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Calcium supply effects on wheat cultivars differing in salt resistance with special reference to leaf cytosol ion homeostasis

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Salinity causes changes in cytosolic Ca^{2+}, $[Ca^{2+}]_{cyt}$, Na^+, $[Na^+]_{cyt}$ and pH, pH_{cyt}, which induce specific reactions and signals. Reactions causing a rebalancing of the physiological homeostasis of the cytosol could result in plant resistance and growth. Two wheat cultivars, *Triticum aestivum*, Seds1 and Vinjett, were grown in nutrient solution for 7 days under moderate salinity (0 and 50 m*M* NaCl) with and without extra addition of 5 m*M* $CaSO_4$ to investigate the seedling-ion homeostasis under salinity. In the leaf protoplasts $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ and pH_{cyt} were detected using acetoxymethyl esters of the ion-specific dyes, Fura 2, SBFI and BCECF, respectively, and fluorescence microscopy. In addition, both cultivars were grown for 3 weeks at 0, 50 and 125 m*M* NaCl with, or without, extra addition of 5 m*M* $CaSO_4$ to detect overall Na^+ and Ca^{2+} concentrations in leaves and salinity effects on dry weights. In both cultivars, salinity decreased $[Ca^{2+}]_{cyt}$, while at extra Ca^{2+} supplied, $[Ca^{2+}]_{cyt}$ increased. The $[Ca^{2+}]_{cyt}$ increase was accompanied by increase in the overall Ca^{2+} concentrations in leaves and decrease in the overall Na^+ concentration. Moreover, irrespective of Ca^{2+} treatment under salinity, the cultivars reacted in different ways; $[Na^+]_{cyt}$ significantly increased only in cv. Vinjett, while pH_{cyt} increased only in cv. Seds1. Even at rather high total Na^+ concentrations, the cytosolic concentrations were kept low in both cultivars. It is discussed whether the increase of $[Ca^{2+}]_{cyt}$ and pH_{cyt} can contribute to salt tolerance and if the cytosolic changes are due to changes in overall Ca^{2+} and Na^+ concentrations.

Introduction

Approximately 400 million hectares throughout the world is affected by soil salinity, which is a serious problem for agriculture (FAO 2008) and mainly constrains plant growth by high Na^+ and Cl^- concentrations, as well as osmotic stress, which at an early stage may cause toxicity. However, these effects can be mitigated if the stress is not severe (Munns and Tester 2008). Nutritional deficiencies like K^+ and Ca^{2+} usually occur under salinity, although an adequate level of Ca^{2+} can be beneficial for reducing the Na^+ uptake by the plant (Genc et al. 2010, Tavakkoli et al. 2011). Therefore, addition of gypsum ($CaSO_4$) is the most commonly used way to ameliorate the harmful effects of Na^+ (Anil et al. 2007), although other sources, such as $CaCl_2$ are used. An

Abbreviations – BCECF-AM, acetoxy methyl ester of 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; Fura 2-AM, acetoxy methyl ester of calcium binding benzofuran; SBFI-AM, acetoxy methyl ester of sodium-binding benzofuran isophthalate.

adequate Ca^{2+} addition could maintain membrane function by reducing the Na^+/Ca^{2+} ratio in plants, which leads to less toxicity and facilitates nutrient uptake into the cytosol (Hepler 2005). Salinity may also disturb normal Ca^{2+} functions without disturbing overall Ca^{2+} tissue concentrations. It can do so, because $[Ca^{2+}]_{cyt}$ is in the n*M* range, whereas apoplastic and vacuolar Ca^{2+} is in the m*M* range (Mühling and Läuchli 2002, Kader et al. 2007, Conn et al. 2011).

The mechanism of the regulation of total Ca^{2+} concentration and $[Ca^{2+}]_{cyt}$ levels under NaCl stress is still unclear (Yang et al. 2007). From investigations so far, it can be concluded that the change in $[Ca^{2+}]_{cyt}$ is not uniform (see references in Kader and Lindberg 2010) and closely linked to instant changes in the pH_{cyt} (Kader and Lindberg 2010). Both $[Ca^{2+}]_{cyt}$ and pH_{cyt} levels could differ with time of salinity. Moreover, interactions between sodium and calcium are well documented. Investigations by Davenport et al. (1997) and Husain et al. (2004) showed that a change of the calcium supply affected the salinity response of wheat in different ways in salt-tolerant and sensitive cultivars. Short-term influx studies using Na^{22} showed that sodium influx was lower in the presence of 3.11 m*M* compared with 0.06 m*M* calcium activities (Davenport et al. 1997). In several wheat genotypes increasing external Ca^{2+} concentration reduced the accumulation of total Na^+ concentration in the shoot (Husain et al. 2004).

Few studies concern cytosolic ion changes under salinity. Carden et al. (2003) investigated cytosolic changes in Na^+, K^+ and pH in cortex root cells of barley under salt stress. By use of triple-barreled microelectrodes they found that a more tolerant variety maintained a tenfold lower Na^+ activity than a more sensitive variety after 5 days, but that the activities were similar after 8 days. Moreover, after 8 days the cytosolic K^+ activity was decreased only in the sensitive variety. A low Na^+/K^+ ratio is a trait for salt tolerance (Maathuis and Amtmann 1999). The cytosolic pH did not differ significantly between varieties or time.

In order to understand the plant's reactions under salinity, we investigated the pH_{cyt}, $[Ca^{2+}]_{cyt}$, and $[Na^+]_{cyt}$ homeostasis in leaves of wheat, as well as the overall concentrations of Na^+, Ca^{2+} and K^+ under salinity stress. These parameters were investigated, with and without an extra Ca^{2+} addition, in the Swedish cv. Vinjett, with unknown salt-sensitivity, and in the Egyptian cv. Seds1. The latter cultivar takes up less sodium in the leaves than cv. Vinjett and may be an excluder. This cultivar is recommended for cultivation on saline soils in Egypt (Egyptian Minister of Agriculture, http://www.caae-eg.com/new/index.php/component/content/article/85-2010-11-04-18-42-40/758-2012-10-11-13-23-00.html). The aim was to clarify any relations between the overall and the cytosolic ion concentration changes. We hypothesized that the $[Ca^{2+}]_{cyt}$ and pH_{cyt} could be maintained by improving the overall Ca^{2+} concentration under salt stress by adding extra Ca^{2+} and that such addition could restrict Na^+ accumulation concerning both overall and the cytosolic levels, which could improve salt resistance.

Material and methods

Cultivation

Seeds of wheat (*Triticum aestivum* L. cvs. Vinjett and Seds1) were surface-sterilized with 10% chlorine solution for 15 min and then rinsed with distilled water five to six times. Thereafter they were soaked in 5 m*M* $CaSO_4$ solution for 3 h and then rinsed with distilled water five to six times. Then the seeds were placed under dark conditions on a Mira cloth (LIC, Stockholm, Sweden) covering a metal net. The net was placed on beakers containing a complete nutrient solution according to Shishova and Lindberg (2004) [2 m*M* KNO_3, 1 m*M* $Ca(NO_3)_2$, 1 m*M* $MgSO_4$, 1 m*M* KH_2PO_4, 0.5 m*M* Na_2HPO_4, 2.5 μ*M* H_3BO_3, 0.3 μ*M* $CuSO_4$, 0.5 μ*M* $ZnSO_4$, 2.0 μ*M* $MnSO_4$, 0.01 μ*M* $(NH_4)_6Mo_7O_{24}$ and 200 μ*M* Fe-EDTA]. After seed germination (3–4 days), NaCl (0, 50 and 125 m*M* Na) and/or $CaSO_4$ (0 and 5 m*M* Ca) were added in portions during 3 days until the final desired concentrations were reached. The seedlings were cultivated for 3 weeks in a climate-controlled chamber at $20 \pm 1^{\circ}C$, light 14 h $days^{-1}$ at an irradiance 118 W m^{-2} at the top of the shoots and relative humidity 50–60%. The nutrient solution was renewed twice a week.

Determinations of $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ and pH_{cyt}

Protoplast isolation and dye loading

Leaf protoplasts of both wheat cultivars (Vinjett and Seds1) were isolated after cultivation for 7 days with salinity (0 and 50 m*M* NaCl) and/or extra Ca^{2+} (0 and 5 m*M* $CaSO_4$) treatments, by an enzymatic method as described by Edwards et al. (1978) with some modifications according to Lindberg and Strid (1997). The isolated protoplasts were loaded in darkness at $22 \pm 1^{\circ}C$ for 3 h with calcium binding benzofuran dye (Fura 2) in the acetoxymethyl ester form (Fura 2-AM, Molecular Probes, Leiden, the Netherlands) for $[Ca^{2+}]_{cyt}$ determination according to Shishova and Lindberg (2004), and for 4 h with sodium-binding benzofuran isophthalate dye (SBFI-AM) for $[Na^+]_{cyt}$ determination according to Kader and Lindberg (2005). For pH_{cyt}

determination, the protoplasts were loaded in darkness at 4°C for 50 min, with the tetra (acetoxymethyl) ester of bis-carboxyethyl-carboxyfluorescein (BCECF-AM) according to Lindberg and Strid (1997).

Fluorescence measurements and calibration

Before measurements, samples were kept in darkness at room temperature for 30 min to allow the protoplasts to recover after centrifugation. An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochem, Germany), supplied with an electromagnetic filter-exchanger, xenon lamp (XBO 75), photometer, microprocessor (MSP 201) and a personal computer was used to determine fluorescence intensity of the protoplasts after dye excitation at 340/380 nm for the Fura 2 and SBFI dyes and at 485/436 nm for the BCECF dye. Emission wavelengths were 510–550 nm. All measurements were performed with a Planneofluar ×40/0.75 objective (Zeiss) for phase contrast. The ratio measurements were performed only with protoplasts of similar size and properly loaded only in the cytosol. Adjustment for signals and noise was made automatically. The effect of different dye concentration can be eliminated by means of ratio imaging (Tsien and Poenie 1986). Micro-slides were covered with 0.2% poly-L-lysine (MW 150 000–300 000, Sigma) in order to attach protoplasts to their surfaces. For measurements, only protoplasts of similar size, with a dense cytoplasm were selected. The cell viability was always checked before, and after, the fluorescence measurements by measuring the presence of fluorescence inside the cells, because the dye hydrolysis is a good viability indicator (Gualtieri 1992), and also by checking the protoplasmic streaming and any visible change in size and shape of the protoplasts (Kader et al. 2007).

The in situ calibrations were provided with single protoplasts loaded with Fura 2-AM for detection of $[Ca^{2+}]_{cyt}$ as described by Shishova and Lindberg (2004), with SBFI-AM for $[Na^+]_{cyt}$ as described by Kader and Lindberg (2005) and with BCECF-AM for pH_{cyt} as described by Kader et al. (2007).

Determination of dry weights and overall ion concentrations

Plant samples were taken after 7, 14 and 21 days of salinity (0, 50 and 125 m*M* NaCl) and/or Ca^{2+} (0 and 5 m*M* $CaSO_4$) treatments. The leaves and whole plant dry weights were measured. The dry leaves were wet digested using HNO_3: $HClO_4$ (7:3 V/V). Thereafter, the Na^+ and Ca^{2+} contents were determined using atomic absorption spectrophotometer (SpectrAA-100, Varian, Springvale, Australia).

Statistics

All experiments were performed three times with plants from different cultivations (biological replicates). In each biological replicate growth characters were measured in 10 plants and for the atomic absorption measurements three replicates were used. For the protoplast experiments more than 20 different single protoplasts were measured. All collected data were statistically analyzed using two factorial (salinity and/or calcium additions) completely randomized design (CRD) and the means were compared using the least significant difference test (LSD) at 5% level of probability to indicate treatment differences (Snedecor and Cochran 1980).

Results

Influence of calcium treatments on cytosolic ion concentrations and pH in leaf protoplasts under saline conditions

The $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ and pH_{cyt} were detected in the living protoplasts isolated from the leaves of both cultivars 7 days after start of full salinity treatment and with or without extra Ca^{2+} addition during the cultivation. No visible difference between the cultivars was found in the efficiency of taking up the different dyes. Also, as dual wavelength photometry was used, variation in dye concentration in the cytosol is of less importance. Our results show that, the $[Ca^{2+}]_{cyt}$ significantly decreased from 50 to 20 n*M* in the leaf protoplasts of both cultivars when treated with 50 m*M* NaCl (Fig. 1A), but significantly increased in cv. Seds1 from 40 to 90 n*M* and in cv. Vinjett from 40 to only 60 n*M*, when Ca^{2+} was supplied (Fig. 1A).

Among the different behaviors between cultivars upon salinity, in cv. Seds1 there was no significant change in the $[Na^+]_{cyt}$, irrespective of extra Ca^{2+} addition, while $[Na^+]_{cyt}$ significantly increased in cv. Vinjett (Fig. 1B). Another difference existed between the cultivars; the pH_{cyt} increased in cv. Seds1, irrespective of extra Ca^{2+} addition, but did not change in cv. Vinjett (Fig. 1C). On the other hand, when Ca^{2+} was supplied under nonsaline conditions, no significant differences were obtained in the $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ or pH_{cyt} of both cultivars (Fig. 1A–C).

Moreover, upon 50 m*M* NaCl treatment the $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratio (Fig. 1D) was higher in cv. Seds1 (551%) than in cv. Vinjett (332%), while when Ca^{2+} was supplied the cytosolic $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ decreased in both cultivars to approximately equivalent levels under both nonsaline and saline conditions and almost to the same level as for the control plants.

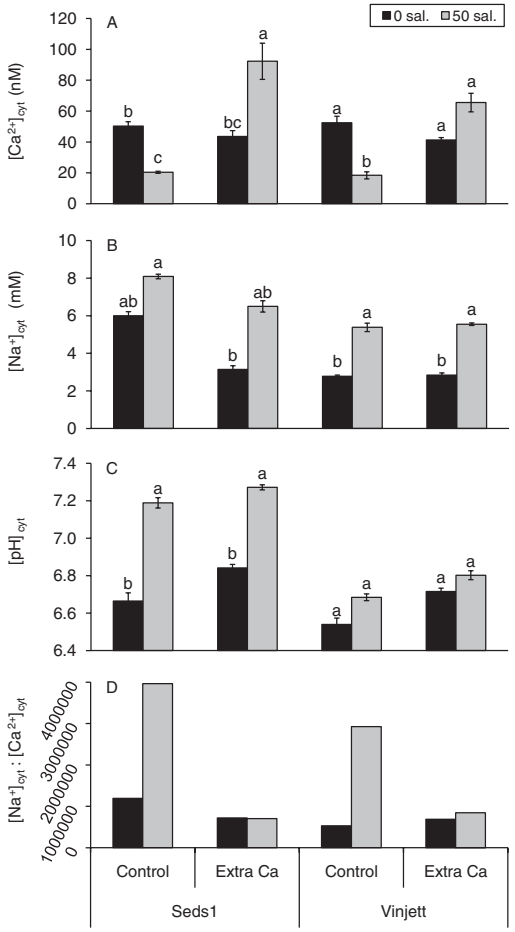

Fig. 1. The $[Ca^{2+}]_{cyt}$ (A), $[Na^{+}]_{cyt}$ (B), pH_{cyt} (C) and ratio of $[Na^{+}]_{cyt}/[Ca^{2+}]_{cyt}$ (D) in wheat leaf protoplasts of cvs. Seds1 and Vinjett, cultivated under different salinity levels for 7 days, with and without extra addition of calcium (5 m*M*) to the nutrient solution. Significant differences between the treatments within the same date and for each cultivar were shown at $P < 0.05$ by LSD. The error bars indicate the mean SE ($n \geq 60$).

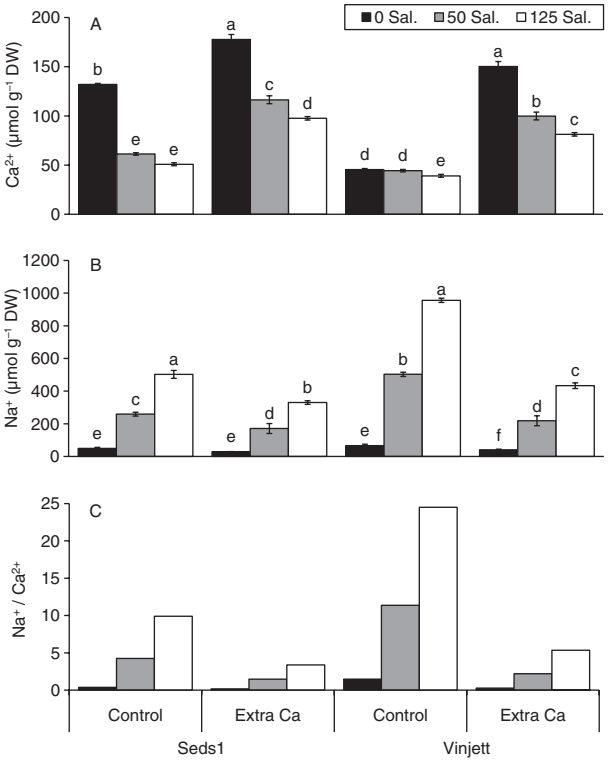

Fig. 2. Total concentrations of Ca^{2+} (A), Na^{+} (B) and ratio of Na^{+}/Ca^{2+} (C) in leaves of wheat, cvs. Seds1 and Vinjett, cultivated under different salinity levels for 7 days, with and without extra addition of calcium (5 m*M*) to the nutrient solution. Significant differences between the treatments for each cultivar were shown at $P < 0.05$ by LSD. The error bars indicate the mean SE ($n = 9$).

Influence of calcium treatment on total (overall) concentrations of Na^{+} and Ca^{2+} in leaves under saline conditions

The Ca^{2+} concentration significantly decreased by increasing salinity level (Fig. 2A), whereas Na^{+} concentrations increased in leaves of both cultivars by increasing salinity level, irrespective of extra Ca^{2+} addition (Fig. 2B). However, under saline conditions, cv. Seds1 obtained lower Na^{+} and higher Ca^{2+} concentrations than cv. Vinjett. Moreover, the extra Ca^{2+} addition under saline conditions decreased Na^{+} accumulation and significantly increased Ca^{2+} concentrations in the leaves of both cultivars with some superiority of cv. Seds1 and also restricted the translocation of Na^{+} from roots to shoots (for shoot and root ion concentrations after 7, 14 and 21 days of growth see the Figs S1 and S2).

In both cultivars the overall Na^{+}/Ca^{2+} ratio gradually increased by increasing saline conditions irrespective of extra Ca^{2+} addition (Fig. 2C). Such increases were more pronounced in cv. Vinjett than in cv. Seds1. In addition, when Ca^{2+} was supplied, the overall Na^{+}/Ca^{2+} ratio decreased under the same salinity level.

Salinity and/or calcium treatments effects on dry weights

In both cultivars the dry weights were more reduced at 125 m*M* Na than at 50 m*M* Na (Fig. 3), and more after 21 days than after 7 and 14 days, with different reduction intensity between cultivars. After treatment with 125 m*M* NaCl, the reduction in cv. Seds1 reached less than 20–25% (Fig. 3A), and was nonsignificant at 50 m*M* NaCl. In cv. Vinjett the reduction in shoots and whole plant dry weights was 40–50% at 125 m*M* Na (Figs 3B and S3), but at 50 m*M* Na, the whole plant reduction was around 25% (Fig. 3B). After 21 days of salinity with extra

Ca^{2+} addition (5 m*M*), plant growth was more improved in cv. Vinjett than in cv. Seds1 (Fig. 3A,B). (The statistical difference can be seen in the Fig. S3)

Discussion

To our knowledge little information still exists on cytosolic ion behavior of the leaves under stress, especially ion-activity changes caused by salinity stress for several days. This is due to the difficulty to do direct noninvasive measurements of ion activities in living cells. In the present investigation, treatment with 50 m*M* NaCl during 7 days showed that less Ca^{2+} was taken up into the cytosol of leaf protoplasts of both cultivars than without NaCl (Fig. 1A), while when Ca^{2+} was supplied under salinity $[Ca^{2+}]_{cyt}$ increased. These results partly corroborate those presented by Halperin et al. (2003). As more Ca^{2+} is taken up into the cytoplasm in the presence of NaCl, than in its absence, the uptake may depend on a sufficient overall Ca^{2+} concentration in both the cultivars (Figs 1A and 2A). We can, thus, propose that the changes of $[Ca^{2+}]_{cyt}$ activity are both salinity and Ca^{2+} dependent.

After 7 days of salinity treatment the $[Na^+]_{cyt}$ in cv. Seds1 (6–8 m*M*) was only slightly higher than in cv. Vinjett (2–6 m*M*). Also in root cells of barley there was little difference in the $[Na^+]_{cyt}$ after 8 days of salinity (Carden et al. 2003). Moreover, in cv. Seds1 the $[Na^+]_{cyt}$ was associated with pH_{cyt} alkalization under salinity, irrespective of extra Ca^{2+} addition (Fig. 1B,C). On the other hand, in cv. Vinjett salinity treatment increased $[Na^+]_{cyt}$ and did not affect pH_{cyt} (Fig. 1B,C). Thus, these cultivars might have different mechanisms to limit Na^+ toxicity (Blumwald et al. 2000, Kader and Lindberg 2005, Munns and Tester 2008, Tavakkoli et al. 2011), and to resist salinity, which was confirmed by our investigation on dry weights (Fig. 3). In cv. Seds1 (Fig. 3A) the dry weights of leaves were not significantly affected by moderate salinity, while in cv. Vinjett (Fig. 1B) dry weights of leaves significantly decreased after 21 days, showing that the latter cultivar is more sensitive to salt exposure (for statistical difference, see Fig. S3). The latter results are in agreement with results reported by Hanafy Ahmed et al. (2008) and Genc et al. (2010).

The obtained increase of the $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ in both cultivars under salinity (Fig. 1D) was a consequence of both lower $[Ca^{2+}]_{cyt}$ and higher $[Na^+]_{cyt}$ concentrations (Fig. 1A,B) and was very close to the overall Na^+/Ca^{2+}-ratio trends of the leaves (Fig. 2C). The extra Ca^{2+} addition rebalanced both ratios to approximately equivalent levels and almost equal to the control (Fig. 1D). This mechanism, which can be identified as a Ca^{2+} regulatory effect, was surprising, because the $[Na^+]_{cyt}$ concentration is in m*M*, while the $[Ca^{2+}]_{cyt}$ concentration is in n*M*. Thus, the $[Ca^{2+}]_{cyt}$ increases may be effective enough in limiting $[Na^+]_{cyt}$ toxicity and could be an explanation for salinity resistance in wheat during this early stage of growth. Then the ion toxicity is still less harmful but can increase by time (Munns and Tester 2008) depending on the genetic variation between cultivars and if the overall Ca^{2+} concentration is too low.

In more salt-sensitive plants the transport of Ca^{2+} into the shoots is less pronounced under salt stress, and the

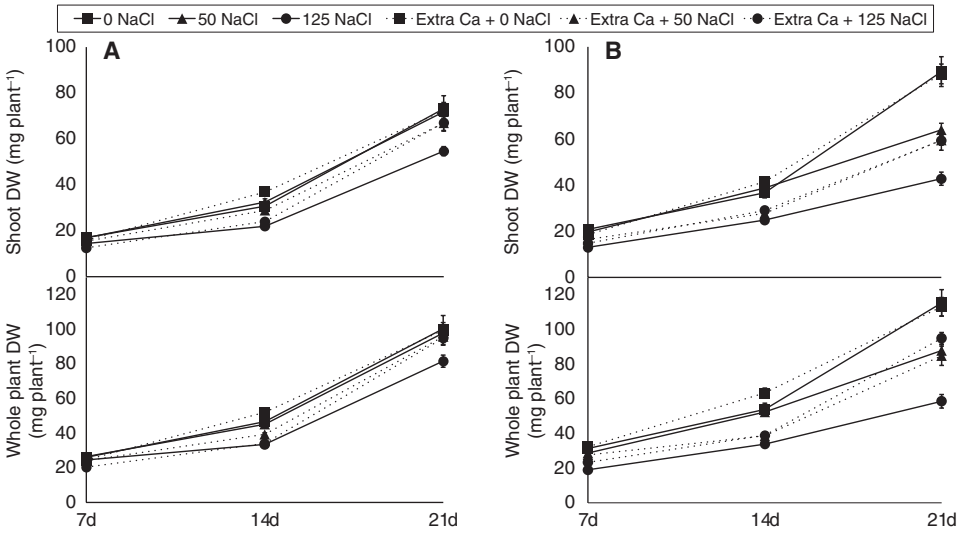

Fig. 3. Dry weights (mg plant^{-1}) of shoot and whole plant of wheat, cvs. Seds1 (A) and Vinjett (B), cultivated under different salinity levels for 7, 14 and 21 days, with and without extra addition of calcium (5 m*M*) to the nutrient solution. The error bars indicate the mean SE (n = 30).

ability of plants to retain Ca^{2+} may be associated with their salt resistance (Unno et al. 2002). Therefore, the detrimental effects attributed to salinity stress on dry weights of the two wheat cultivars after 21 days (Fig. 3) might be partially due to the high overall Na^+/Ca^{2+}-concentration ratio (Fig. 2C), which plays a significant role in inhibiting plant growth (Cramer 2002). This growth inhibition could start during the early growth stage (after 7 days) by the high cytosolic and overall Na^+/Ca^{2+}-concentration ratios (Figs 1D and 2C). Both of them were rebalanced when Ca^{2+} was supplied.

From our observations we can suggest two different mechanisms for salt resistance in wheat:

1 Prevention of uptake: The plant may decrease the permeability of the plasma membrane to Na^+ to minimize Na^+ influx into the cytosol (Anil et al. 2007, Senadheera et al. 2009). In this investigation it is likely that the extra Ca^{2+} addition improved the membrane selectivity for Ca^{2+} over Na^+ in cv. Seds1 and restricted the increase of Na^+ at both cytosolic and overall levels (Figs 1B and 2B). Despite the relatively high overall Na^+ concentrations, the cytosolic concentrations were very low in both cultivars. Physiological data indicate that Ca^{2+} at 0.5 m*M* or higher concentration inhibits the nonselective cation channels, NSCCs (Demidchik and Tester 2002, Kader and Lindberg 2005), which are the dominant transporters for Na^+ influx into cells (Demidchik et al. 2002). Moreover, other investigations showed that uptake of Na^+ into the xylem can be regulated by the sodium transporters AtHKT1:1 and OsHKT1:5a in *Arabidopsis* and rice, respectively, leading to a reduced Na^+ translocation into the shoot (Davenport et al. 2007, Plett et al. 2010 and references therein, Munns et al. 2012). Recent findings by Munns et al. 2012 showed that a similar *HKT1:5* gene, when transformed to durum wheat also caused reduced transport of Na^+ into the leaves and increased yield of this cultivar. Those HKTs genes and several other genes could be upregulated or downregulated by the $[Ca^{2+}]_{cyt}$ level by its influence on transcriptional levels (Galon et al. 2010). In that context and from our present results, it can be suggested that, the reported increase of the leaves overall Ca^{2+} concentration by extra Ca^{2+} addition restricts the Na^+ influx into the plant, the Na^+ translocation to the leaves and the Na^+ influx into the leaf cytosols by improving the overall Ca^{2+} concentration in the leaves, which in turn decreases both cytosolic and overall Na^+/Ca^{2+} ratios. This in turn should improve salinity resistance (Maathuis and Amtmann 1999, Munns and Tester 2008, Senadheera et al. 2009, Genc et al. 2010, Tavakkoli et al. 2011, Munns et al. 2012).

2 Retained ion homeostasis: An effective sequestering of Na^+ into the intracellular compartments like the vacuole, or a fast efflux into the apoplast, are important for salt resistance (Blumwald et al. 2000, Kader and Lindberg 2005, Anil et al. 2007). In wheat also an increase of $[Ca^{2+}]_{cyt}$ can prevent elevation of the $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratio under salinity or keep it at a constant nontoxic level (Fig. 1D). Under salinity (50 m*M*) even without extra Ca^{2+} addition, cv. Seds1 did not significantly change the $[Na^+]_{cyt}$ as did cv. Vinjett (Fig. 1B).

A simplified model is shown in Fig. 4. The increase of external Ca^{2+} increases the overall Ca^{2+} concentration in the leaves and restricts the influx of Na^+ into the cytosol. Under salinity treatment with extra Ca^{2+} addition the increase of Na^+ level in the cytosol triggers downstream reactions in cv. Seds1 starting by an increase of $[Ca^{2+}]_{cyt}$, which may activate the *salt overly sensitive* (SOS) pathway by activation of the Na^+/H^+ antiporter at the plasma membrane (SOS1) (Zhu 2002), as well as at the tonoplast (NHX) (Roos et al. 2006, Kader and Lindberg 2010, Yarra et al. 2012). The Na^+ extrusion from the cytosol by any way is associated with H^+ influx into the cytosol, which in turn activates the H^+-ATPases (Blumwald et al. 2000, Hamilton et al. 2002, Kader et al. 2007) leading to a pH_{cyt} increase with time, as reported for cv. Seds1 (Fig. 1C). In the more sensitive cv. Vinjett, $[Na^+]_{cyt}$ significantly accumulated with no significant pH_{cyt} changes in contrast to the more resistant cultivar cv. Seds1. Further studies on the quantity and activity of the relevant antiporters are needed to better

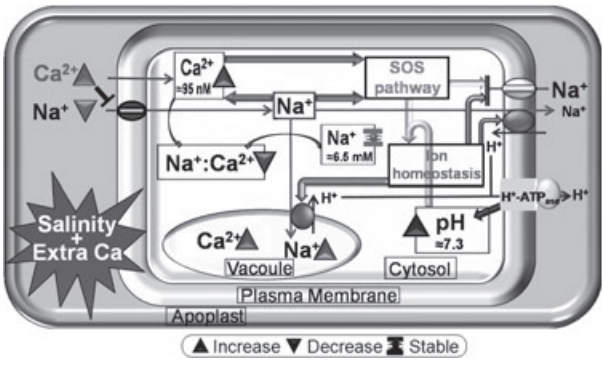

Fig. 4. A proposed model for the $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ and pH_{cyt} changes in the leaf protoplasts of cv. Seds1 after 7 days of salinity and extra calcium addition to the nutrient solution.

understand the plant behavior under salinity with extra Ca^{2+} addition.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Total concentrations of Ca^{2+} and Na^+ ($\mu mol\ g^{-1}$ DW) in leaves of wheat, cvs. Seds1 (A) and Vinjett (B), cultivated under different salinity levels, with and without extra addition of calcium.

Fig. S2. Total concentrations of Ca^{2+} and Na^+ ($\mu mol\ g^{-1}$ DW) in roots of wheat, cvs. Seds1 (A) and Vinjett (B), cultivated under different salinity levels, with and without extra addition of calcium.

Fig. S3. Fresh and dry weights ($mg\ plant^{-1}$) of shoot and whole plant of wheat, cvs. Seds1 (A) and Vinjett (B), cultivated under different salinity levels, with and without extra addition of calcium.

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