrient solution by adding 16.66 mM per week, leading to a final concentration of 50 mM in 3 weeks. The more salt stress resistant 'Norland' and 9506 genotypes were treated in increments of 25 mM NaCl per week, reaching a final concentration of 75 mM in 3 weeks.

Accordingly, pre-treatments applied in this study were: 0 mM $CaCl_2$ and 0 NaCl; 20 mM $CaCl_2$ for one week followed by three weeks of NaCl salt acclimation; 20 mM $CaCl_2$ applied for four weeks; and 20 mM $CaCl_2$ for one week followed by three weeks of NaCl salt acclimation combined with $CaCl_2$ (20 mM). The salt pre-treatments were applied three times per day starting on the first day after transferring rooted plants to the hydroponic sand-based system. The EC and pH of the solutions were monitored weekly. The EC ranged from 1.22 for 0 mM NaCl to 13.44 dS m^{-1} for 100 mM NaCl + 20 mM $CaCl_2$, whereas the pH ranged between 7.6 for 0 mM NaCl, to 6.63 for 100 mM NaCl + 20 mM $CaCl_2$.

After exposure to the pre-treatments, two weeks of salinity stress were applied. According to preliminary trials, LD50 was attained at 150 mM NaCl for the ABA(-) and its ABA(+) sibling while LD50 was reached at 180 mM NaCl for the more stress resistant 'Norland' and 9506 genotypes. The salt stress treatments were applied three times per day via the irrigation system.

A range of phenotypical and physiological traits were measured after two weeks of salt stress. Leaf necrosis using a rating scale of the lowest non-wilted leaf and the youngest expanded fourth or fifth leaves was scored from 1-5: 1- (0% leaf area necrosis), 2- (1-25% leaf area necrosis), 3- (26-50% leaf area necrosis), 4- (51-75% leaf area necrosis), 5- (76-100% leaf area necrosis) (Shaterian *et al.* 2005b).

Leaf water content (fourth and fifth leaves from apex) was also measured after the two weeks of salt stress. Leaves were sampled between 9:00 a.m. and 12:00 p.m. Water content was determined by measuring leaf fresh weight (FW), then drying in a hot air oven (DW) for 48 hrs at 75°C and was calculated by the formula: (FW-DW)/DW. Plant height (stem collar to shoot tip) was monitored on a weekly basis over two weeks of salt stress to calculate growth rate:

Growth rate = height at T_2 - height at T_1/T_2 - T_1. Where, T_1 = before stress and T_2 = after stress.

Osmotic potential (OP) was measured at the end of the salt stress treatment. Sampling was done between 10:00 a.m. and 12:00 p.m. on the fourth and fifth fully expanded leaves. The tissue samples (50 gr) were collected in Eppendorf test tubes and kept frozen at $-20^{\circ C}$ until analysis. Samples were thawed and homogenized in the tubes at room temperature. The sap was then loaded into a Wescor vapour pressure osmometer (Wescor-5000, U.S.A.) chamber for analysis. The Ψs was calculated by the formula: $\Psi s = - CiRT$.

Stomatal conductance was also recorded after two weeks of salt stress. Using the abaxial leaf surface of the fourth and fifth fully expanded leaves stomatal conductivity was assessed between 10:00 a.m. and 11:00 a.m. in morning with a Steady State Porometer (Li-Cor Inc. Li 1600, Lincoln, Nebraska, 68504. U.S.A.). At the termination of the project, shoot and root fresh and dry weight and shoot and root water content were measured as described above. The roots were washed with tap water to remove any sand or residual salt and rinsed with distilled water for ion analysis. Total Ca^{2+}, Na^+ and K^+ concentrations in the shoots and roots were measured at the termination of the trial using atomic absorption spectrophotometry (Perkin Elmer-3100, Waltham, Mass., 02451, U.S.A.) according to Thomas *et al.* (1967), and expressed on a dry weight basis.

A randomized complete block design in a factorial (4 pre-treatments × 4 genotypes) experiment with 4 replicates was utilized with 3 plants per replicate. Treatments were compared in a 2 way ANOVA and the interaction table is presented. Means were compared using the least significant difference (LSD) test at P=0.05. All data were analyzed by GLM program of SAS (SAS Institute, 2002-2003, Canada).

Grafting experiment

A reciprocal grafting experiment was performed using the ABA(-), ABA(+), and the 9506 genotypes. Shoot tip cuttings of the three genotypes were prepared and after five weeks, the rooted cuttings were transferred to 400-mL pots filled with Ottawa sand. The ABA(-) and its ABA(+) sibling were reciprocally grafted onto the 9506 resistant rootstock [ABA(-)/9506], [ABA(+)/9506] and [9506/ABA(-)], [9506/ABA(+)]. The graft unions were covered by a paraffin-embedded plastic film (Parafilm, American National Can Menasha) to avoid desiccation. The grafted plants were transferred to a mist chamber where they were grown for two weeks. During this time, the plants were irrigated three times daily with water containing 1.27 g L-l 20-20-20 N-P-K plus micro-nutrients (Plant Products Co. Ltd., 314 Orinda Road, Brampton, Ontario, Canada). To provide enough humidity for healing of the graft unions, the grafted plants were covered by clear plastic bags for the first week after grafting. All plants were sprayed with fungicide three times per week to prevent foliar diseases. After a week, the plastic bags were removed and the plants were transferred to the greenhouse and acclimatized to the new conditions. To maintain only one grafted scion, any sprouts growing from the nodes of the rootstock were removed. Plants were grown in the sand and hydroponic system as previously described.

Sodium chloride and calcium chloride pre-treatments

Treatments were applied to the grafted plants to determine if there is a difference between NaCl and $CaCl_2$ acclimation pre-treatments on scion/rootstock responses. Based on the salt tolerance of the rootstock, appropriate levels of NaCl were applied as previously described. To study the effect of NaCl and $CaCl_2$ pre-treatments, different concentrations of NaCl and $CaCl_2$ at the same EC levels were utilized. In summary, the salt pre-treatments applied in this experiment consisted of:

1) a) 50 mM NaCl (EC = 5.0) or b) 75 mM NaCl (EC = 7.39):
a) 16.66 mM NaCl per week reaching 50 mM in 3 weeks applied to grafts on ABA(-), ABA(+) rootstocks
b) 25 mM NaCl per week reaching 75 mM in 3 weeks applied to the 9506-resistant rootstocks

2) a) 27 mM $CaCl_2$ (EC = 5.0) or b) 37 mM $CaCl_2$ (EC = 7.39)
a) 9 mM $CaCl_2$ per week, reaching 27 mM in 3 weeks applied to the ABA(-), ABA(+) rootstocks
b) 37 mM $CaCl_2$ (12.33 mM per week, reaching 37 mM in 3 weeks) treated on 9506-resistant rootstocks

Leaf necrosis, leaf water content, leaf osmotic potential, leaf stomatal conductance, shoot and root dry weight, and shoot and root water content were evaluated as previously described at the end of two weeks of salt stress. Degree of leaf greenness (which is an approximate estimate of chlorophyll content) was also measured (on the same leaf as necrosis) by a SPAD Meter (Model Minolta-502).

Based on the relative salt tolerance of genotypes and rootstocks, two weeks of salinity stress were initiated after three weeks of salt pre-treatment. The salt stress consisted of 150 mM NaCl for the ABA(-) and ABA(+) genotypes/rootstocks and 180 mM NaCl for the 9506-resistant genotype/rootstock. The experiment was conducted as a randomized complete block design in a factorial experiment (7 graft combinations × 2 pre-treatments) with five replicates and one plant per replicate. Treatments were compared in a 2-way ANOVA and the interaction table is presented. Means were compared using the least significant difference (LSD) test at P=0.05. All data were analyzed using the GLM program of SAS (SAS Institute, 2002-2003).

RESULTS AND DISCUSSION

Leaf necrosis

In the absence of salt pre-treatments, both the ABA(-) and the ABA(+) genotypes expressed the same degree of leaf necrosis after exposure to salt stress (**Fig. 1**). Therefore, ABA does not appear to be directly involved in alleviating the symptoms of leaf necrosis in these genotypes. The responses to both $CaCl_2$ and NaCl salt pre-treatments were also similar for these two genotypes. However, the pre-treatments were more effective at reducing leaf necrosis when applied to the ABA(+) genotype compared to the ABA(-) and thus ABA may be involved in salt acclimation. A similar result was found with another ABA-deficient mutant,

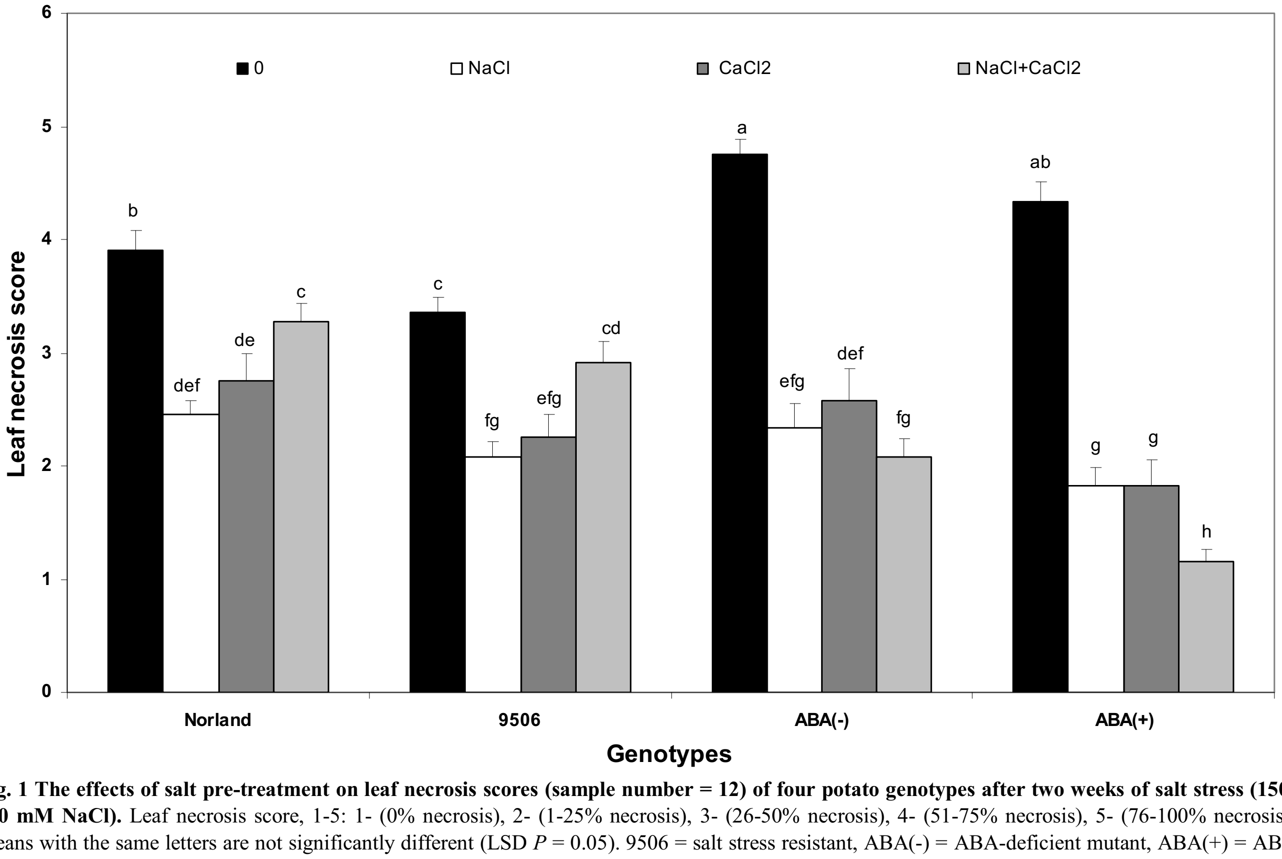

Fig. 1 The effects of salt pre-treatment on leaf necrosis scores (sample number = 12) of four potato genotypes after two weeks of salt stress (150-180 mM NaCl). Leaf necrosis score, 1-5: 1- (0% necrosis), 2- (1-25% necrosis), 3- (26-50% necrosis), 4- (51-75% necrosis), 5- (76-100% necrosis). Means with the same letters are not significantly different (LSD *P* = 0.05). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 1 The effects of salt pre-treatments on shoot fresh and dry weight and shoot water content of four potato genotypes after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	'Norland'	9506	ABA(-)	ABA(+)	Mean
Shoot fresh weight (g)					
0	110.86 de	120.34 d	35.83 i	81.59 fg	87.15
NaCl	238.43 b	261.97 a	56.54 h	174.46 c	182.85
$CaCl_2$	84.60 fg	128.10 d	40.51 hi	98.27 ef	87.87
NaCl + $CaCl_2$	79.26 g	81.66 fg	46.04 hi	125.88 d	83.21
Mean	128.28	148.01	44.73	120.05	
Shoot dry weight (g)					
0	28.72 de	41.61 b	15.99 gh	21.39 fg	26.92
NaCl	34.42 c	51.15 a	10.87 hi	26.79 def	30.80
$CaCl_2$	26.29 ef	32.08 cd	10.20 i	17.62 g	21.54
NaCl + $CaCl_2$	20.53 g	21.45 fg	9.68 i	20.44 g	18.02
Mean	27.49	36.57	11.68	21.56	
Shoot water content (g H2O/g dry weight)					
0	2.88 bc	1.92 ab	1.40a	2.84abc	2.26
NaCl	5.95f	4.14cde	4.96def	5.54ef	5.14
$CaCl_2$	2.25ab	5.66f	2.76 abc	4.64 def	3.82
NaCl + $CaCl_2$	2.93 bc	3.74cd	5.42 ef	5.34ef	4.35
Mean	3.5	3.86	3.63	4.59	

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = Salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

tomato (*Lycopersicon esculentum*) (*sitiens*), in which the percentage of leaf injury was greater than its wild type at moderate salinity (Mäkelä *et al.* 2003).

NaCl and $CaCl_2$ pre-treatment effects

Leaf stomatal conductivity was reduced in the more salt resistant 9506 and 'Norland' genotypes under all pre-treatments (**Table 1**). However, none of the pre-treatments affected stomatal conductivity in either of the most salt sensitive ABA(-) and ABA(+) genotypes. There was no differential stomatal conductivity response between the $CaCl_2$ and NaCl pre-treatments except in the 9505 genotype in which stomatal conductivity increased under $CaCl_2$. Leaf osmotic potential generally became less negative under both $CaCl_2$ and NaCl pre-treatments when measured after 2 weeks of salt stress, except for the 9506 genotype in which there was no effect. The relative difference in osmotic potential response between $CaCl_2$ and NaCl pre-treatments was genotype-dependent in which $CaCl_2$ increased (more negative) osmotic potential in 'Norland' while osmotic potential became less negative in ABA(-) compared to the NaCl salt pre-treatment. Leaf water content generally exhibited no differential response to any of the pre-treatments in any genotypes except in the most sensitive ABA(-) genotype in which the combined NaCl + $CaCl_2$ pre-treatment induced a significantly higher water content compared to the stressed plants without any pre-treatment.

A more consistent distinction between $CaCl_2$ and NaCl pre-treatments was observed in shoot and root responses (**Tables 2, 3**). By the end of the salt stress event, shoot fresh and dry weights were most elevated under the NaCl pre-treatment compared to the $CaCl_2$ salt pre-treatment in all genotypes except the ABA(-) genotype in which none of the pre-treatments induced a differential response. Shoot water content, root fresh and dry weights were consistently elevated under the NaCl pre-treatment but results were less consistent under the $CaCl_2$ pre-treatment. The observed enhancement of shoot water content appears to be associated with stem water since leaf water content did not significantly increase under the NaCl pre-treatment, even though leaf osmotic potential generally became less negative under this pre-treatment (**Table 1**). Saxena *et al.* (1989), who compared different salts including NaCl, $CaCl_2$, Na_2SO_4, and Na_2CO_3 on the germination and growth stages of alfalfa (*Medicago sativa*), also found NaCl to be the most effective in inducing salt tolerance. Root water content showed relatively little response to either $CaCl_2$ or NaCl pre-treatments.

Table 2 The effects of salt pre-treatments on root fresh and dry weights and root water content of four potato genotypes after two weeks salt stress (150-180 mM NaCl).

Pre-treatments	'Norland'	9506	ABA(-)	ABA(+)	Mean
Root fresh weight (g)					
0	2.01 fghi	2.55 def	1.55 i	1.93 ghi	2.01
NaCl	3.28 bc	3.57 b	2.46 efg	3.54 b	3.21
CaCl2	1.88 ghi	3.05 bcd	1.97 fghi	2.79 cde	2.42
NaCl + $CaCl_2$	1.79 hi	2.91 fgh	1.98 fghi	4.78 a	2.86
Mean	2.24	3.02	1.99	3.26	
Root dry weight (g)					
0	0.63 de	0.89 cd	0.45 e	0.57 de	0.63
NaCl	1.25 ab	1.47 a	0.72 d	1.25 ab	1.17
$CaCl_2$	0.56 de	1.24 ab	0.64 de	1.10 bc	0.88
NaCl + $CaCl_2$	0.57 de	0.76 cde	0.64 de	1.46 a	0.85
Mean	0.75	1.09	0.61	1.09	
Root water content (g H2O/g dry weight)					
0	2.21 cd	1.42 a	2.33 cde	2.02 bcd	1.99
NaCl	1.64 ab	1.86 abc	2.43 de	2.80 e	2.18
$CaCl_2$	2.37 de	1.51 a	2.03 bcd	1.86 abc	1.94
NaCl + $CaCl_2$	2.11 bcd	2.09 bcd	2.12 cd	3.63 f	2.48
Mean	2.08	1.72	2.22	2.57	

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = Salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 3 The effects of salt pre-treatments on leaf stomatal conductivity, leaf osmotic potential, and leaf water content of four potato genotypes after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	'Norland'	9506	ABA(-)	ABA(+)	Mean
Leaf stomatal conductivity (cm s^{-1})					
0	0.048 b	0.074 a	0.014 fg	0.016 fg	0.038
NaCl	0.036 c	0.029 cd	0.011 g	0.017 fg	0.023
$CaCl_2$	0.036 c	0.047 b	0.011 g	0.020 ef	0.028
NaCl + $CaCl_2$	0.027 de	0.025 de	0.010 g	0.017 fg	0.019
Mean					
Leaf osmotic potential (s = Mpa)					
0	-1.94 efgh	-1.31 a	-2.42 i	-2.04 gh	-1.92
NaCl	-1.27 a	-1.30 a	-2.02 fgh	-1.65 bcd	-1.56
$CaCl_2$	-1.74 cdef	-1.39 ab	-1.73 cde	-1.68 bcde	-1.58
NaCl + $CaCl_2$	-1.68 bcde	-1.81 defg	-2.14 hi	-1.49 abc	-1.82
Mean					
Leaf water content (g H_2O/g dry weight)					
0	5.37 abc	4.10 abc	1.72 ab	2.73 abc	3.48
NaCl	6.29 bc	4.42 abc	4.11 abc	6.18 bc	5.25
$CaCl_2$	7.47 c	5.52 abc	5.92 bc	7.18 c	6.52
NaCl + $CaCl_2$	7.88 c	6.32 c	7.55 c	7.27 c	7.25
Mean					

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = Salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

The role of ABA, $CaCl_2$ and the K^+/Na^+ ratio

ABA appears to be related to managing the K^+/ Na^+ ratio in the shoots (**Table 4**). The ABA(-) genotype was unable to reduce shoot Na^+ or increase shoot K^+ levels under any of the pre-treatments compared to its ABA(+) counterpart, resulting in a non-significant difference in the K^+/ Na^+ ratio in the ABA(-) genotype as compared to an elevated ratio in the ABA(+) genotype. Accumulation of Na^+ in the roots with a reduction of Na^+ in the shoots appeared to be a consistent mode of salt stress resistance in all genotypes tested in this study and may have been the main factor resulting in reduced leaf necrosis of the pre-treated plants. Roots, the first organ of the plant to become exposed to salinity (Koyro 1997), have been shown to play a role in exclusion of salt from the leaves (Heimler *et al.* 1995; Saqib *et al.* 2005). Shaterian *et al.* (2005b) reported that early-maturing potatoes such as 'Norland' tended to be Na^+ includers in the leaves compared to late-maturing types, which tend to be leaf Na^+ excluders. NaCl salt-adapted plants generally have significantly lower Na^+ in their leaves compared to non-

Table 4 The effects of salt pre-treatment on shoot and root Ca^{2+}, Na^+, K^+ content (mg/g dry matter) and K^+/Na^+ ratio of four potato genotypes after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	**'Norland'**	**9506**	**ABA(-)**	**ABA(+)**	**'Norland'**	**9506**	**ABA(-)**	**ABA(+)**
	Shoot				**Calcium**		**Root**	
0	7.92 g	6.19 h	4.45 i	4.49 i	4.62 def	4.46 def	2.34 gh	2.82 fgh
NaCl	4.17 i	5.87 h	4.21 i	4.82 i	5.76 d	3.75 efg	2.50 gh	1.81 h
$CaCl_2$	26.17 a	20.51 b	17.68 d	21.18 b	10.75 b	11.37 b	14.24 a	10.72 b
NaCl + $CaCl_2$	20.74 b	19.31 c	11.92 f	14.46 e	10.70 b	9.78 bc	8.32 c	4.73 de
	Shoot				**Sodium**		**Root**	
0	28.40 c	24.75 d	17.08 fg	35.71 a	21.57 e	18.20 f	13.98 h	16.70 g
NaCl	16.06 g	22.90 d	15.58 g	23.96 d	27.40 b	21.81 e	17.87 f	27.89 b
$CaCl_2$	23.98 d	16.44 fg	17.09 fg	20.29 e	34.83 a	20.91 e	21.42 e	21.35 e
NaCl + $CaCl_2$	34.97 a	31.24 b	15.17 g	18.61 ef	25.62 c	15.84 g	21.51 e	22.86 d
	Shoot				**Potassium**		**Root**	
0	54.89 bc	44.13 fgh	42.48 ghi	41.00 hi	7.21 g	3.63 k	3.80 jk	4.08 j
NaCl	55.06 bc	48.43 de	39.81 i	44.78 efgh	9.08 e	3.27 l	12.28 c	21.19 a
$CaCl_2$	59.84 a	48.62 de	45.45 efg	46.97 ef	5.27 h	2.97 l	4.53 i	8.74 f
NaCl + $CaCl_2$	58.93 ab	46.25 efg	45.07 efgh	52.54 cd	4.52 i	2.97 l	11.02 d	15.07 b
	Shoot				**Potassium/Sodium**		**Root**	
0	1.93 fgh	1.78 ghi	2.48 cde	1.14 j	0.33 f	0.19 ij	0.27 g	0.24 h
NaCl	3.42 a	2.11 efg	2.56 bcd	1.86 ghi	0.33 f	0.15 i	0.68 b	0.75 a
$CaCl_2$	2.72 bc	2.96 b	2.65 bcd	2.31 def	0.15 i	0.14 i	0.21 i	0.41 e
NaCl + $CaCl_2$	1.68 hi	1.48 ij	2.97 b	2.82 bc	0.17 k	0.19 ij	0.51 d	0.66 c

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

adapted plants (Montero *et al.* 1997; Djanaguiraman *et al.* 2006). Huang and Liau (1998) and Umezawa *et al.* (2000) found higher Na^+ concentration in the root of soybean (*Glycine max*) acclimated with salt than non-acclimated plants. The effect of salt pre-treatment on Na^+ accumulation in soybean and *Sorghum bicolor* appeared to be related to a re-translocation mechanism of Na^+ from the shoot (Amzallag and Lerner 1994, 1995; Umezawa *et al.* 2000) to the root.

In the more salt stress resistant potato genotypes, greater accumulation of potassium in the shoots was observed (**Table 4**). K^+ is the most abundant and highly mobile cation in both the xylem and phloem. Potassium may serve to balance Na^+ thereby increasing the viability of leaves (Marschner 1995; Pardo *et al.* 2006). The concurrent increase in shoot K^+ associated with a reduction in Na^+ is a consistent mechanism of salt stress avoidance (Greenway 1962; Munns *et al.* 2000; Zhu *et al.* 2001). The reduction of Na^+ in the shoot of 'Norland' coincided with the increase of the K^+ ion concentrations in the root. A similar interplay between root K^+ levels and shoot Na^+ levels was observed by Bañuls *et al.* (1997) on *Citrus sinensis* and Anil *et al.* (2005) on rice (*Oryza sativa*). K^+ plays an important role in enzymes, modulating ionic charge, balance of cytoplasm, and vacuole osmotic pressure (Maathuis and Amtmann 1999).

The $CaCl_2$ pre-treatment appeared to shift the most salt stress resistant 9506 genotype from a shoot Na^+ includer to a Na^+ excluder (**Table 4**). In fact, the calcium-binding protein SOS3 (Salt-Overly-Sensitive 3) and the protein kinase SOS2 are activated by a NaCl-induced calcium signal which, in turn, activates the $Na^+/H+$ antiport SOS1 (Zhu 2003). The results of studies by Ehret *et al.* (1990) and Melgar *et al.* (2006) also indicated that Ca^{2+} participated in a Na^+ exclusion mechanism mainly by preventing transport of Na^+ to the shoot. Anil *et al.* (2005), working on two salt-sensitive and salt-tolerant genotypes of rice, demonstrated Ca^{2+}-dependent reduction in Na^+ transport to the shoot, which correlated with a decline in Na^+ bypass flow in the transpiration stream. The salt tolerant genotypes maintained lower Na^+ in its apoplast relative to the salt-sensitive genotypes by developing a regulatory mechanism of sequestration into intracellular compartments.

That the $CaCl_2$ pre-treatment also enhanced shoot K^+ except in the ABA(-) genotype, and that the ABA(-) genotype was not able to exclude Na^+ from the shoot relative to the ABA(+) and other genotypes (**Table 4**) further suggests that ABA and $CaCl_2$ are requirements for this mode of salt stress defense. Addition of calcium to the salt solution increased K^+ uptake and transport (Dabuxilatu and Ikeda 2005) and improved the K^+/ Na^+ selectivity (Bolat *et al.* 2006). There is also evidence that K^+ levels can play a role in the homeostasis of Na^+ (Liu *et al.* 2000) and that calcium supply ($CaCl_2$) may take part in the Na^+ exclusion mechanism mainly by preventing Na^+ transport to the shoot (Melgar *et al.* 2006). Moreover, calcium content positively correlated with increasing levels of ABA in the plant (Staxen *et al.* 1999). Regulation of Na^+ exclusion, as well as reduced Na^+ accumulation, has been reported to be induced by ABA in several studies (Sibole *et al.* 2003; Yan *et al.* 2006). The effect of exogenous ABA on Na^+ exclusion and reduced Na^+ accumulation has been studied (Wang 1998; Sarwat *et al.* 2000). It has been proposed that in *A. chinense*, external ABA could promote the uptake of K^+ and reduce the toxicity of Na^+ (Wang *et al.* 1996; Wang 1998). Exogenous ABA has also been shown to increase K^+ ion content by nearly twofold in xylem sap (Chen *et al.* 2006) mediated by K^+ channels (Roberts and Snowman 2000).

While the combined NaCl + $CaCl_2$ pre-treatment reduced leaf necrosis after salt stress in both 'Norland' and 9506 genotypes compared to the control, the combined treatment was the least effective of all pre-treatments and also induced the highest Na^+ accumulation in shoot tissue. The negative effect of mixed salts may have also been provoked by an increase in the Cl^- ion. The greater reduction in the osmotic potential of the solution may have prevented the root cells from obtaining water and essential nutrients (Çiçek and Çakirlar 2002) or may have caused changes in membrane permeability (Cramer *et al.* 1985; Tobe *et al.* 2004). The high EC of the combined solution may have limited the supply of water to the plant resulting in a significant reduction of water in the leaf. Similar to our finding, significant reduction in plant growth parameters have been reported when EC of mixed salts exceeded 11 dS m^{-1} for wheat (*Triticum aestivum*) (Wilson *et al.* 2002) and 16.4 dS m^{-1} for pomegranate (*Punica granatum*) (Asrey and Shukla 2003).

Grafting responses

Grafting relatively salt sensitive scions [(ABA(-) and ABA(+)] onto the 9506 resistant rootstock was unable to decrease leaf necrosis beyond the salt pre-treatment effect alone. There was generally no difference between the NaCl and $CaCl_2$ pre-treatment effects in the leaf necrosis response, except in the most salt sensitive ABA(-) genotype in which the NaCl pre-treatment was more effective than the $CaCl_2$

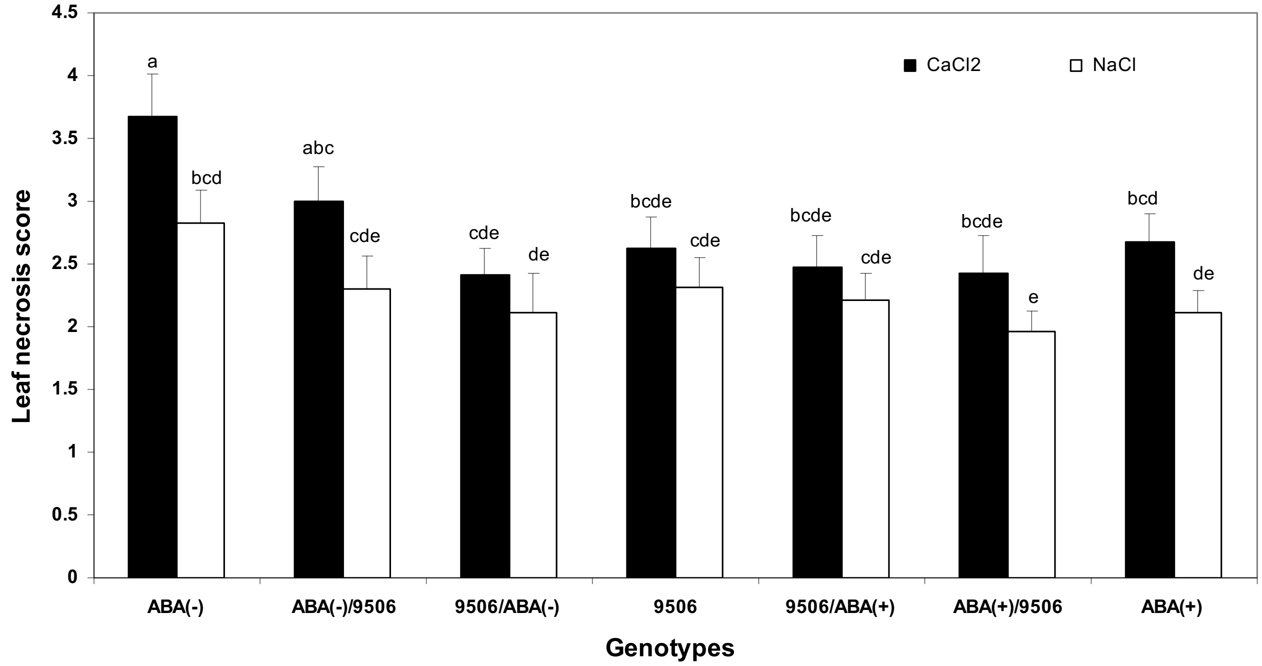

Fig. 2 The effect of sodium chloride and calcium chloride pre-treatments (sample number = 5) on leaf necrosis of potato genotypes and their graft combinations (scion/rootstock) measured after two weeks of salt stress (150-180 mM NaCl). Leaf necrosis score: 1-5. 1- (0% necrosis), 2- (1-25% necrosis), 3- (26-50% necrosis), 4- (51-75% necrosis), 5- (76-100% necrosis). Means with the same letters are not significantly different (LSD *P* = 0.05). 9506 = Salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

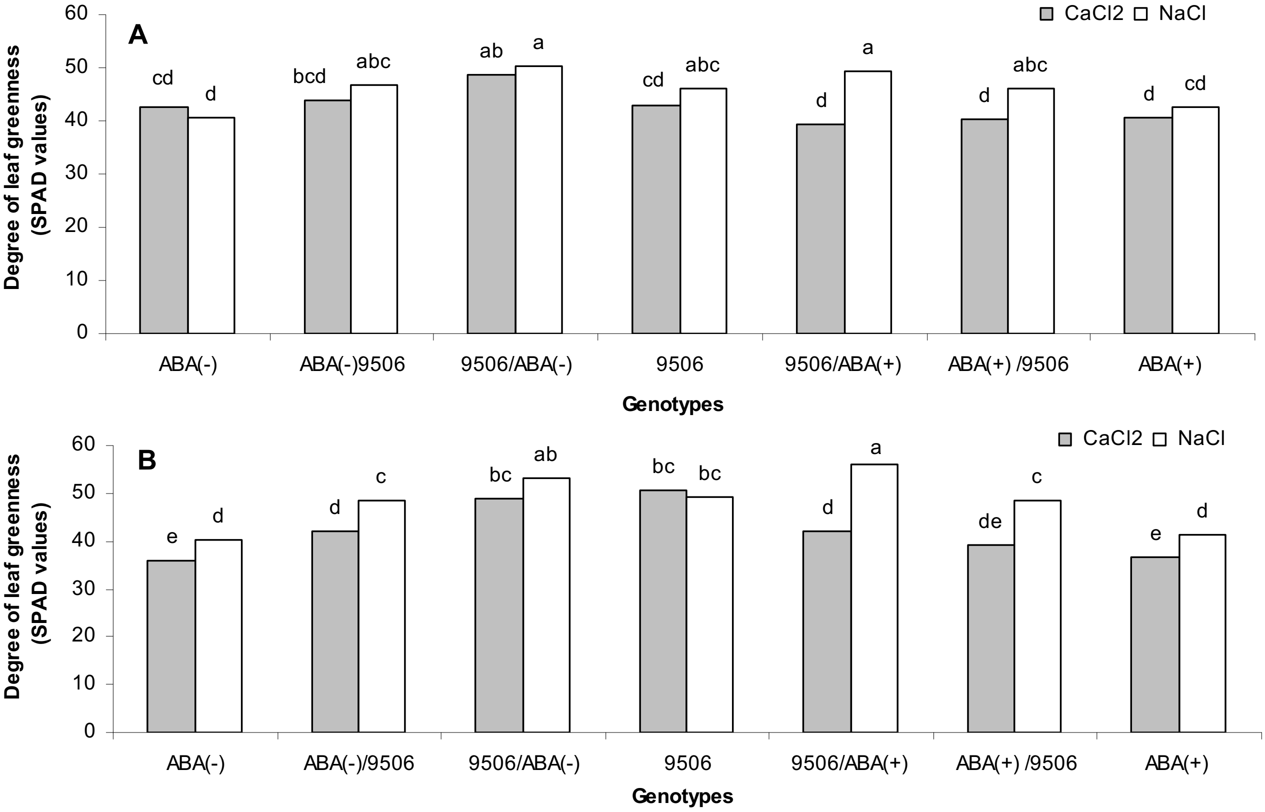

Fig. 3 The effect of sodium chloride and calcium chloride pre-treatments (sample number = 5) on the degree of leaf greenness of different grafted and non-grafted potato genotypes (A) before salt stress and (B) after two weeks of salt stress (150-180 mM NaCl). Means with the same letters are not significantly different (LSD *P* = 0.05). 9506 = Salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

pre-treatment in alleviating leaf necrosis caused by subsequent exposure to salt stress (**Fig. 2**).

By contrast, leaf greenness, a reflection of chlorophyll content, was higher following the NaCl pre-treatment compared to the $CaCl_2$ pre-treatment and this effect was even more pronounced after the salt stress, particularly in the relatively more salt sensitive genotypes (**Fig. 3**). The 9506 salt stress resistant rootstock increased leaf greenness beyond the NaCl pre-treatment effect alone in both ABA(-) and ABA(+) scions (**Fig. 3**). In a similar work with rice, Djanaguiraman *et al.* (2006) found that plants that had been gradually salt treated contained higher chlorophyll content than did control plants (one of the reviewers wanted, control plants did). Romero *et al.* (1997) and Fernández-García *et al.* (2002) also showed that leaf pigment and chlorophyll content in grafted plants exposed to salinity conditions were

Table 5 The effect of sodium chloride and calcium chloride pre-treatments on leaf stomatal conductivity (SC) (cm s^{-1}) of scion/rootstock combinations of different potato genotypes before and after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	ABA (-)	ABA (-)/9506	9506/ABA (-)	9506	9506/ABA (+)	ABA (+)/9506	ABA (+)
Before stress							
$CaCl_2$	0.04 c	0.02 a	0.06 e	0.04 c	0.02 a	0.04 c	0.05 d
NaCl	0.04 c	0.03 b	0.06 e	0.04 c	0.07 f	0.04 c	0.05 d
After stress							
$CaCl_2$	0.02 b	0.01 a	0.04 d	0.03 c	0.02 b	0.02 b	0.03 c
NaCl	0.03 c	0.02 b	0.06 f	0.03 c	0.05 e	0.03 c	0.04 d

Means with the same letters are not significantly different ((LSD $P = 0.05$). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 6 The effect of sodium chloride and calcium chloride on leaf osmotic potential (s = MPa) of scion/rootstock combinations of different potato genotypes before and after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	ABA (-)	ABA (-)/9506	9506/ABA (-)	9506	9506/ABA (+)	ABA (+)/9506	ABA (+)
Before stress							
$CaCl_2$	-2.13 g	-1.89 f	-1.49 bcd	-1.47 bcd	-1.45 bcd	-1.53 cd	-1.61 cde
NaCl	-2.34 g	-1.56 cde	-1.40 abc	-1.66 de	-1.24 a	-1.31 ab	-1.64 de
After stress							
$CaCl_2$	-2.72 f	-2.22 de	-1.75 ab	-2.04 cd	-2.16 de	-1.64 ab	-1.70 ab
NaCl	-2.32 e	-1.70 ab	-1.52 a	-1.84 bc	-1.74 ab	-1.67 ab	-1.85 bc

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 7 The effect of sodium chloride and calcium chloride on leaf water content (%) of scion/rootstock combinations of different potato genotypes before and after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	ABA (-)	ABA (-)/9506	9506/ABA (-)	9506	9506/ABA (+)	ABA (+)/9506	ABA (+)
Before stress							
$CaCl_2$	56.67 e	62.95 de	82.62 a	83.94 a	77.93 ab	78.36 ab	78.14 ab
NaCl	69.60 cd	70.75 c	79.42 ab	82.19 a	78.70 ab	81.11 a	73.76 bc
After Stress							
$CaCl_2$	42.98 i	51.53 h	68.69 bcdef	69.88 abcde	66.37 cdef	64.60 def	57.03 gh
NaCl	61.31 fg	73.00 abc	77.32 a	76.19 ab	68.66 bcdef	71.85 abcd	63.85 efg

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 8 The effect of sodium chloride and calcium chloride on shoot dry weight (g) and shoot water content (%) of scion/rootstock combinations of different potato genotypes after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	ABA (-)	ABA (-)/9506	9506/ABA (-)	9506	9506/ABA (+)	ABA (+)/9506	ABA (+)
Shoot dry weight							
$CaCl_2$	1.72 f	4.19 ef	16.23 b	13.82 b	7.20 cd	7.14 cd	4.01 ef
NaCl	2.75 f	4.25 ef	19.43 a	15.22 b	9.14 c	9.42 c	5.42 de
Shoot water content							
$CaCl_2$	60.05 f	64.63 f	88.58 a	81.66 cd	81.67 bcd	85.53 abc	81.48 cd
NaCl	70.55 e	77.48 d	84.68 abc	87.11 ab	78.60 d	87.84 a	85.93 abc

Means with the same letters are not significantly different (LSD $P = 0.05$).9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

Table 9 The effect of sodium chloride and calcium chloride on root dry weight (g) and root water content (%) of scion/rootstock combinations of different potato genotypes after two weeks of salt stress (150-180 mM NaCl).

Pre-treatments	ABA (-)	ABA (-)/9506	9506/ABA (-)	9506	9506/ABA (+)	ABA (+)/9506	ABA (+)
Root dry weight							
$CaCl_2$	0.57 e	1.14 bcd	1.33 abc	1.33 abc	0.65 e	1.23 bcd	0.86 de
NaCl	0.60 e	1.42 abc	1.75 a	1.58 ab	1.10 cd	1.20 bcd	1.19 bcd
Root water content							
$CaCl_2$	42.86 g	69.57 de	84.36 a	70.39 d	72.56 cd	70.07 de	63.46 e
NaCl	54.52 f	70.14 de	88.37 a	70.52 d	78.62 bc	74.44 c	67.87 de

Means with the same letters are not significantly different (LSD $P = 0.05$). 9506 = salt stress resistant, ABA(-) = ABA-deficient mutant, ABA(+) = ABA normal sibling.

determined by the genotype of the rootstock.

Whenever a difference in either leaf stomatal conductivity (**Table 5**), leaf osmotic potential (**Table 6**), leaf water content (**Table 7**), shoot (**Table 8**) and root (**Table 9**) dry weight or water content was detected, NaCl pre-treatment consistently increased these parameters compared to the $CaCl_2$ pre-treatment. The higher leaf stomatal conductivity in the NaCl salt-acclimated potato genotypes and graft combinations during subsequent salt stress observed in this study is in accord with the results of Djanaguiraman *et al.* (2006) on salt-acclimated rice plants.

Although both acclimation treatments were applied at the same EC level, the NaCl pre-treatment generally produced a higher level of acclimation compared to the $CaCl_2$ pre-treatment. NaCl acclimation was more effective in alleviating negative salt stress responses compared to $CaCl_2$. A similar Na^+ specific effect on growth enhancement of *Suaeda maritime* following application of NaCl was also observed (Yeo and Flowers 1980). This differential salt-specific response was particularly pronounced in stomatal conductivity and osmotic potential measurements after stress as well as leaf water content.

ABA levels in the rootstock also had a significant effect on influencing stomatal conductivity. The 9506 rootstock, which had the highest levels of endogenous ABA (Etehadnia *et al.* 2008), induced greater stomatal closure in the ABA(-) and ABA(+) scions both before and after salt stress compared to non-grafted plants (**Table 5**). Similarly, under NaCl pre-treatment when 9506 scions were grafted onto the ABA(-) or ABA(+) rootstocks containing relatively lower levels of endogenous ABA (Etehadnia *et al.* 2008), the stomatal conductivity of the 9506 scion increased both before and after subsequent salt stress. These results are consistent with several reports that elevated ABA levels will induce stomatal closure (Wright 1978; Montero *et al.* 1998; Sibole *et al.* 2000; Mishra *et al.* 2006) but is in contrast to Jones *et al.* (1987) and Holbrook *et al.* (2002) who found stomatal closure in scions to be independent of the ABA levels in the rootstock.

The osmotic potential of the ABA(-) scion was increased (less negative) when grafted onto the 9506 rootstock. This 9506 rootstock effect was not observed on the ABA(+) scion (**Table 6**). The 9506 rootstock also significantly elevated both leaf water content of the ABA(-) and ABA(+) genotypes after salt stress under both pre-treatments (**Table 7**). Chen *et al.* (2003) in a similar work with tomato reported that shoot growth of ABA-deficient mutant tomato (*flacca*) scions grafted onto ABA-normal rootstocks was superior to growth of flacca grafted onto its own rootstock, regardless of the salinity level. In the reciprocal graft, the ABA(-) and ABA(+) as rootstocks also altered the shoot water content of the 9506 scion. The effects of rootstalks in increasing leaf water content (Santa-Cruz *et al.* 2001, 2002; Estañ *et al.* 2005) and increasing leaf osmotic potential of the scion (which was more significant at high salinity levels) (Santa-Cruz *et al.* 2002) indicate that graft unions are not physical barriers to water transport from rootstock to scion (Fernández-García *et al.* 2002). Indeed, graft unions are structurally and chemically very functional in herbaceous plants such as tomato (Fernández-García *et al.* 2004). Our non-destructive micro-imaging NMR studies on shoot water flow of grafted and non-grafted potato plants grown under normal and salt stress conditions were also consistent with these results (Etehadnia *et al.* 2008).

While the NaCl pre-treatment in combination with the 9506 as a rootstock increased the shoot water content of ABA(-) after salt stress (**Table 8**), a more dramatic response was observed by the scion impact on root growth (**Table 9**). When the salt stress resistant 9506 scions were grafted onto the ABA(-) rootstocks, by the end of the salt stress treatment, the root dry weight and root water content of the ABA(-) rootstock increased on average 80% with no significant difference between the NaCl and $CaCl_2$ salts. Grafting experiments with salt-sensitive and salt-tolerant genotypes of mango (*Mangifera indica* L.) and chickpea (*Cicer arietinum*) (Dua 1997; Schmutz and Lüdders 1999) revealed that scions also play a vital role in salt tolerance of grafted plants. In chickpea, when a salt tolerant scion was grafted onto a sensitive rootstock, the scion remained tolerant to salinity but in the reciprocal grafts, the scion of the sensitive genotype on the tolerant rootstock died a few days after salinization (Dua 1997). Chen *et al.* (2003), working on tomato, also found that when grafted plants had ABA-normal shoots (Ws), they produced more biomass than did scions that were ABA-deficient mutants (*flacca*). They suggested that this might be due to higher photosynthetic rates in the more vigorous salt-tolerant scions which resulted in greater potential for partitioning of assimilates to the rootstock.

CONCLUSION

Overall, the NaCl acclimation pre-treatment was the more effective compared to $CaCl_2$ in protecting plants against subsequent salt stress resistance in both grafted and non-grafted plants. This was associated with a specific Na^+ ion effect rather than a non-specific EC-dependent response. The salt resistant rootstock with higher endogenous ABA levels positively influenced water content in the more salt sensitive scions after salt stress as reflected through stomatal conductivity, osmotic potential, shoot growth and water content of the scions. Grafting salt stress resistant scions onto the ABA(-) rootstock also led to increased root growth. Grafting of salt- resistant scions under salt acclimation treatments may be an alternative approach to increasing stress resistance in commercially important potato cultivars. While the $CaCl_2$ acclimation pre-treatment was not as effective as NaCl pre-treatment, the importance of calcium in stress acclimation cannot be excluded, particularly since low levels of Ca^{2+} existed in the background nutrient and NaCl salt stress solutions. Further, both calcium and ABA appear to be involved in increasing the ratio of K^+/Na^+ in the shoots which was associated with elevated salt stress resistance.

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