

Salt Stress in Rice: Adaptive Mechanisms for Cytosolic Sodium Homeostasis

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Abstract

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This thesis concerns salt stress in plants, particularly sodium (Na^+) stress. A high concentration of Na^+ in the cytosol interferes with many K^+ -binding enzymes and thus, hinders the metabolic activities in cells and is termed as Na^+ (ionic) toxicity. Two important aspects of Na^+ toxicity in rice (*Oryza sativa* L. indica cvs. Pokkali and BRRI Dhan29) cells are described: i) how Na^+ is transported into and out of cells, and ii) how cells react by changing cytosolic Ca^{2+} and pH for activation of the adaptive responses. Under salt stress the ability to reduce Na^+ -influx into the cytosol, and subsequently increase the compartmentalization of cytosolic Na^+ into the vacuole, appeared to be the significant salt-tolerance determinant in the salt-tolerant rice cv. Pokkali. These mechanisms were either absent, or less efficient, in the salt-sensitive rice cv. BRRI Dhan29.

A lower Na^+ -uptake in cv. Pokkali, compared to that in cv. BRRI Dhan29, depended on different types of transporters involved in these two cultivars. In cv. BRRI Dhan29, the transporters mediating Na^+ -influx were K^+ -selective channels and NSCCs, whereas NSCCs were the main pathways for Na^+ -uptake in cv. Pokkali. Apart from the lower Na^+ -uptake, cv. Pokkali seemed to take up Na^+ only transiently and extrude it mainly into the vacuole. Moreover, to maintain a low cytosolic Na^+/K^+ ratio, Pokkali also might have increased K^+ -uptake under salt stress.

The study indicates that Na^+ must be sensed inside the cytosol, before any changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{pH}]_{\text{cyt}}$ occur. Sensing of Na^+ differentially induced $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{pH}]_{\text{cyt}}$ changes in the two rice cultivars. Also, the sources for the changes of $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{pH}]_{\text{cyt}}$ were different in the two rice cultivars. Internal stores in cells like vacuole and ER appeared to be the major sources for $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in cv. Pokkali, whereas the apoplast was more important in cv. BRRI Dhan29. The $[\text{pH}]_{\text{cyt}}$ was differentially shifted in the two rice cultivars, in response to salt stress, and was coupled to different H^+ -ATPases.

Key words: salt stress, Na^+ toxicity, Pokkali, BRRI Dhan29, fluorescence microscopy, Na^+ transport, confocal microscopy; Ca^{2+} and pH signaling.

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*“Water, water, everywhere,
And all the boards did shrink;
Water, water, everywhere,
Nor any drop to drink.”*

From: The Rime of the Ancient Mariner
By Samuel Taylor Coleridge

To my sister *Nazma Ferdousi* and my brother-in-law *Abdul Wahab* who have been my real mentor
to come so far!

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. D'Onofrio, C., Kader, M.A. & Lindberg, S. 2005. Uptake of sodium in quince, sugar beet and wheat protoplasts determined by the fluorescent sodium-binding dye benzofuran isophthalate. *Journal of plant physiology* 162, 421-428.
- II. Kader, M.A. & Lindberg, S. 2005. Uptake of sodium in protoplasts of salt-sensitive and salt-tolerant cultivars of rice, *Oryza sativa* L. determined by the fluorescent dye SBFI. *Journal of experimental botany* 56, 3149-3158.
- III. Kader, M.A., Lindberg, S., Seidel, T., Gollack, D. & Yemelyanov, V. Sodium sensing induces different changes in free cytosolic calcium concentration and pH in salt-tolerant and -sensitive rice cultivars. (Submitted).
- IV. Kader, M.A., Seidel, T., Gollack, D. & Lindberg, S. Expressions of OsHKT1, OsHKT2 and OsVHA are differentially regulated in a salt-sensitive and a salt-tolerant rice cultivars. *Provisionally accepted for publication in the Journal of experimental botany*.

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Introduction

Soil salinity

A soil becomes saline when it contains high concentrations of dissolved mineral salts. This may cause salt stress in plants. A various number of salts e.g. sodium chloride (NaCl), sodium sulphate (Na_2SO_4), sodium nitrate (NaNO_3), magnesium sulphate (MgSO_4), magnesium chloride (MgCl_2), potassium sulphate (K_2SO_4), calcium carbonate (CaCO_3) etc. could be dissolved in saline soil, although NaCl causes most of the salt problems for higher plants in nature. Soil salinity is often measured as electrical conductivity (EC) expressed as milliSiemens/m (for lower salinity) or deciSiemens/m (for higher salinity). A higher salt concentration shows a higher electric conductivity. A soil is called saline when its electrical conductivity exceeds 4 dS/m (USDA, 1954).

Reasons for soil salinity

The origin of salinity in a particular location can be natural (primary salinization) or human-induced (secondary salinization). The source of primary salinization is mostly seawater, since it contains around 500 mM NaCl (Taiz & Zeiger, 2002). Therefore, the area in close vicinity of the sea is vulnerable to salinity. Especially, this concerns areas with tidal water flowing over the areas. The problem becomes acute when this tidal water goes away and soil becomes dry. It may also happen in areas that come in contact with seawater through rivers, canals and creeks. Moreover, cyclones, like that occurred in Bangladesh in 1991 or exceptionally high tides, for example the recent tsunami occurred in Indonesia and Thailand push the saline water front further inland and into the groundwater. Primary salinization also occurs in areas formed from parent rocks with a high concentration of salts.

Apart from these primary sources, soil in a particular area may become saline because of some secondary sources, such as irrigation. A salinization developed in an area that is irrigated over long periods is very often exacerbated by a high evaporation. In many parts of the world, irrigation has been adopted as an important tool to modernize agriculture and improve crop yield, especially in the dry areas. The other sources for secondary salinization are residual salts from soil and water amendments, animal wastes, chemical fertilizers, applied sewage sludges, the disposal of oil and gas field brines etc. From an agricultural point of view salinity is also termed as dryland salinity (occurring on land not subject to irrigation) and irrigated land salinity.

Salt affected areas

The Environment Program of United Nations estimates that approximately 20% of the world's agricultural land and nearly 50% of all irrigated land is adversely affected by soil salinity (Flowers & Yeo, 1995). It is a worldwide problem, but most acute in North and Central Asia, Australia and South America (Pessarakli,

1999). Some of the most serious problems occur in semi-arid regions associated with the great river systems of South-East Asia. In Bangladesh, over 30% of the net cultivable area lies in the coastal zone of Bay of Bengal, of which approximately 53% is affected by varying degrees of salinity (http://www.apctt.org/publication/pdf/tm_dec_grow.pdf#search='Growing%20rice%20in%20saline%20soils'; 21-June-2006). The salt affected area in the coastal zone of the country was about 0.83 million ha in 1966-76, which expanded to 3.1 million ha over the last two decades (<http://www.fao.org/ag/agl/agll/spush/topic2.htm>; 20-June-2006). In addition, more area in that zone is expected to become saline affected in future due to increase in sea water level as a consequence of the greenhouse effect.

The other concern is that the area under irrigation is increasing worldwide day-by-day leaving more area to be affected by salinity stress. As estimated by FAO, about 20-30 million ha of irrigated lands worldwide were seriously damaged in 2002 due to the build-up of salts (Martinez-Beltran & Manzur, 2005). Moreover, in the same investigation it was also reported that every year 0.25-0.50 million ha of irrigated lands worldwide are lost from production due to salts build-up.

Plant species with varying capacity to grow under salt environment

Depending on the ability to grow in saline environment plants can be grouped as halophytes and glycophytes. Halophytes are native to saline soils (around 500 mM NaCl) and able to complete their life cycle in that environment (Colmer, Flowers & Munns, 2006). Glycophytes or non-halophytes, on the other hand, can not survive at a high salt concentration. Most of the agricultural crops are glycophytes, although some of them like sugar beet, barley, wheat etc. can tolerate salt to some extent. Rice is one of the most sensitive plants to salinity with the critical tolerance level 40 mM (Glenn, Brown & Khan, 1997).

Salt stress and its effects on agricultural production

Salt stress is very often referred to as 'Silent Killer' of natural production, since it usually kills plants and soil organisms in the affected areas. It is also termed as 'White Death' because it conjures up white images of lifeless shining lands studded with dead trees. Salt stress is one of the most deleterious environmental factors limiting agricultural productivity worldwide. The effects of salt stress on agriculture can be dramatic. The area in the close vicinity of the seashore is prone to salt stress and thus, agricultural production in those areas is reduced. Salt problem in agricultural crops, however, commonly develops in the irrigated areas when salts from the irrigation water build up in the root zone. Out of the total world's cropland, nearly 17% is under irrigation, but irrigated agriculture contributes to more than 30% of the total agricultural production (Hillel, 2000). Since the cropland under irrigation has been substantially increasing as discussed earlier, the salt stress in irrigated agriculture is a major concern for world food security.

Influences of salt stress on crop growth and yield

Salt stress reduces crop growth and yield in different ways. However, NaCl, the dominant salt in nature, elicits two primary effects on plants: osmotic stress and ionic toxicity. Under normal condition the osmotic pressure in plant cells is higher than that in soil solution. Plant cells use this higher osmotic pressure to take up water and essential minerals in root cells from the soil solution. Under salt stress the osmotic pressure in the soil solution exceeds the osmotic pressure in plant cells due to the presence of high salt, and thus, reduces the ability of plants to take up water and minerals like K^+ and Ca^{2+} (Glenn, Brown & Khan, 1997; Munns, James & Läuchli, 2006). On the other hand, Na^+ and Cl^- ions can enter into the cells and have their direct toxic effects on cell membranes, as well as on metabolic activities in the cytosol (Greenway and Munns, 1980; Hasegawa *et al.*, 2000; Zhu, 2001). These primary effects of salinity stress cause some secondary effects like reduced cell expansion, assimilate production and membrane function, as well as decreased cytosolic metabolism and production of reactive oxygen intermediates (ROs). As a result, in extreme case plants may die under salt stress.

Na^+ toxicity

Growth inhibition by Na^+ toxicity is one of the principal adverse effects of salt stress in plants. The sodium ion (Na^+) is very harmful in cell for most plants when it is present in the cytosol at a concentration higher than the adequate level (1-10 mM). The potassium ion (K^+), on the other hand, is one of the essential and most abundant monovalent cations in cells, and needs to be maintained within 100-200 mM range in the cytosol for efficient metabolic functioning (Walker, Sanders & Maathuis, 1996; Taiz & Zeiger, 2002; Cuin *et al.*, 2003). As a co-factor in cytosol, K^+ activates more than 50 enzymes, which are very susceptible to high cytosolic Na^+ and high Na^+/K^+ ratios (Munns, James & Läuchli, 2006). Therefore, apart from low cytosolic Na^+ , maintenance of a low cytosolic Na^+/K^+ ratio is also critical for the function of cells (Rubio, Cassmann & Schroeder, 1995; Zhu, Liu & Xiong, 1998). At saline conditions, Na^+ competes with K^+ for uptake through common transport systems, since Na^+ and K^+ are physicochemically similar monovalent cations. Thus, elevated levels of cytosolic Na^+ , or in other way high Na^+/K^+ ratios, exert metabolic toxicity by a competition between Na^+ and K^+ for the binding sites of many enzymes (Bhandal & Malik, 1988; Tester & Davenport, 2003). Moreover, at a high concentration, Na^+ can displace Ca^{2+} from the plasma membrane, resulting in a change in plasma membrane permeability. This can be reflected by a leakage of K^+ from the cells (Cramer, Epstein & Läuchli, 1989). This high uptake of Na^+ and leakage of K^+ result in an imbalance in the Na^+/K^+ ratio in the cytosol, which, in turn, leads to many imbalances in enzymatic reactions of the cell. As a consequence of these primary effects, secondary stresses, such as oxidative damage, often occur. In extreme cases these adverse effects contribute to plant growth inhibition and even plant death.

Mechanisms of Na⁺-uptake in plants

Under salt stress, the plant root is the first organ to combat with high Na⁺ in the soil solution. Root tip seems to be the major nutrient uptake region rather than the elongated mature segments of the root (Golldack *et al.*, 2003). Root epidermal cells including root hairs are primary sites for the uptake of inorganic ions through plasma membrane. Sodium ion, like any other mineral nutrients to be taken up by cells, may pass the plasma membrane either in the root epidermal cells or cortical cells. Structurally the plant plasma membrane is a lipid bilayer, which surrounds cell cytoplasm and, in principle, is impermeable to solutes. There are, however, many transport proteins, specific for one or a group of solutes, spanning the lipid bilayer of the plasma membrane and facilitate the movement of solutes in and out of cytosol. In plant cells, plasma membrane potential is negative inside (~-120 to -200 mV), but Na⁺ is a positive ion. Therefore, Na⁺ mainly enters the cytosol through the passive way. When it enters in epidermal cells or cortical cells, Na⁺ may follow a symplastic pathway or apoplastic pathway before it encounters the endodermis layer (Fig. 1). Since the endodermis with the Casparian strip is an effective barrier for apoplastic Na⁺ movement (Pitman, Läuchli & Stelzer, 1981; Peterson & Enstone, 1996; White, 2001; Karahara *et al.*, 2004), Na⁺ enters it symplastically. Thus, membrane transport of root epidermal and cortex cells is a decisive tool for the uptake or rejection of toxic ions like Na⁺ from the environment. Afterwards, Na⁺ enters into the xylem through xylem loading and follows the long-distance xylem transport. Finally, Na⁺ reaches to all cells including metabolically active mesophyll cells following xylem unloading. For nutrients, leaf mesophyll cells exert another membrane transport to entering into the other long-distance pathway in plants, the phloem (Sondergaard, Schulz & Palmgren, 2004) and thus, Na⁺ may also be recirculated in different cells and/or tissues.

Transporters involved in Na⁺-uptake

As stated earlier, the potassium ion (K⁺) needs to be maintained within 100-200 mM range in the cytosol for efficient metabolic functioning. Under this necessity of quite high level of K⁺, plants compromise with the environment, which has a great variation in the concentration of K⁺, by having a large number of K⁺-transporter genes (Maathuis *et al.*, 1997; Mäser *et al.*, 2001, Mäser, Gierth & Schroeder, 2002; Véry & Sentenac, 2002, 2003). Sodium exploits some of these K⁺ transporters to enter the cytosol.

Different uptake mechanisms for Na⁺ into plant cells have been suggested. Non-selective cation channels (NSCCs) are proposed to be the dominant pathways for Na⁺-influx in many plant species (Davenport & Tester, 2000; Demidchik & Tester, 2002; Demidchik, Davenport & Tester, 2002). However, the molecular identity of these NSCCs is still unknown. High-affinity potassium transporters (HKTs) were also suggested to mediate a substantial Na⁺-influx in some species (Uozumi *et al.*, 2000; Horie *et al.*, 2001; Golldack *et al.*, 2002; Mäser *et al.*, 2002; Gárciadeblás *et al.*, 2003). In rice, nine HKT homologues (OsHKT1-9) have been identified of which only OsHKT5 is considered as a non- functional gene, due to the existence

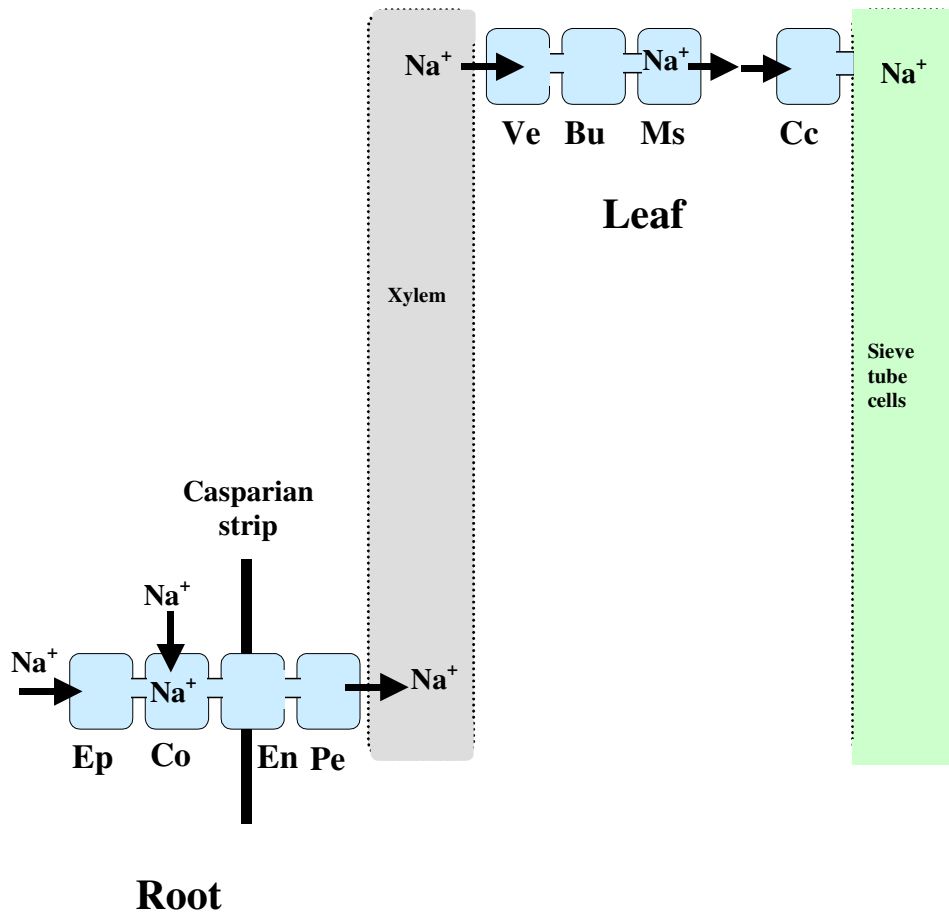


Fig. 1 Mechanisms of Na^+ -uptake in plant roots and its transport in leaf (adopted from Sondergaard, Schulz & Palmgren, 2004). Abbreviations: Ep, epidermis; Co, cortex; En, endodermis; Pe, pericycle; Ve, vessel-associated cell; Bu, bundle sheath cell; Ms, mesophyll cell; Cc, companion cell.

of three stop codons in its mRNA (Gárciadeblás *et al.*, 2003). All of these functional genes encode proteins with distinct transport activities, which might be expressed in various tissues and/or organs. Horie *et al.* (2001) suggested that OsHKT1 encodes a Na⁺-transporter and OsHKT2 a Na⁺/K⁺-coupled transporter. Gárciadeblás *et al.* (2003) also showed that OsHKT1 could be a high affinity Na⁺-transporter and OsHKT4 a low affinity Na⁺-transporter. OsHKT8 has recently been shown to be a Na⁺-transporter, but contributing to the increased ability of salt tolerance by maintaining shoot K⁺ homeostasis under salt stress (Ren *et al.*, 2005; Rus, Bressan & Hasegawa, 2005). This is analogous to the function of AtHKT1 gene in *Arabidopsis*, which is a Na⁺-transporter, and interestingly, plays a very important role in controlling the cytosolic Na⁺ detoxification (Berthomieu *et al.*, 2003; Rus *et al.*, 2004; Sunarpi *et al.*, 2005). AtHKT1 functions in mediating tolerance to salt stress by unloading Na⁺ from xylem vessels to xylem parenchyma cells and thus, protecting the plant leaves from salt stress (Sunarpi *et al.*, 2005). This transporter might also be responsible for unloading of Na⁺ from the phloem (Berthomieu *et al.*, 2003). Therefore, it is very likely that the HKT gene family in rice has an important role for plant ion homeostasis even though some of the members evidently transport Na⁺. Apart from these NSCCs and HKTs, other transport proteins that might be involved in mediating Na⁺ influx under salinity stress are HAK/KT/KUP-type transporters, inward-rectifying potassium channels, and low-affinity cation transporters of the LCT-1 type (Schachtman *et al.*, 1992, 1997; Maathuis *et al.*, 1997; Amtmann & Sanders, 1999; Golldack *et al.*, 2003).

Perception of Na⁺ stress

The sensing of Na⁺ and the subsequent signal transduction to switch on adaptive responses are critical steps for plants exposed to NaCl-dominated salt environments. Under high salinity both osmotic stress and ionic stress are perceived at the cellular level. Until now, however, very little information is known about the sensors in plants for ionic toxicity and osmotic stress under high salt, although several osmosensors are reported to be involved in plant signal perception at osmotic stress (Urao *et al.*, 1999; Reiser, Raitt & Saito, 2003; Tamura *et al.*, 2003). In recent years, a substantial progress has been made regarding plant response under salt stress including the SOS (Salt-Overly-Sensitive) pathway in *Arabidopsis* (Zhu, 2002). But, how crop plants sense Na⁺ toxicity in cells, and whether they sense Na⁺ inside or outside the plasma membrane are still unknown. However, it is speculated that SOS1, a plasma membrane Na⁺/H⁺ antiporter, senses Na⁺ by its long C-terminal tail, predicted to reside in the cytoplasm (Zhu, 2003; Zhang, Creelman & Zhu, 2004; Shabala *et al.*, 2005). It is also speculated that Na⁺ sensors control the [Ca²⁺]_{cyt} level that exerts at least two roles in salt tolerance; a pivotal signaling function (the SOS signaling pathway) for the regulation of Na⁺ homeostasis leading to plant adaptation, and a direct inhibitory effect on the Na⁺ entry system.

Changes in $[Ca^{2+}]_{cyt}$ under salt stress

Cytosolic free Ca^{2+} $[Ca^{2+}]_{cyt}$ plays a key role in a wide range of cellular events including the signal transduction for the activation of adaptive responses against biotic and abiotic stresses. (Trewavas & Malho, 1998; Knight, 2000; Sanders *et al.*, 2002; Gao *et al.*, 2004; Henriksson & Henriksson, 2005). Perception of Na^+ in cells elicits a transient change in $[Ca^{2+}]_{cyt}$ levels. In many studies high concentration of NaCl has been reported to reduce Ca^{2+} concentration in root cells of *Arabidopsis* (Cramer & Jones, 1996; Halperin, Gilroy & Lynch, 2003), and corn root protoplast, within minutes of application of 100 mM NaCl (Lynch & Läuchli, 1988). On the other hand, many studies revealed an increase in $[Ca^{2+}]_{cyt}$ after salt stress (Bittisnich, Robinson & Whitecross, 1989; Lynch, Polito & Läuchli, 1989; Knight, Trewavas & Knight, 1997; Halfter, Ishitani & Zhu, 2000; Zhu, 2001; Gao *et al.*, 2004; Henriksson & Henriksson, 2005). The results are also contrasting in studies whether osmotic stress increases or decreases $[Ca^{2+}]_{cyt}$ (Cramer & Jones, 1996; Knight, Trewavas & Knight, 1997; Kiegle *et al.*, 2000). From the results so far, it can be concluded that the change in $[Ca^{2+}]_{cyt}$ is not uniform and varies with species, cell type or tissue type (Cramer & Jones, 1996). Specific Ca^{2+} signatures are also important for plant cells to follow the subsequent events in the signaling process. Such processes may change with the particular stress (Kiegle *et al.*, 2000), the rate of stress development (Plieth *et al.*, 1999), pre-exposure to the stress (Knight, Trewavas & Knight, 1997) and the tissue type (Kiegle *et al.*, 2000).

The changes in $[Ca^{2+}]_{cyt}$ either can be attributed to the apoplast, or to the internal stores like ER, golgi bodies, mitochondria or vacuole (Sanders *et al.*, 2002). Calcium-permeable channels in the plasma membrane, which are activated by membrane depolarization, are thought to lead to elevation of $[Ca^{2+}]_{cyt}$ in many species after the perception of a range of stimuli (Gelli & Blumwald, 1993; Gelli, Higgins & Blumwald, 1997; Hamilton *et al.*, 2000; Véry & Davis, 2000; White, 2000; Sanders *et al.*, 2002). The generation of Ca^{2+} in the cytosol further modulates other messengers like inositol phosphate, which induces a further Ca^{2+} elevation in the cytosol through the opening of inositol-(1, 4, 5)- triphosphate (IP_3)- regulated Ca^{2+} channels (Sanders *et al.*, 2002). There are some studies showing that salt stress induces a rapid increase in IP_3 concentration in the cytosol (Takahashi *et al.*, 2001; DeWald *et al.*, 2001). By use of the inhibitors verapamil and nifedipine for plasma membrane Ca^{2+} -permeable channels (Polevoi *et al.*, 1996; Babourina, Shabala & Newman, 2000; White *et al.*, 2002; Shishova & Lindberg, 2004) and LiCl for inhibition of Ca^{2+} release from internal stores, like vacuole or ER, through IP_3 -regulated Ca^{2+} -permeable channels (Gillaspy *et al.*, 1995; Knight, Trewavas & Knight, 1996, 1997; Liang, Shen & Theologis, 1996), the major sources for $[Ca^{2+}]_{cyt}$ dynamics could be suggested.

Changes in cytosolic and vacuolar pH under salt stress

The $[Ca^{2+}]_{cyt}$ and pH homeostasis in cells are closely linked (Bush, 1993). Upon shifting of $[Ca^{2+}]_{cyt}$ under salt stress, cells are challenged with the excess of other monovalent ions in the cytosol like H^+ (Plieth, Sattelmacher & Hansen, 1997; Plieth *et al.*, 1999; Gao *et al.*, 2004). Moreover, transient shifts in intracellular and

apoplastic pH are essential steps in several signal transduction processes, and pH is involved in cell signaling, either directly, or in cross talk with plant hormones, or Ca^{2+} (Gilroy & Trewavas, 1994; Ward, Pei & Schroeder, 1995; Blatt & Grabov, 1997; Roos, 2000; Felle, 2001; Gao *et al.*, 2004). Rising of vacuolar pH was obtained in salt-sensitive plants upon exposure to salt stress (Okazaki *et al.*, 1996; Gruwel *et al.*, 2001). Recently vacuolar Ca^{2+} and pH were suggested to control the activity of the tonoplast Na^+/H^+ antiporter (Yamaguchi *et al.*, 2005).

Na^+ homeostasis in the cytosol

Under saline conditions the protection of Na^+ -sensitive metabolic mechanisms in cell cytosol partly depends on the ability to keep cytosolic Na^+ levels low. Apart from the low concentration of cytosolic Na^+ , it is also critical for most of the plants to maintain a correct ratio of cytosolic Na^+/K^+ in order to survive under high NaCl condition (Carden *et al.*, 2003). For plant cells, the most important way of keeping cytosolic Na^+ concentration at a low level is to minimize Na^+ -influx into the cytosol. Sodium entry into plant cells may be restricted either by down-regulating or inactivating the Na^+ -influx channels and transporters in cells. Once Na^+ enters the cytosol at a toxic level, plant cells can deal with the internal Na^+ by sequestering it either into the apoplast or into the vacuole. Cytosolic Na^+ can also be compartmentalized in some other subcellular organelles like the ER and golgi bodies (Jou *et al.*, 2006).

A substantial progress has been made for the last few years regarding the SOS pathway, which plays an important role for maintaining Na^+ homeostasis in the cytosol and thus, to keep this compartment metabolically active under high salt. A possible model (Fig. 2) was outlined for the SOS pathway in *Arabidopsis* (Zhu, 2003; Zhang, Creelman & Zhu, 2004). In this model, an unknown sensor, probably in the plasma membrane, senses salt stress and a signal is transmitted to some type of calcium channel, causing an influx of calcium into the cytosol. However, the control of the activity of tonoplast Na^+/H^+ antiporter (NHX) by SOS3-SOS2 kinase complex, as proposed in this model, has been questioned very recently. It was suggested that the entire C-terminal hydrophilic region of this antiporter resides in the vacuolar lumen (Yamaguchi *et al.*, 2003). Hence, it was proposed that the activity of this protein might be controlled by the changes in vacuolar Ca^{2+} and pH (Yamaguchi *et al.*, 2005).

Apoplastic sequestration of cytosolic Na^+

The plasma membrane SOS1 Na^+/H^+ antiporter is the primary transport system responsible for cellular Na^+ -efflux (Zhu, 2002, 2003). It sequesters Na^+ from cytosol into the apoplast through a secondary active transport energized by a H^+ ATPase in the same membrane. Overexpression of SOS1 increases the salt tolerance in *Arabidopsis* (Zhu, 2002; Shi *et al.*, 2003). In this species it controls Na^+ loading into the xylem of the root and thus, restricts the accumulation of Na^+

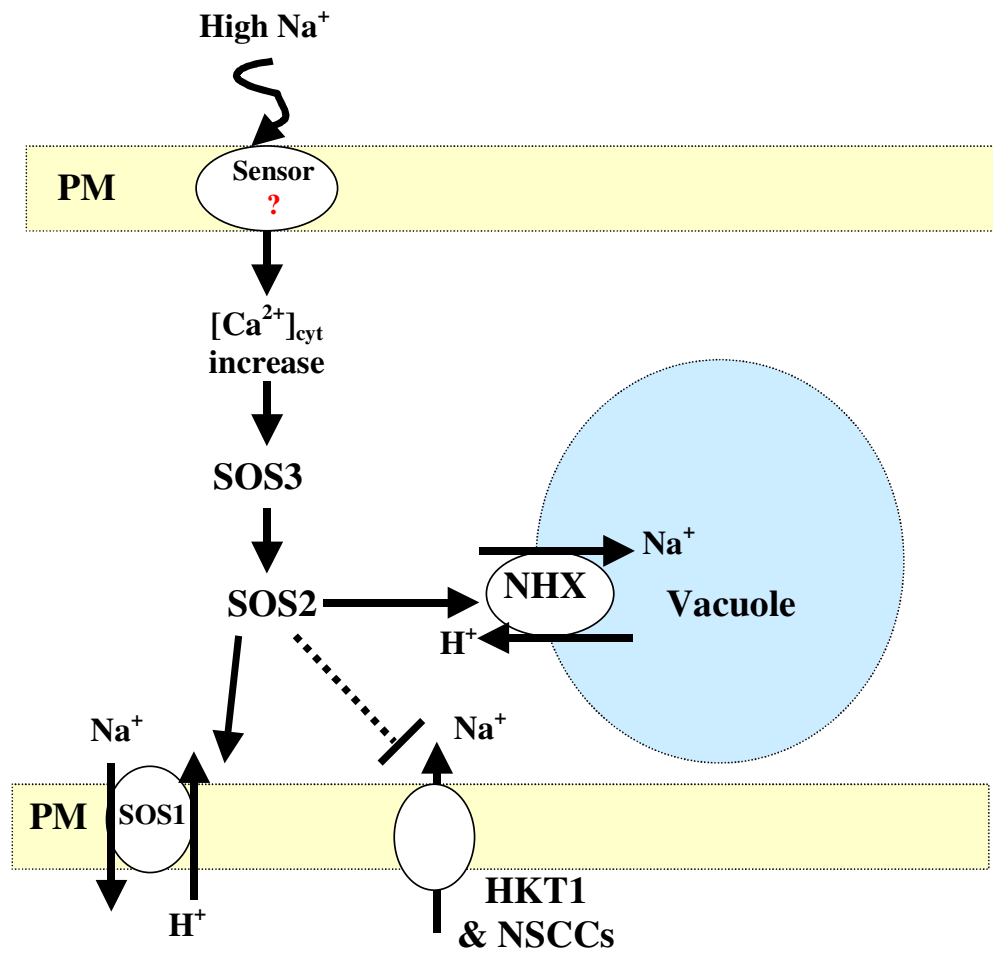


Fig. 2 The proposed model for the regulation of cytosolic Na^+ homeostasis under salt stress by the activation of the SOS signaling pathway (adopted from Zhu, 2003; Zhang, Creelman & Zhu, 2004). In this model, SOS3 is a calcium sensor, which perceives the [Ca^{2+}]_{cyt} changes and activates SOS2 kinase. This SOS3-SOS2 complex then activates SOS1 protein, which is a Na^+/H^+ antiporter in the plasma membrane. It is also suggested that this SOS3-SOS2 complex induces the activity of vacuolar Na^+/H^+ antiporter (NHX) and inhibits Na^+ uptake, either by downregulating HKTs and NSCCs, or inactivating these proteins.

into the shoot (Shi *et al.*, 2002). In addition, SOS1 is localized in the epidermis, particularly in the root tip where ion exclusion is a primary mechanism for the salinity tolerance of cells (Shi *et al.*, 2002). Apoplastic sequestration of Na^+ , however, does not play a role in salt tolerance in rice, since most Na^+ in the rice shoot comes through apoplastic streaming (Yeo *et al.*, 1999).

Vacuolar compartmentalization of cytosolic Na^+

Vacuolar compartmentalization is an efficient strategy for plant cells to cope with salinity stress (Fukuda *et al.*, 1998, 2004; Apse *et al.*, 1999; Blumwald, 2000; Chauhan *et al.*, 2000; Hamada *et al.*, 2001; Tester & Davenport, 2003). When compartmentalized into the vacuole, Na^+ is no more toxic for cells (Flowers & Läuchli, 1983; Subbarao *et al.*, 2003) and also an advantage for growth and osmotic adjustment (Zhu, 2003; Rodriguez-Navarro & Rubio, 2006), particularly since the vacuole may occupy more than 95% of the volume of mature cells. The candidate protein for compartmentalization of Na^+ into the vacuole is the tonoplast Na^+/H^+ -antiporter (NHX), which is energized by the vacuolar H^+ -ATPase (VATPase or VHA) to do so.

VHA, as well as pyrophosphatase, is in general required for the maintenance of intracellular pH and ion homeostasis (Padmanaban *et al.*, 2004). VHA is a protein complex of 13 different subunits with a bipartite structure similar to F-ATP-synthases (Seidel, Golldack & Dietz, 2005). This protein is composed of the membrane integral sector V_0 with subunits VHA-a, c, d and e and the cytoplasmically exposed V_1 sector containing the subunits VHA-A to VHA-H (Fig. 7 in Paper IV). The sector V_0 catalyses the proton transport whereas V_1 is involved in ATP binding and hydrolysis.

Through the hydrolysis of ATP, VHA generates a proton motive force that energizes secondary transports across the vacuolar membrane, like that of Na^+ , under high salt. VHA was shown to be important for salt tolerance in *Saccharomyces cerevisiae* (Hamilton, Taylor & Good, 2002) and in many plant species (Golldack & Dietz, 2001; Kluge *et al.*, 2003; Senthilkumar *et al.*, 2005; Vera-Estrella *et al.*, 2005).

Changes in vacuolar area upon salt stress

Increases in the volume of vacuoles under salt stress could be an important salt adaptation processes (Mimura *et al.*, 2003). Vacuoles, comprising up to 95% volume of mature mesophyll cells (Epimashko *et al.*, 2004), exert an efficient way for the cells to deal with excess cytosolic Na^+ . The pumping of Na^+ into the vacuole by the vacuolar Na^+/H^+ antiporter has an impact on cellular osmolarity by increasing the concentration of Na^+ in the vacuole. A higher Na^+ -concentration in the vacuole may lead to inflow of water and thus, may cause an increase in vacuolar volume under salt stress. In meristematic cells from barley roots, as well as in cells of Mangrove plants, there was a rapid vacuolization upon exposure to salt (NaCl) stress, but at the expense of the cytoplasmic volume (Mimura *et al.*

2003, Gárbarino and Dupon, 1988). Thus, increases in vacuolar volume may serve both in detoxifying cytosolic Na⁺ and increasing cell osmotic pressure.

Aim of the study

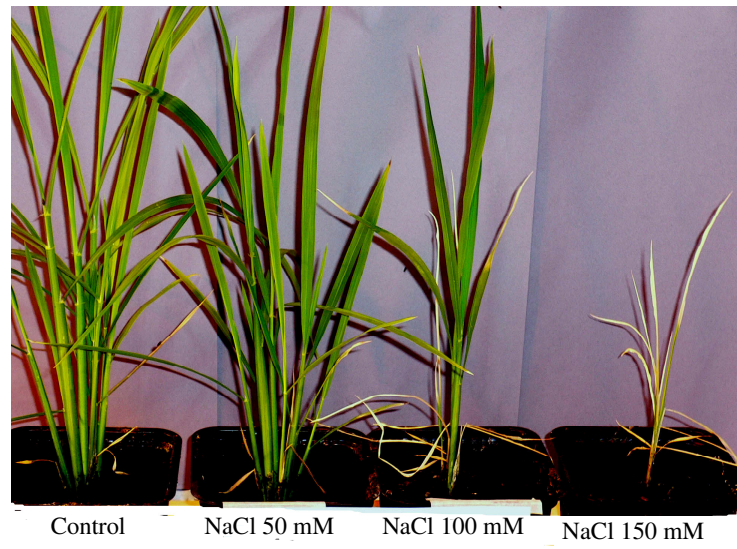
The aim of the present work was to study the mechanisms of salt tolerance in plants. Rice (*Oryza sativa* L.) is the most important food crop in Asia. Rice cultivated in the coastal area of Bangladesh, which comprises 30% of the net cultivable area of the country, is largely affected by varying degrees of salinity (http://www.apctt.org/publication/pdf/tm_dec_grow.pdf#search='Growing%20rice%20in%20saline%20soils'; 21-June-2006). Rice is very sensitive to salt stress. Some traditional cultivars of rice have been found to be more tolerant to salinity but are not suitable for cultivation for their low yield potentiality. Considering the significance of the salt problem in the country, Bangladesh Government recently approved research approaches towards developing high-yielding transgenic rice that can be grown in the salt affected areas. To be able to produce such crops, it is necessary to know how tolerant plants are able to adapt to salt stress and the genes for salt tolerance. It is of fundamental importance to understand the mechanisms by which rice plants take up Na⁺ in cells and how they deal with excess Na⁺ in the cytosol. On the other hand, in terms of adaptive responses, it is of prime importance to understand how rice cells perceive Na⁺ stress and how the signal is transmitted to the downstream cellular machinery. In order to achieve the aim, the objectives of this work were as follows:

- i. To elucidate the mechanisms of Na⁺ uptake in root and leaf cells.
- ii. To investigate how cells perceive Na⁺ stress and subsequently transduce the signal through cytosolic Ca²⁺ and pH changes, and vacuolar pH changes, for the adaptation of downstream responses.
- iii. To delineate the mechanisms of maintaining cytosolic Na⁺ homeostasis upon exposure to salt stress.

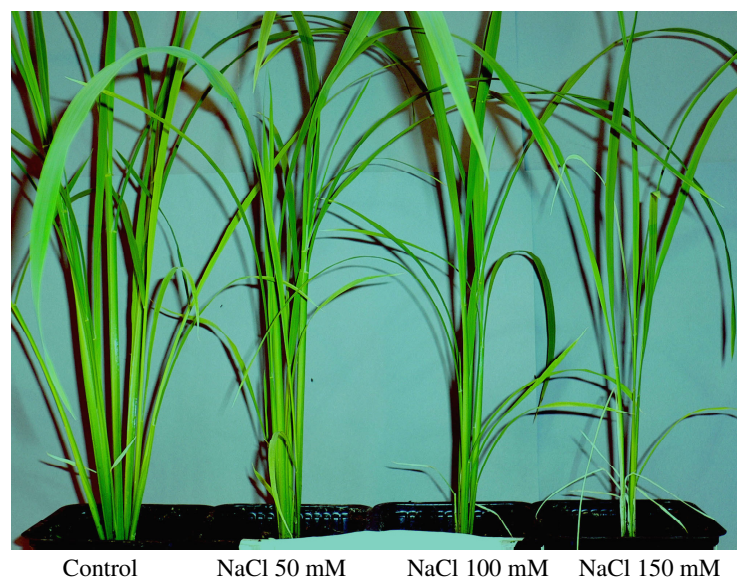
Materials and Methods

Plant materials

To understand the underlying mechanisms for salt tolerance in plants we mainly used a salt-sensitive rice (*Oryza sativa* L. indica) cultivar BRRI Dhan29 and a salt-tolerant rice cultivar Pokklai (Fig. 3). BRRI Dhan29 is currently one of the most popular high yielding rice cultivars among the farmers in Bangladesh due to its high yield potential, but very sensitive to salt stress. On the other hand, Pokkali is tolerant to salt stress to some extent, but not suitable for cultivation for its low yield potentiality.



BRRI Dhan29



Pokkali

Fig. 3 Rice seedlings (cvs. BRRI Dhan29 and Pokkali) cultivated under varying concentrations of NaCl. Photos taken two weeks after the stress.

In this work, we also used to some extent wheat (*Triticum aestivum* L.), sugar beet (*Beta vulgaris* L.) and quince (*Cydonia oblonga* Mill.) species having different tolerance to salt stress.

Isolation of protoplasts

Protoplasts from leaves, and to some extent roots, of the abovementioned species were prepared following a method described by Shishova & Lindberg (1999) with some modifications. The enzymes cellulase (lyophilized powder; 10 units mg⁻¹ solid) from *Trichoderma resei* (Sigma, EC 3.2.1.4) and maserase (lyophilized powder; 0.6 units mg⁻¹ solid), Macerozyme R-10 from *Aspergillus japonicus* (Serva, EC 3.2.1.4) were used to isolate the protoplasts from leaf/root sliced to 0.5 mm pieces. The concentration of these enzymes varied depending on the species used.

Loading of protoplasts with ion-specific dyes

To monitor the dynamics of cytosolic ions like Na⁺, Ca²⁺ and H⁺ under salt stress the isolated protoplasts were loaded with the ion-specific dyes SBFI-AM for Na⁺, Fura 2-AM for Ca²⁺ and BCECF-AM for H⁺. For the measurement of vacuolar H⁺ concentration, the isolated protoplasts were loaded with 6-CFDA.

Before any dye loading the protoplasts were washed twice in the loading medium containing 0.5 M sorbitol (Sigma, St Louis, MO, USA), 0.1 mM CaCl₂, 0.2% (w/v) polyvinylpyrrolidone (PVP, Sigma, St Louis, MO, USA) and a buffer (pH 5.5: medium A) containing 5 mM TRIS (Labassco, Germany) and 5 mM MES (Sigma, St Louis, MO, USA).

The SBFI-AM (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulphoxide (DMSO, Merck, Eurolab AB, Stockholm, Sweden (<0.1% water) to give a 5 mM stock solution. Two microlitres of the stock solution was diluted with 6.75 µl ethanol (Kemetyl, Stockholm, Sweden) and 1.25 µl pluronic F-127 (Molecular Probes) as described by Poenie *et al.* (1986), and added to 1 ml of protoplast suspension to get a final concentration 10 µM. The Fura 2-AM (Molecular Probes, Leiden, The Netherlands) solution was prepared by mixing 2 µL of Fura 2-AM stock solution (5mg / ml) in dry (<0.1% v/v water) DMSO (dimethyl sulfoxide), 1.25 µL of pluronic F-127 (Molecular Probes) solution (20% w/v) in DMSO and 6.75µL of ethanol (99.5% v/v) (Sebastiani, Lindberg & Vitagliano, 1999). From the above mixed Fura 2-AM dye solution, 5 µL was added to 1 ml of protoplast suspension. Protoplasts were loaded with BCECF-AM in the same way as for Fura 2-AM, but without any pluronic. For vacuolar 6-CFDA loading the protoplasts were stained with 3 µL of 5 mM 6-carboxyfluorescein diacetate (6-CFDA, Sigma-Aldrich) in DMSO through PEG-mediated osmotic shock (Seidel, Golldack & Dietz, 2005).

Dye loading was performed in darkness in medium A at room temperature for 3 to 4 h with SBFI-AM, at 22°C for 2 to 3 h with Fura 2-AM and at 4°C for 1 h with

BCECF-AM. After loading, the samples were centrifuged and pellets were resuspended into 1 ml of a solution similar to medium A, but with TRIS-MES buffer at pH 7 (medium B). Before measurements, samples were kept in darkness at room temperature for 25 min.

Fluorescence measurements in cytosol and vacuole

For cytosolic fluorescence measurements an epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochen, Germany), supplied with an electromagnetic filter-exchanger (Zeiss), Xenon lamp (Zeiss XBO 75), photometer (Zeiss 01), microprocessor (MSP 21, Zeiss), and a personal computer was used. The fluorescence intensity was determined after excitation at 340/380 nm for SBFI and Fura 2 and at 485/436 nm for BCECF. Emission wavelengths were 530-550 nm for SBFI, 500-530 nm for Fura 2 and 510-550 nm for BCECF.

A confocal microscope (Leica SP2, Heidelberg) was used to measure 6-CFDA fluorescence from the vacuoles. The dye was excited sequentially by using the 458 and 488 nm lines of an argon-ion laser and the signal was detected in the range of 500-530 nm after passing the short pass filter RSP500 (Leica).

FRET measurements

The constructs of VHA subunits VHA-a, VHA-c, VHA-E and VHA-B of *Mesembryanthemum crystallinum* fused to fluorescent proteins YFP and CFP were introduced into isolated rice protoplasts by transfection. Fluorescence resonance energy transfer was measured by confocal microscopy (Leica SP2, Heidelberg) following the method described by Seidel, Golldack & Dietz, 2005).

Expression analysis

Expressions of OsHKT1, OsHKT2 and OsVHA at transcripts level were studied by real-time RT-PCR and *in situ* PCR (details are written in Paper IV).

Results and Discussion

Na⁺-uptake in salt-sensitive and -tolerant species (Paper I)

The Na⁺-uptake in mesophyll protoplasts was compared in three different plant species having variation in tolerance level to salt stress, such as quince (*C. oblonga* Mill), sugar beet (*B. vulgaris* L.) and wheat (*T. aestivum* L.). Quince is a highly tolerant halophyte whereas sugar beet is less tolerant halophyte and wheat is a glycophyte, less tolerant to salinity than sugar beet. It was found that little Na⁺ was taken up in quince compared to that in sugar beet or wheat. Na⁺-uptake into the cytosol of these three species was in the following order: wheat > sugar beet > quince. Moreover, quince protoplasts took up Na⁺ only temporarily and seemed to have a mechanism for fast extrusion of cytosolic Na⁺. The main efflux of Na⁺ from the cytosol in quince seemed to occur at the tonoplast, not at the plasmalemma.

Protoplasts from quince seedlings pre-cultivated with 200 mM NaCl showed no Na⁺-uptake at all. Moreover, Na⁺-uptake in quince, as well as in wheat, was inhibited substantially by 1.0 mM extra cellular Ca²⁺. In these three species, the mechanisms for maintaining a low cytosolic Na⁺, either by decreasing Na⁺-uptake into the cytosol, and/or by increasing Na⁺ compartmentalization into the vacuole, seem to be important for their different tolerance levels.

Na⁺-uptake differs between salt-sensitive and -tolerant rice cultivars (Paper I & II)

The salt-tolerant rice cv. Pokkali also was able to take up less Na⁺ into the cytosol compared to the salt-sensitive cv. BRRI Dhan29. The total cytosolic Na⁺-concentration in Pokkali protoplasts was approximately half of that in BRRI Dhan29, after addition of 5, 50 and 100 mM extra cellular NaCl. Furthermore, the uptake of Na⁺ in Pokkali protoplasts upon extra cellular NaCl addition was transient, whereas Na⁺ uptake in BRRI Dhan29 protoplasts was stable, especially after higher salt addition, like 50 and 100 mM NaCl. In Pokkali, Na⁺-uptake was even lower in root protoplasts, compared to that in shoot protoplasts, although the cytosolic Na⁺ taken up was nearly stable after each addition of NaCl. On the other hand, Na⁺-uptake was similar in root and shoot protoplasts of BRRI Dhan29. After pretreatment of seedlings in saline conditions, protoplasts from Pokkali showed reduced Na⁺-uptake, whereas BRRI Dhan29 did not show any variation in Na⁺ uptake compared to protoplasts from untreated plants. Under saline condition, the ability to keep a low cytosolic Na⁺-concentration appears to be an important trait of the salt-tolerant rice cv. Pokkali, in the same way as in the salt-tolerant halophytic plant quince. Pokkali seems to maintain a low cytosolic Na⁺ by reducing Na⁺-uptake of into the cytosol, and also by extruding Na⁺ from the cytosol. The ability to maintain a low concentration of Na⁺ in the cytosol has been shown to be an important salt-tolerance criterion in many studies (Maathuis & Sanders, 2001; Flowers & Hajibagheri, 2001; Carden *et al.*, 2003; Golldack *et al.*, 2003).

Transporters involved in Na⁺-uptake into the cytosol (Paper II & IV)

The major proteins involved in Na⁺-uptake in cells are NSCCs and HKTs (Amtmann & Sanders, 1999; Uozumi *et al.*, 2000; Horie *et al.*, 2001; Demidchik & Tester, 2002; Demidchik, Davenport & Tester, 2002; Golldack *et al.*, 2002; Mäser *et al.*, 2002; Garciadeblás *et al.*, 2003). The functions of NSCCs are inhibited by Ca²⁺ at 0.5 mM or higher concentrations (Amtmann & Sanders, 1999; Demidchik & Tester, 2002). These channels are insensitive to most organic blockers, which inhibit different classes of cation channels, such as TEA for K⁺-selective channels (Demidchik & Tester, 2002).

In the present study, there was a substantial Na⁺-influx probably mediated by NSCCs in wheat and quince, since 1.0 mM extra cellular Ca²⁺ blocked Na⁺ influx into the cytosol in these species. NSCCs were also found to play substantial role in Na⁺-uptake in both salt-sensitive and -tolerant cultivars of rice. In Pokkali protoplasts Na⁺-uptake was inhibited in the presence of inhibitors for NSCCs like 1 mM Ca²⁺, Zn²⁺ or La³⁺. All of these inhibitors exerted a partial inhibition of the uptake of Na⁺ in cv. BRRI Dhan29. On the other hand, inhibitors for K⁺-selective channels, such as 1.0 mM TEA or 10 mM Cs⁺ did not cause any inhibition of Na⁺-uptake in cv. Pokkali, but caused a significant reduction in cv. BRRI dhan29. The inhibitor studies suggested that different channels or transporters in these cultivars mediate the Na⁺-influx into the cytosol. Both K⁺-selective channels and NSCCs may be involved in mediating Na⁺-uptake in the salt-sensitive rice cv. BRRI Dhan29 whereas NSCCs are the main pathways for Na⁺-influx in cv. Pokkali. Pokkali seemed to lack the Na⁺-transport through K⁺-selective channels or transporters. However, the results from expression studies showed that the Na⁺-transport protein OsHKT1 was induced only 24 h after the stress in cv. Pokkali and 1 and 6 h after the stress in cv. BRRI Dhan29. Moreover, the induction was higher in cv. BRRI Dhan29 compared to that in cv. Pokkali. In Pokkali, however, the transcript of OsHKT1 showed a down-regulation 48 and 72 h after the stress. Although OsHKT1 is a Na⁺ transporter, it specifically mediates Na⁺-uptake in rice roots when the plants are K⁺ deficient (Garciadeblás *et al.*, 2003), and is induced by low-K⁺ conditions (Horie *et al.*, 2001). Since the experimental plants in this study were grown with an optimal K⁺ concentration in the growth medium, deficiency of K⁺ in cells under control condition is unlikely. Under salt stress, Na⁺ competition at binding sites for K⁺ may result in K⁺ deficiency (Maathuis & Amtmann, 1999), and thus, cause the induction of OsHKT1 in both the cultivars. It can not be excluded that under high NaCl, when excess Na⁺ enters the cytosol, the optimal ratio of cytosolic Na⁺/K⁺ increases causing cells to sense a K⁺ deficiency, and thus, induces the OsHKT1. For example 48 h after the stress, the salt-tolerant cv. Pokkali started to down-regulate the OsHKT1 expression both in roots and shoots. A down-regulation of the OsHKT1 in leaf mesophyll cells in Pokkali under salt stress, as shown by *in situ* PCR expression analyses, might hinder Na⁺-influx into these metabolically very important cells and thus, confer tolerance to salt stress. The down-regulation of OsHKT1 in response to salt stress was also shown by Horie *et al.* (2001) and Golldack *et al.* (2002).

Na⁺ stress is sensed inside the cytosol (Paper III)

Salt stress, like many other abiotic and biotic stresses, induces a transient change in cytosolic Ca²⁺ [Ca²⁺]_{cyt} concentrations (mostly increase with different amplitudes) soon after the stress is sensed. Upon addition of 100 mM NaCl, there was a transient increase in [Ca²⁺]_{cyt} in the presence of 0.1 mM extra cellular Ca²⁺. This change in [Ca²⁺]_{cyt} may occur due to influx of Na⁺ in cells and thus, is caused by Na⁺ stress. Addition of sorbitol of the same osmolarity as 100 mM NaCl did not cause any [Ca²⁺]_{cyt} increase. In the presence of 1.0 mM extra cellular Ca²⁺ or 1.0 mM Zn²⁺, NaCl addition did not result in any increase in [Ca²⁺]_{cyt} in Pokkali protoplasts. In cv. Pokkali, NSCCs are the main pathways for cytosolic Na⁺ influx. NSCCs mediated Na⁺-influx is inhibited by a wide range of inorganic cations like Ca²⁺, Zn²⁺ and La³⁺ (Demidchik & Tester, 2002). Addition of 100 mM NaCl to Pokkali protoplasts, in the presence of 1.0 mM extra cellular Ca²⁺ or Zn²⁺, does not cause any Na⁺-influx in the cytosol, and subsequently no response in [Ca²⁺]_{cyt} dynamics. Therefore, it is likely that at this condition the cells do not sense Na⁺ until Na⁺ enters the cytosol. Thus, it can be suggested that Na⁺ stress is sensed inside the cytosol. This is consistent with the current idea that sensing of Na⁺ occurs by the long C-terminal tail of the SOS1 protein resided in the cytosol (Zhu, 2003; Zhang, Creelman & Zhu, 2004; Shabala *et al.*, 2005). Rice SOS1 protein is a functional equivalent of the *Arabidopsis* SOS1 (Pardo *et al.*, 2006).

Salt stress induces [Ca²⁺]_{cyt} changes (Paper III)

Soon after sensing Na⁺ stress inside the cytosol, possibly through the SOS1 protein, changes in [Ca²⁺]_{cyt} dynamics evidently occurred in rice. In the presence of 0.1 mM extra cellular Ca²⁺, addition of 100 mM NaCl induced a transient increase in free cytosolic Ca²⁺ both in cvs. BRRI Dhan29 and Pokkali, but an one-phase increase in cv. BRRI Dhan29 and a two-phase increase in cv. Pokkali. The opposite effect was obtained when 200 mM sorbitol (of the same osmolirity) was added. Osmotic stress mimicked by sortbitol, in fact, caused a decrease in [Ca²⁺]_{cyt} concentration. Therefore, the increase in [Ca²⁺]_{cyt} depended on ionic stress.

The increase in [Ca²⁺]_{cyt} in cv. BRRI Dhan29 became more pronounced in the presence of 1.0 mM Ca²⁺, compared to that in the presence of 0.1 mM extra cellular Ca²⁺. This might indicate, that in this cultivar, a substantial amount of Ca²⁺ enters the cytosol from apoplast upon salt stress, which was proven by the inhibitor study. Pretreatment of BRRI Dhan29 protoplasts with verapamil and nifedipine (believed to block Ca²⁺ influx from the apoplasts; Polevoi *et al.*, 1996; Babourina, Shabala & Newman, 2000; White *et al.*, 2002) lowered the increase in [Ca²⁺]_{cyt} concentration. On the other hand, LiCl, believed to block Ca²⁺ influx from intracellular stores (Gillaspy *et al.*, 1995; Knight, Trewavas & Knight, 1996, 1997; Liang, Shen & Theologis, 1996), little affected the changes of [Ca²⁺]_{cyt} in this cultivar.

The increase in [Ca²⁺]_{cyt} could be blocked also in Pokkali by use of inhibitors both for plasma membrane Ca²⁺-channels and IP₃-regulated Ca²⁺-channels in the internal membranes. However, pretreatment of protoplasts with LiCl blocked the

maximum amplitude of $[Ca^{2+}]_{cyt}$ increase and average $[Ca^{2+}]_{cyt}$ increase by 77% and 72%, respectively, compared to the non-treated control protoplasts. On the other hand, the blocking of average $[Ca^{2+}]_{cyt}$ increase by inhibitors of plasma membrane Ca^{2+} -channels were only 28% and 30% for varapamil and nifedipine, respectively, compared to non-treated control protoplasts. It is, therefore, likely that internal cell organelles in cv. Pokkali like vacuole, ER etc. contribute substantially more than the apoplast to $[Ca^{2+}]_{cyt}$ increases.

Changes in cytosolic and vacuolar pH occur upon salt stress (Paper III)

Salt stress, ionic stress in particular, induced changes of cytosolic and vacuolar pH in different ways in the salt-tolerant cv. Pokkali and salt-sensitive cv. BRRI Dhan29. Upon addition of 100 mM NaCl, the cytosolic pH increased in the salt-tolerant cv. Pokkali, whereas it decreased in the salt-sensitive cv. BRRI Dhan29. On the other hand, NaCl addition significantly decreased the vacuolar pH in cv. Pokkali and increased it a little in cv. BRRI Dhan29, within 5 to 10 min after salt addition. Therefore, it is likely that salt addition to cv. Pokkali causes proton transport from the cytosol into the vacuole, probably by activation of the vacuolar H^+ -ATPase (VHA), as suggested by the inhibitor study. When the protoplasts of cv. Pokkali were pretreated with NH_4NO_3 , an inhibitor of VHA, a significantly lower increase in cytosolic pH was obtained. On the other hand, no significant changes were obtained in vacuolar pH, and thus, of VHA activity in BRRI Dhan29 upon salt addition. This indicates that there is no movement of protons between the cytosol and vacuole. This result is consistent with the expression of rice vacuolar H^+ -ATPase (OsVHA) in cvs. Pokkali and BRRI Dhan29. Upon salt stress there was an immediate induction of OsVHA in cv. Pokkali, but not in cv. BRRI Dhan29. However, the significant decrease in vacuolar pH at 24 h after salt addition in cv. BRRI Dhan29 may reflect an activation of VHA, which is consistent with the induction of OsVHA in this cultivar 24h after NaCl stress. It is, however, likely that immediately after NaCl stress proton movement occurs mainly within the apoplast and cytosol in this cultivar, since NH_4VO_3 caused an inhibition of the plasma membrane H^+ -ATPase and thus, a lower shift in cytosolic pH. Osmotic stress (mimicked by sorbitol), however, did not cause any significant alterations in cytosolic pH of these cultivars (data not shown).

The increase in cytosolic pH in the salt tolerant cultivar is consistent with the result obtained in our lab for quince, one very salt-tolerant halophyte (C. D'Onofrio and S. Lindberg, unpublished result). The increase in cytosolic pH is, however, contrasting with the result of Halperin, Gilroy and Lynch (2003) and Gao *et al.* (2004). Halperin, Gilroy and Lynch (2003) did not find any change in cytosolic pH in *Arabidopsis* roots, whereas Gao *et al.* (2004) found a decrease in cytosolic pH in *Arabidopsis* upon salt stress. Our result concerning the decrease in cytosolic pH in the salt-sensitive cv. BRRI Dhan29 is, however, consistent with Gao *et al.* (2004). Increases in vacuolar pH have been reported in some salt-sensitive species (Okazaki *et al.*, 1996; Gruwel *et al.*, 2001). Therefore, the

increase of the cytosolic pH and decrease of the vacuolar pH under salt stress in cv. Pokkali could be the trait for salt-tolerant plants.

Salt stress and structural changes of OsVHA (Paper IV)

The signaling for regulation of the activity of VHA is transduced from the V_1 domain to V_0 via conformational changes in VHA-a (Landolt-Marticorena *et al.*, 1999). But, there is no report so far for the structural changes of VHA upon salt stress. However, some data of ATP hydrolysis and proton transport indicated that changes in the coupling ratio of VHA occur upon salt stress (Ratajczak, 2000). In the V_1 domain, the FRET-measurement between the peripheral stalk subunit VHA-E and the catalytic head subunit VHA-B in cv. Pokkali showed a significant higher efficiency than in cv. BRRI Dhan29. This indicates structural differences between these two proteins in these cultivars. But the FRET efficiency between VHA-E and VHA-B was not affected by salt stress in any of the rice cultivars. On the other hand, in the transmembrane sector V_0 the FRET efficiency between the proteolipid VHA-c and the C-terminal transmembrane domain of VHA-a significantly increased in cv. BRRI Dhan29 due to salt stress, but decreased a little in cv. Pokkali. The increased FRET efficiency between VHA-E and VHA-B in cv. Pokkali compared to cv. BRRI Dhan29, and the differential changes of FRET efficiencies between VHA-a and VHA-c upon salt stress in these two cultivars might be correlated with their differential Na^+ -compartmentalization into the vacuole and thus, differential salt tolerance.

Vacuoles in cells increase their volume upon salt stress (Paper IV)

The vacuolar area in leaf cells (protoplasts) was also investigated upon salt stress. Vacuoles, comprising up to 95% of cell volume in mature cells, are thought to be a good sink for compartmentalization of cytosolic Na^+ . Therefore, changes in the vacuolar area could be evident under salt stress. However, there was no significant change in the vacuolar area in the salt-tolerant rice cv. Pokkali upon salt stress, both at a short-term (5-10 min) and a long-term (24 h) treatments. On the other hand, the vacuolar area in the salt-sensitive rice cv. BRRI Dhan29 decreased after 5-10 min NaCl stress and then increased after 24 h stress compared to the control. Without stress treatment the vacuolar area was, however, larger in cv. Pokkali than in cv. BRRI Dhan29. This might indicate that the salt-tolerant rice cv. Pokkali by its nature is more capable of compartmentalizing Na^+ into the vacuole than salt-sensitive cv. BRRI Dhan29.

Excess cytosolic Na^+ is differentially treated in salt-sensitive and -tolerant rice cultivars (Paper I, II and IV)

At salt stress, the ratio of cytosolic Na^+/K^+ is disrupted in many higher plants, since the concentration of Na^+ is much higher than at normal condition. Salt-tolerant species recover this Na^+/K^+ homeostasis mostly by reducing the cytosolic Na^+ -

concentration or by increasing cytosolic K^+ -concentration. However, cytosolic K^+ -concentration is also harmful when it exceeds the normal range (Greenway & Osmond, 1972). Once Na^+ enters the cytosol, salt-tolerant plants either can pump it out of the cells, or compartmentalize it into various sub-cellular organelles like the vacuole.

In the salt-tolerant halophyte quince, the mechanisms to combat salt stress include a lower Na^+ -uptake in cells and a compartmentalization of cytosolic Na^+ into the vacuole. Both these mechanisms also appear in salt-tolerant rice cv. Pokkali, but not in the salt-sensitive cv. BRRI Dhan29. At the on-set of salt stress, cv. Pokkali took up Na^+ through NSCCs, but not by K^+ -selective channels. Immediately after salt exposure, Pokkali reduced the Na^+ -uptake by K^+ -selective channels, possibly by inducing a conformational change in the K^+ -selective channels. At later stage, Pokkali might reduce the Na^+ -uptake by downregulating the expression of Na^+ -transporters like OsHKT1. However, at the onset of salt stress, Pokkali compartmentalizes cytosolic Na^+ into the vacuole, as suggested from the inhibitor study using NH_4VO_3 , an inhibitor of plasma membrane H^+ -ATPase, or NH_4NO_3 , an inhibitor of vacuolar H^+ -ATPase, or both NH_4VO_3 and NH_4NO_3 . The vacuolar compartmentalization of Na^+ is pronounced in the salt-tolerant cv. Pokkali, but not in the salt-sensitive cv. BRRI Dhan29. Although apoplastic sequestration of cytosolic Na^+ seemed to work in the salt-sensitive cv. BRRI dhan29, this is not an efficient strategy for salt tolerance in rice. It has been shown previously that most of the Na^+ in rice leaves comes through apoplastic streaming (Yeo *et al.*, 1999).

Vacuolar compartmentalization of cytosolic Na^+ in cv. Pokkali, but not in cv. BRRI Dhan29, corresponds with the expression of vacuolar H^+ ATPase (OsVHA) under salt stress in these two rice lines. OsVHA was induced both in root and shoot tissues immediately after salt stress in cv. Pokkali, but not until 6 h after the stress in cv. BRRI dhan29. The induction was also higher in cv. Pokkali than that in cv. BRRI Dhan29. In both the lines, however, the induction was higher in the shoot tissue than root tissue.

This study suggests the involvement of OsHKT2 in salt-stress response, especially in the salt-tolerant cv. Pokkali. There was a substantial induction (some 15-folds higher compared to control) of OsHKT2 in the shoot of salt-tolerant cv. Pokkali and to a lesser extent in the root of the same cultivar, but not in the salt-sensitive cv. BRRI Dhan29. Although OsHKT2 (K^+ - Na^+ coupled transporter) does not mediate K^+ -influx from a high K^+ solution in the absence of Na^+ , it confers tolerance to salinity under high Na^+ , probably by increased ability of K^+ -uptake, as shown in *Saccharomyces cerevisiae* (Horie *et al.*, 2001). In the present study, the induction of OsHKT2 in epidermis, exodermis and vascular tissue in roots might indicate its involvement in K^+ -uptake. The expression of OsHKT2 in the phloem and the transition from phloem to mesophyll cells, along with mesophyll cells, may indicate its involvement in the recirculation of K^+ within the mesophyll cells through the phloem. In addition to metabolites such as sugars, salts also can use the phloem pathway to be redistributed from old source leaves towards young and expanding sink leaves (Sondergaard, Schulz & Palmgren, 2004). Thus, the

induction of OsHKT2 in the salt-tolerant cv. Pokkali might confer salt tolerance by increasing its expression in leaf, through contributing to a low cytosolic Na^+/K^+ ratio, as suggested by Horie *et al.* (2001). The upregulation of K^+ -transporter genes upon salt stress possibly reflects the plants ability to maintain certain cytosolic K^+ levels to survive under salt stress (Su *et al.*, 2001, 2002; Pilot *et al.*, 2003; Maathuis, 2006). Since K^+ , at a high concentration, also is inhibitory for enzymatic functions in the cytosol (Greenway & Osmond, 1972), the induction of OsHKT2 in Pokkali shoot decreased after some stress period.

The ability to maintain ionic homeostasis under salt stress was shown to be an important salt-tolerance determinant in barley, than in the moderate salt-sensitive rice cultivar IR64 (Ueda *et al.*, 2006). The present study revealed that the regulatory mechanism for controlling K^+/Na^+ homeostasis in cells of the salt-tolerant cv. Pokkali seemed to be working by increasing the K^+ uptake (by inducing the expression of OsHKT2) along with the mechanism for compartmentalizing of cytosolic Na^+ into the vacuole (by inducing the expression of OsVHA), and later on by reducing Na^+ influx (by decreasing the expression of OsHKT1). These mechanisms were not as efficient in the salt-sensitive cv. BRRI Dhan29 as in cv. Pokkali.

Conclusion

As shown in Fig 4, the ability to maintain a low cytosolic Na^+ under salinity stress appears to be an important salt tolerance determinant in plants. Salt-tolerant rice cv. Pokkali possesses some mechanisms contributing towards the cytosolic Na^+ homeostasis, which are either absent, or less efficient, in the salt-sensitive cv. BRRI Dhan29. By a reduced Na^+ -uptake cv. Pokkali keeps a lower level of cytosolic Na^+ than cv. BRRI Dhan29. The difference depends on that K^+ -selective channels take part in the Na^+ -uptake in cv. BRRI Dhan29, but not in cv. Pokkali. The induction of a Na^+ -transporter, OsHKT1, 24h after salt stress in cv. Pokkali, is against this concept. It is, however, not unlikely that at the onset of salt stress cv. Pokkali hinders Na^+ -uptake, mediated by K^+ -selective channels, by changing the structure of these proteins. Moreover, later on (48 h after the stress) cv. Pokkali down-regulates the expression of OsHKT1, possibly to block the Na^+ -uptake. Pokkali might also keep a low ratio of cytosolic Na^+/K^+ by increasing the K^+ -uptake most likely through the up-regulation of OsHKT2. Non-selective cation channels (NSCCs), however, play a vital role in Na^+ -uptake both in cv. Pokkali and cv. BRRI Dhan29.

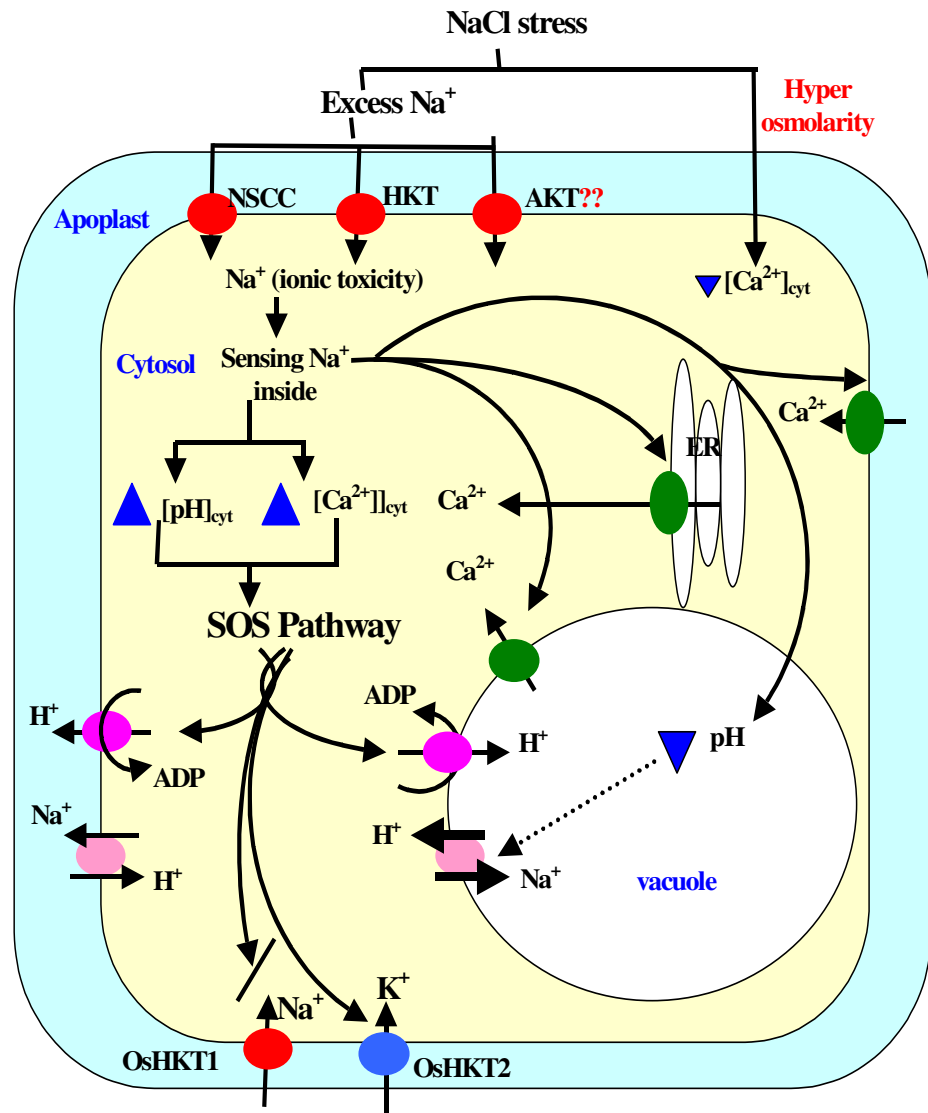


Fig. 4 Possible model for perception of Na⁺ stress and subsequent changes in $[Ca^{2+}]_{cyt}$ and $[pH]_{cyt}$ and vacuolar pH for the adaptation of Na⁺ homeostasis in cytosol.

Once Na^+ enters into the cytosol to a toxic level, Na^+ is sensed inside the cytosol and subsequently induces the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase. Both the apoplast and intracellular parts of the cell contribute towards the shift in $[\text{Ca}^{2+}]_{\text{cyt}}$ concentration in cv. Pokkali. In BRRI Dhan29, the apoplast part seems to be the major source for $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics. It is likely, that the $[\text{pH}]_{\text{cyt}}$ dynamics are connected with $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in the stress signaling. Salt stress increases $[\text{pH}]_{\text{cyt}}$ in cv. Pokkali, whereas it decreases $[\text{pH}]_{\text{cyt}}$ in cv. BRRI Dhan29. Mainly the tonoplast H^+ -ATPase contributes towards the $[\text{pH}]_{\text{cyt}}$ increase in cv. Pokkali and the decrease in vacuolar pH. On the other hand, the plasma membrane Na^+/H^+ antiporter causes $[\text{pH}]_{\text{cyt}}$ decrease in cv. BRRI Dhan29.

The most important mechanism in cv. Pokkali for salt tolerance is to keep cytosolic Na^+ at a low level by compartmentalizing it into the vacuole. Once Na^+ reaches the toxic level in the cytosol, Pokkali cells compartmentalize it into the vacuole, as shown by inhibitor analysis. This corresponds with the induction of vacuolar H^+ ATPase (OsVHA) in cv. Pokkali immediately after salt stress. This enzyme energizes the tonoplast Na^+/H^+ antiporter. Vacuolar compartmentalization of Na^+ is also present in cv. BRRI Dhan29, but to a lesser extent and much later than in cv. Pokkali.

Future Perspectives

Salt-tolerance in plants is a multigenic trait with many quantitative trait loci (QTLs) associated with ion transport and tolerance (Flowers, 2004). For example, the recent identification of a major QTL for salt tolerance in rice encodes a Na^+ -transporter OsHKT8 and plays a very important role in cytosolic Na^+ detoxification (Ren *et al.*, 2005). Thus, plants need to possess a wide range of adaptations for osmotic stress as well as ionic toxicity to be tolerant under high salt. This multigenic trait of salt tolerance, in turn, makes plant breeder's job challenging to develop salt-tolerant crop species/cultivars. That's why the effort from last ten year's research using transgenic plants to improve salt-tolerance has not been established in the field yet (Flowers, 2004). Nevertheless, an understanding of how plant sense salt stress and subsequently an understanding of how cellular and physiological changes allow plants to be adept at dealing salt stress have been very important goals for salt stress research over the years. In this study, it has been shown how Na^+ stress is sensed in cells, how the sensing elicits signaling by changing $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{pH}]$, and how the adaptive mechanisms are activated for maintaining cytosolic Na^+ homeostasis. The differences in terms of activation of adaptive responses to salt stress between the salt-tolerant and –sensitive rice cultivars, as shown in this study, provided some significant insights about salt tolerance in this very important cereal crop.

It has been shown that Na^+ stress in rice is sensed inside the cytosol, and is consistent with the current speculation about sensing Na^+ stress in *Arabidopsis* by the long C-terminal tail of SOS1 protein in the cytosol. It, however, still remains to be shown whether rice SOS1 protein could be the sensor for Na^+ stress. A further

research effort to prove whether rice SOS1 protein is a Na^+ -sensor would be realistic, since rice SOS1 protein has been shown to be a functional equivalent to the *Arabidopsis* SOS1 protein.

K^+ selective channels/transporters appeared to be the significant determinants for variation in cytosolic Na^+ -influx between rice cvs. Pokkali and BRRI Dhan29. These proteins did not contribute to cytosolic Na^+ -influx in the tolerant cv. Pokkali, whereas they mediated a significant part of total cytosolic Na^+ -influx in the sensitive cv. BRRI Dhan29. It was suggested that these transporters are downregulated under salt stress in the salt-tolerant cultivar, as shown in case of OsHKT1. However, the mechanism by which Na^+ -influx through K^+ -selective transporters is inhibited in cv. Pokkali, at the onset of salt stress, needs to be further studied.

NSCCs seem to play the significant role in cytosolic Na^+ -influx in both the salt-tolerant cv. Pokkali and salt-sensitive cv. BRRI Dhan29, like in many other plant species. However, the molecular identity of these proteins is still not known. Therefore, any progress in characterizing these proteins, in combination with the current paradigm of salt stress research, will deliver the most significant platform to materialize plant breeder's challenge to develop salt-tolerant crop species/cultivars.

In rice, all eight functional HKT members are thought to play an important ion transport network for cytosolic Na^+/K^+ homeostasis. These members are specific in their expression and in Na^+/K^+ transport, for example root uptake, long-distance transport and efflux or compartmentalization. The expression studies of OsHKT1 & OsHKT2 indicted their significant role in cytosolic Na^+/K^+ homeostasis. However, to get the whole story of cytosolic Na^+/K^+ homeostasis in rice, the tissue- and cell-specific expressions of the other members of rice HKT-family needs a proper clarification.

All of these proposed works, in combination with the findings shown in this study, would provide a platform to delineate a conceptual framework for cytosolic Na^+ homeostasis and salt tolerance in rice. This would eventually be an important ground for genetic engineering to develop salt-tolerant high yielding rice cultivars.

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I





Uptake of sodium in quince, sugar beet, and wheat protoplasts determined by the fluorescent sodium-binding dye benzofuran isophthalate

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Summary

The uptake of sodium into protoplasts of quince (*Cydonia oblonga* Mill, clone BA29), sugar beet (*Beta vulgaris* L. cv. Monohill), and wheat (*Triticum aestivum* L. cv. Kadett) was determined by use of the acetoxymethyl ester of the fluorescent sodium-binding benzofuran isophthalate (SBFI-AM). In the presence of 1 mM CaCl₂, little sodium was taken up in the cytosol of quince mesophyll cells compared to cytosols of sugar beet and wheat. Upon addition of 40 mM NaCl, approximately the same amount of sodium was taken up in leaf and root protoplasts of wheat, but no sodium was taken up in quince. However, in calcium-free medium, obtained by addition of ethylene glycol tetra acetic acid (EGTA), quince protoplasts transiently took up sodium in the cytosol when 200–400 mM NaCl was added to the protoplast medium. Moreover, after cultivation of quince in the presence of 200 mM sodium for 4 weeks, the cytosol of isolated protoplasts did not take up any sodium at all from a calcium-free medium. The results show that protoplasts from salt tolerant quince only temporarily take up sodium in the cytosol and that they have a mechanism for fast extrusion of sodium from that compartment. These mechanisms are probably important for the high salt tolerance of quince. Calcium blocks the sodium uptake into the cytosol of both quince and wheat protoplasts.

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Abbreviations: SBFI-AM, acetoxymethyl ester of sodium-binding benzofuran isophthalate; EGTA, ethylene glycol tetra acetic acid

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Introduction

Excess of sodium chloride in the soil is the principal cause of salinity and, because about 10–25% of arable lands are saline, it is one of the most important problems in agriculture (Postel, 1989). It has long been known that calcium, by competition with sodium, can attenuate the toxic effects of Na^+ salinity on plants (LaHaye and Epstein, 1971; Cramer et al., 1985; Ben-Hayyim et al., 1987; Allen et al., 1995). At the cellular level, calcium may reduce the flux of sodium that enters the cytosol (Cramer et al., 1987; Whittington and Smith, 1992; Maathuis and Amtmann, 1999) and sodium also can move calcium from cellular membranes (Cramer et al., 1985; Lynch et al., 1987) and reduce the influx of Na^+ through the low-affinity cation channel (Amtmann et al., 2001). The isolation and use of the *Arabidopsis* *sos3* mutant provided further evidence supporting the role of Ca^{2+} in plant salt tolerance (Liu and Zhu, 1998; Qiu et al., 2002).

The sodium-binding benzofuran isophthalate (SBFI), introduced by Minta and Tsien (1989), was used to determine changes in intracellular sodium (Na^+). This probe has benzofuran fluorophores similar to those of Fura-2 and PBFI and, therefore, is compatible with filters used for these dyes. The specific potassium-binding dye PBFI is most similar to SBFI and was used earlier to determine concentrations of potassium in protoplasts of barley and wheat (Lindberg, 1995; Lindberg and Strid, 1997). At present, there are many investigations where SBFI is used to study Na^+ metabolism in animal and human cells, such as fibroblasts (Harootunian et al., 1989), retinal cells (Agostinho et al., 1996) and neuroblastoma cells (Kopper and Adorante, 2002). Recently, SBFI was used to detect sodium accumulation in root hairs of *Arabidopsis* (Halperin and Lynch, 2003). The main purpose of the present study was to investigate whether the acetoxymethyl ester of SBFI could also be used as a tool for detection of sodium uptake in the cytosol of plant protoplasts.

Another purpose was to compare the uptake of sodium in protoplasts of the salt-tolerant quince with the uptake in protoplasts of less tolerant wheat and sugar beet. Roots of quince are used as rootstocks for cultivation of fruit trees, especially pear trees on salty soils, but little is known about the mechanism for salt tolerance in this plant. We determined the uptake of sodium into protoplasts of quince and wheat in the absence and presence of free calcium concentrations. Such information may explain the different levels of salinity resistance in these species.

Materials and methods

Cultivation

Shoots of Quince BA29 (*Cydonia oblonga* Mill) were propagated in vitro on DKW salt medium (Driver and Kuniyuki, 1984) supplemented with 1 mg l^{-1} thiamine hydrochloride, 100 mg l^{-1} myo-inositol, 1.5 mg l^{-1} BA (6-benzylaminopurine), 1.5 mg l^{-1} BAr (6-benzylaminopurine riboside), 30 g l^{-1} sucrose, and with and without 200 mM NaCl . The cultivations were repeated four times with or without 200 mM sodium. The medium was adjusted to pH 5.2 with KOH before addition of 4 g l^{-1} of agar and 5 g l^{-1} of pectin. The medium was sterilized by autoclavation at 120°C for 20 min.

Seeds of sugar beet (*Beta maritima* L. cv. Monohill) from Hammenhög, Sweden were developed in moist vermiculite for 1 week. The seedlings were then transferred to plastic holders in 1-L beakers containing a complete mineral nutrition as described by Lindberg (1990).

Seeds of wheat (*Triticum aestivum* L. cv. Kadett) from Svalöf Weibull AB, Sweden were treated and cultivated in a complete nutrient solution as described by Shishova and Lindberg (1999).

Cultures of quince were maintained at $22 \pm 1^\circ\text{C}$ under white light ($60 \mu\text{mol m}^{-2} \text{ s}^{-1}$) with a 16-h photoperiod and were transferred to fresh medium every third week. Sugar beet and wheat seedlings were cultivated at $20 \pm 1^\circ\text{C}$ in the light and at $17 \pm 1^\circ\text{C}$ during darkness in a growth chamber at the same photoperiod and light condition as for quince. The relative humidity was 50–60% during the cultivation of sugar beet and wheat.

Protoplast isolation

All protoplasts from leaves were isolated from mesophyll cells. The protoplasts from quince were prepared as described by D'Onofrio et al. (1999). The young leaves of the first three shoot apical nodes were harvested, sliced transversely into pieces smaller than 1 mm, and treated with $50 \mu\text{g ml}^{-1}$ cellulase from *Trichoderma reesei* (Sigma, EC 3.2.1.) and $10 \mu\text{g ml}^{-1}$ pectinase from *Rhizopus* sp. (Sigma, EC 3.2.1.) in a CPW salt medium (Frearson et al., 1973) containing 0.45 M sucrose, 0.05% (w/v) polyvinyl pyrrolidone (PVP; Sigma), 0.05% bovine serum albumin (BSA; Sigma), 1 mM CaCl_2 , and 5 mM Tris[hydroxymethyl] amino methane hydrochloride and Mes, 2-[N-morpholino] ethane sulfonic acid (Tris-Mes), pH 5.7 for 21 h at 27°C in darkness. At the end of the incubation the suspension was filtered through a nylon net with

50 μm pores. One ml of the CPW medium supplemented with 0.4 M sucrose and 0.1 M sorbitol was stratified on the filtrate. Then 0.5 ml CPW medium containing 0.5 M sorbitol was gently stratified on top of the latter medium. After centrifugation at 160g for 15 min, the protoplasts were clustered in a ring in the intermediate medium.

Protoplasts were isolated from 3-week-old sugar beet leaves and from 6 to 7-day-old wheat leaves as described by Shishova and Lindberg (1999). Leaves were sliced in 0.5 mm pieces and treated with 1% (w/v) cellulase from *Tricoderma resei* (Sigma, EC 3.2.1.4) and 0.3% (w/v) macerage, Maceroenzyme R-10 (Serva, EC 3.2.1.4) for 3 h. Protoplasts were also isolated from root tips of 6–7-day-old wheat seedlings. The top 2 mm of roots were sliced in 0.5 mm pieces and treated with 2% (w/v) cellulysin cellulase from *T. viride* (Calbiochem, LabKemi, Sweden, EC 3.2.1.4) and 0.1% (w/v) pectolyase Y23 from *Aspergillus japonicus* (Kemila, Sollentuna, Sweden, EC 3.2.1.4) for 3 h as described by Lindberg and Strid (1997).

Dye loading

Before loading, the protoplasts were washed twice in the loading medium containing 0.5 M sorbitol, 1 mM CaCl_2 , 0.2% (w/v) PVP and 5 mM Tris-Mes buffer (pH 5.5; medium A). The SBFI-AM (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulfoxide (< 0.1% water) to give a 5 mM stock solution. Two μl of the stock solution was diluted with 6.75 μl ethanol and 1.25 μl pluronic F-127 (Molecular Probes) as described by Poenie et al. (1986), and added to 1 ml of protoplast suspension. Loading was performed in medium A for 3 h at 20 °C in darkness. After loading, the protoplasts were washed twice in a solution similar to medium A, but with Tris-Mes buffer at pH 7 (medium B).

Fluorescence measurements

An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochen, Germany), supplied with an electromagnetic filter-exchanger (Zeiss), xenon lamp (Zeiss XBO 75), photometer (Zeiss 01), microprocessor (MSP 201, Zeiss) and a personal computer, was used to determine fluorescence intensity after excitation at 340/380 nm. Emission wavelengths were 500–530 nm. All measurements were made with a Planneofluar $\times 40/0.75$ objective (Zeiss) for phase contrast. Adjustment of signals and noise was done automatically. By means of ratio microscopy (Tsien and Poenie, 1986; Bright et al., 1987), the effect of different dye concentration can be

eliminated. Microslides were covered with poly L-lysine (MW 150,000–300,000, Sigma) in order to enable protoplasts to become attached to their surface.

Standard determinations of $[\text{Na}^+]$ using SBFI-AM were made in situ with the different types of protoplasts in Tris-Mes buffer (pH 7) containing three different concentrations of Na^+ (added as NaCl): 0, 200 and 400 mM for quince and 0, 50 and 200 mM for wheat and sugar beet. Ten μM gramicidin (Sigma) was added to the buffer in order to equilibrate intra- and extra-cellular Na^+ (Negulescu and Machen, 1990). To obtain iso-osmotic solutions, KCl was added to the solution to give a final concentration of 400 or 200 mM $[\text{Na}^+ + \text{K}^+]$ for quince, and wheat and sugar beet, respectively. Also, 5 μM nigericin (Sigma) was added to avoid pH effects (Negulescu and Machen, 1990). Measurements were undertaken 5–10 min after addition of gramicidin and nigericin. The fluorescence ratio at 340/380 nm increased in a linear way with extra-cellular concentration of Na^+ (not shown).

The cytosolic fluorescence intensity ratio at 340/380 nm was determined in single protoplasts before and after addition of NaCl (40, 200 and 400 mM final concentrations) to the protoplast suspension. The measurements were made on protoplasts of similar size within each species and in the presence (0.1 or 1.0 mM) and absence of extra cellular-free Ca^{2+} . For measurements in the absence of extra-cellular Ca^{2+} , EGTA was added to a final concentration 5 mM. The fluorescence micrographs of wheat protoplasts were obtained by an axiocam camera (Zeiss) connected to an Axioshop 2 microscope (Zeiss) using a Fitc filter.

Statistics

Each plot is a copy of printer plots and shows representative traces of a specific experiment repeated ≥ 5 times with protoplasts from independent cultivations. Each value is the average of 50 fluorescence-ratio determinations. The table shows data from experiments repeated 5–6 times with protoplasts from independent cultivations.

Results

Loading of the wheat and sugar beet protoplasts with the SBFI-AM dye required 3 h before any fluorescence above the auto-fluorescence was detectable. The protoplasts chosen for measurements showed fluorescence from the cytosol, as shown for a wheat protoplast (Fig. 1A and B). Upon

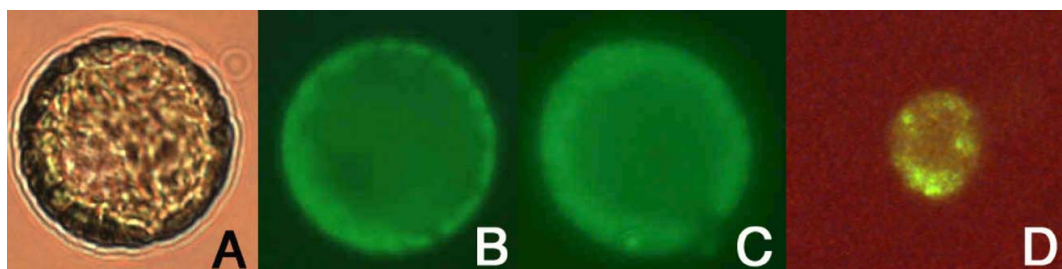


Figure 1. Micrographs showing protoplasts from wheat leaves (A–C) and quince (D) preloaded with SBFI-AM for 3 h in the absence (A, B) or presence of 200 mM NaCl (C, D). Protoplast in transmitted light (A), in fluorescence light using a Fitc filter (B, C) and after excitation at 380 nm (D).

addition of 200 mM NaCl, the fluorescence from the cytosol increased (Fig. 1C). The size of quince protoplasts was smaller than that of wheat protoplasts, approximately 10 nm in diameter (Fig. 1D).

The auto-fluorescence intensity at 340/380 nm excitation of quince leaf protoplasts was 0.20 ± 0.01 but was invisible. After loading of quince protoplasts with SBFI-AM for 3 h, the cytosol fluorescence at a 340/380 nm intensity ratio showed some oscillation, but by non-linear regression analysis using an excel program, the value remained constant at approximately 0.20 ± 0.01 . Thus, there was no endogenous Na^+ present in the cytosol of quince protoplasts, but fluorescence from the cytosol could be seen immediately after the addition of 400 mM NaCl (Fig. 1D).

When 40–200 mM NaCl was added to quince protoplasts in a buffer containing 1 mM Ca^{2+} , the 340/380 fluorescence intensity ratio was stable, but when 400 mM NaCl was added to the protoplasts, the cytosol fluorescence intensity ratio increased somewhat, although only transiently (Fig. 2, Table 1). It was, however, necessary to use regression analyses to determine the sodium concentration in the cytosol. However, in a calcium-free medium, obtained by addition of 5 mM EGTA, the fluorescence increased during 8 min and then decreased again within 3 min (Fig 2). Either EGTA was added prior to or after, 400 mM Na^+ was added; a transient increase in fluorescence (15%) was obtained corresponding to 15 mM Na^+ . There was no difference in fluorescence changes with sodium addition in the presence of 0.1 mM compared to those with zero calcium.

Since it was necessary to use high concentrations of Na^+ in order to detect any influx of this ion into the cytosol of quince, it was necessary to check the viability of the protoplasts after the end of each experiment. Some of the protoplasts exploded after sodium treatment. Therefore, only proto-

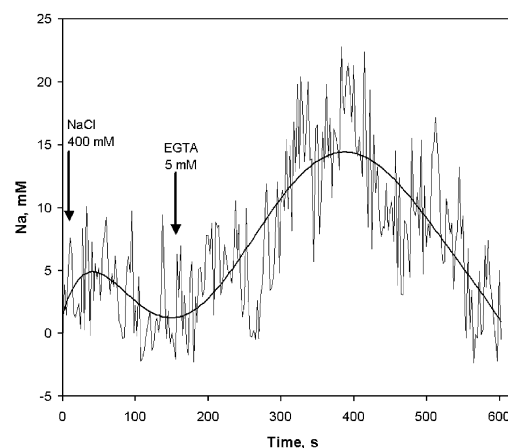


Figure 2. Sodium dynamics in the cytosol of a quince leaf protoplast upon addition of 400 mM NaCl in the absence and presence of 5 mM EGTA. The trace shows typical changes in fluorescence intensity ratio (340/380 nm) of SBFI. The line shows estimated mean values obtained by regression analysis of data. The concentration of sodium was estimated using in situ calibration of quince protoplasts.

plasts with normal fluorescence and with protoplasmic streaming were considered.

When the shoots of quince were cultivated in the presence of 200 mM NaCl for 4 weeks, 70% of them survived. The protoplasts obtained from such shoots did not take up any sodium into the cytosol upon addition of 25–400 mM NaCl to the external medium in the absence of calcium. These protoplasts looked different to the control protoplasts since they were smaller and had smaller vacuoles.

Upon addition of NaCl to root and shoot protoplasts from wheat and sugar beet, another fluorescence pattern was obtained compared with quince (Figs. 3 and 4). When 40 mM NaCl was added either

Table 1. Fluorescence increase (in %) at 340/380 nm in different types of protoplasts upon NaCl addition. Experiments were repeated more than 5 times with protoplasts from independent cultivations

NaCl addition (mM)	Fluorescence increase in %	
	At external CaCl ₂ 0–0.1 mM	At external CaCl ₂ 1 mM
<i>To wheat leaf</i>		
40	18.1±2	6.8±1
200	23.8±9	5.8±0.5
400	6.1±1	3.6±1
<i>To wheat root</i>		
40	15.0±4	7.4±3
400	3.4±0.5	1.6±0.5
<i>To beet leaf</i>		
40	6.2±3	ND
200	6.6±1	ND
<i>To quince leaf</i>		
40	0	0
100	0	0
200	7.5 ^a –>0	0
400	15 ^a –>0	3.8 ^a –>0

ND=not detected. Data show mean values±SE.
^aMaximal increase.

to root or shoot protoplasts of wheat, approximately the same increase in fluorescence was obtained, but upon addition to sugar beet (leaf) protoplasts, only a minor fluorescence increase was obtained compared to the initial value, which was approximately 8 mM (Table 1, Fig. 3). There was a similar increase in fluorescence upon addition of 400 mM NaCl to (leaf and root) wheat protoplast and 200 mM NaCl to sugar beet protoplasts, respectively (Table 1). However, when 200 mM NaCl was added, wheat leaf protoplasts took up more sodium in the cytosol than did the sugar beet protoplasts.

Calcium inhibited the fluorescence increase after addition of NaCl (40 and 400 mM) to the same degree in root and shoot protoplasts of wheat (Table 1). At addition of 200 mM NaCl, the inhibition by calcium was stronger.

Discussion

In the present investigation, the fluorescence oscillation in quince protoplasts loaded with SBFI-AM did not allow us to estimate small concentrations of Na⁺, but by use of regression analysis a better estimation of the sodium concentration was possible (Fig. 2). When 400 mM NaCl was added to

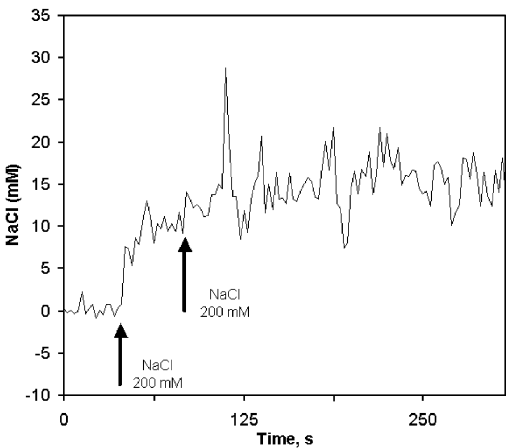


Figure 3. Sodium dynamics in the cytosol of a wheat leaf protoplast upon addition of 200 mM NaCl. Sodium concentration was estimated from changes in fluorescence intensity ratio (340/380 nm) of SBFI. The calcium concentration was 0.1 mM. The concentration of sodium was estimated using in situ calibration of wheat protoplasts.

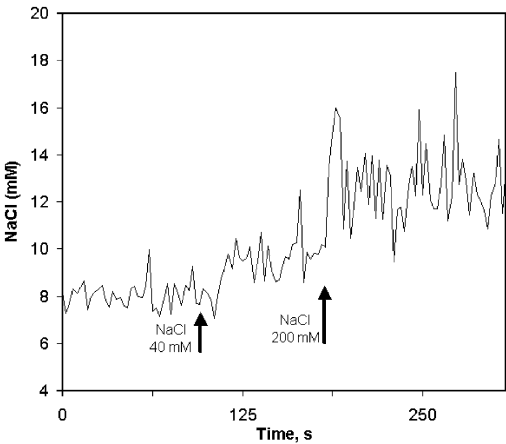


Figure 4. Sodium dynamics in the cytosol a sugar beet leaf protoplast upon additions of 40 and 200 mM NaCl. Sodium concentration was estimated from changes in fluorescence intensity ratio (340/380 nm) of SBFI. The calcium concentration was 0.1 mM. The concentration of sodium was estimated using in situ calibration of sugar beet protoplasts.

the protoplast suspension, in the presence of extra cellular Ca²⁺, the fluorescence increased only slightly (to approximately 5 mM) as a result of Na⁺ uptake into the cytosol. Thereafter, Na⁺ was probably transported out of the cytosol (into the

vacuole or out of the protoplast) and the concentration decreased to the initial value.

The cytosolic calcium concentration increased three times in calcium-free medium compared with calcium medium, but the increase was only transient. Upon addition of 200 mM NaCl the amplitude and duration of the transient increase of cytosol Na^+ was half of that obtained at 400 mM NaCl (Table 1). These results suggest that the maximum cytosolic concentration of Na^+ increases linearly in quince protoplasts with the external NaCl concentration. The results confirm that Ca^{2+} is successfully protecting the influx of Na^+ into the cytosol of quince protoplasts. Even if calcium-free medium rarely exists in nature, even a low concentration (0.1 mM calcium) did not affect the sodium influx.

After cultivation of quince in the presence of 200 mM NaCl, the protoplasts became smaller and did not take up any measurable amount of sodium into the cytosol. The extrusion or prevention of sodium uptake into the cytosol of quince protoplasts seems to be connected with the high salt tolerance of this plant. However, it cannot be excluded, that the high sodium concentration during the cultivation caused a dehydration of the protoplasts and thereby changed their transport capacity.

In addition, in root and shoot protoplasts of wheat and in shoot protoplasts of sugar beet, the increase in fluorescence was 2–4 times higher in the presence of 0.1 mM Ca^{2+} , compared with 1.0 mM Ca^{2+} (Table 1), and corroborates other results with wheat protoplasts (Amtmann and Sanders, 1999). Experiments with root protoplasts from *Arabidopsis* showed that sodium can be transported through non-selective cation channels and that this transport is inhibited by calcium (Demidchik and Tester, 2002). Therefore, it is likely that such channels also mediate part of the sodium influx in quince and wheat.

In protoplasts of sugar beet and wheat, the fluorescence was raised and was stable compared with the quince protoplasts (Figs. 2–4). When 40 or 200 mM NaCl was added (Table 1), the increase in fluorescence was in the following order: wheat > sugar beet > quince, where quince is the most salt-tolerant species and wheat the least tolerant. Thus, the uptake of Na^+ into the cytosol, if the uptake is transient or stable, is of importance for tolerance.

When 400 mM NaCl was added to wheat protoplasts, a smaller increase in fluorescence was obtained compared with addition of 200 mM NaCl. Therefore, the fluorescence increase did not reflect a shrinking of protoplasts at the higher level of

sodium (Table 1). Even if such concentrations of sodium may be toxic to wheat protoplasts after some time, 5 min after sodium addition to the protoplasts, they still looked normal and did not change their approximate size (Fig. 1C).

Sodium uptake into plant cells is suggested to occur by the high-affinity K^+ transporter (HKT1), the low-affinity cation transporter (LCT1), or by non-selective cation channels (Schachtman et al., 1997; Amtmann and Sanders, 1999; Davenport and Tester, 2000; Amtmann et al., 2001; Rus et al., 2001). A sodium efflux from the cytosol has been correlated with Na^+/H^+ transporters at the plasma membrane and the tonoplast (Blumwald and Poole, 1985; Hassidim et al., 1990) and also connected with the potassium transport (Lindberg and Yahya, 1994). In quince protoplasts the influx and efflux reactions did not depend on external pH, since no difference in sodium fluorescence was obtained upon addition of NaCl at external pH 6 or 7. Therefore, it is likely that the main efflux of Na^+ from the cytosol takes place at the tonoplast and not at the plasmalemma in these mesophyll cells. A preferential allocation of sodium into the vacuole was suggested earlier (Yeo, 1998). Under control conditions, the salt-tolerant relative of *Arabidopsis thaliana*, *Thellungiella halophila*, accumulated high concentrations of K^+ in the leaf epidermal cells, but during salt stress epidermal K^+ concentration decreased and Na^+ concentration increased. Such change in the K^+ concentration was not observed in the salt-sensitive *A. thaliana* (Volkov et al., 2003). They concluded that the salt-tolerant species then could maintain relatively high K/Na ratios in metabolically active mesophyll cells. This is in agreement with our results that show low Na^+ influx in the cytosol of mesophyll cells from salt-tolerant quince.

Since the concentration of KCl in the cytosol usually is within the 100–200 mM range, there is competition between sodium and potassium for binding to the dye, especially at lower concentrations of sodium (Minta and Tsien, 1989). On the other hand, the real influx of Na^+ in the presence of 200–400 mM NaCl should be little influenced by the presence of endogenous potassium, since SBFI is approx. 18-fold more selective for Na^+ than for K^+ . The SBFI dye, therefore, is a useful tool to clarify mechanisms for uptake of Na^+ into plant protoplasts.

Conclusion

The dye SBFI can be used to detect sodium uptake into the cytosol of protoplasts. A low sodium uptake

into the cytosol is important for tolerance to sodium.

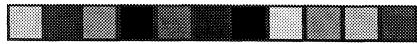
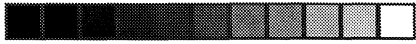
Acknowledgements

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II





RESEARCH PAPER

Uptake of sodium in protoplasts of salt-sensitive and salt-tolerant cultivars of rice, *Oryza sativa* L. determined by the fluorescent dye SBFI

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Abstract

In this study, the uptake of Na^+ into the cytosol of rice (*Oryza sativa* L. cvs Pokkali and BRRI Dhan29) protoplasts was measured using the acetoxymethyl ester of the fluorescent sodium-binding benzofuran isophthalate, SBFI-AM, and fluorescence microscopy. By means of inhibitor analyses the mechanisms for uptake and sequestration of Na^+ in the salt-sensitive indica rice cv. BRRI Dhan29 and in the salt-tolerant indica rice cv. Pokkali were detected. Less Na^+ was taken up into the cytosol of Pokkali than into BRRI Dhan29. The results indicate that K^+ -selective channels do not contribute to the Na^+ uptake in Pokkali, whereas they are the major pathways for Na^+ uptake in BRRI Dhan29 along with non-selective cation channels. However, non-selective cation channels seem to be the main pathways for Na^+ uptake in Pokkali. Protoplasts from Pokkali leaves took up Na^+ only transiently in the presence of extracellular Na^+ at 5–100 mM. Therefore, it is likely that the protoplasts have a mechanism for fast extrusion of Na^+ out of the cytoplasm. Experiments with protoplasts pretreated with NH_4NO_3 and NH_4VO_3 suggest that the salt-tolerant Pokkali extrudes Na^+ mainly into the vacuole. After cultivation of both cultivars in the presence of 10 or 50 mM NaCl for 72 h, the isolated protoplasts from Pokkali took up less Na^+ than the control protoplasts. The results suggest that the salt-tolerance in Pokkali depends on reduced uptake through K^+ -selective channels and a fast extrusion of Na^+ into the vacuoles.

Key words: Benzofuran isophthalate, compartmentalization, fluorescence ratio microscopy, influx, rice, salt stress, sodium uptake.

Introduction

Soil salinity is one of the environmental hazards in agriculture worldwide because it limits crop yield and restricts the use of land previously cultivated. Around 20% of the world's agricultural land and nearly 50% of all irrigated land is adversely affected by soil salinity (Flowers and Yeo, 1995). Moreover, soil salinization due to irrigation is becoming increasingly detrimental to agriculture (Flowers, 1999).

One of the principal adverse effects of high salinity in non-tolerant plants is growth inhibition by toxicity to Na^+ . Maintenance of a high cytosolic $[\text{K}^+]:[\text{Na}^+]$ ratio is critical for the function of cells (Rubio *et al.*, 1995; Zhu *et al.*, 1998). In saline conditions, Na^+ competes with K^+ for uptake through common transport systems and this happens often, since in the environment $[\text{Na}^+]$ is usually higher than $[\text{K}^+]$. Thus, elevated levels of cytosolic Na^+ , or in another way a high $[\text{Na}^+]:[\text{K}^+]$ ratio, exerts metabolic toxicity by competition between Na^+ and K^+ for the binding sites of many enzymes (Bhandal and Malik, 1988; Tester and Davenport, 2003). Protection of this Na^+ -sensitive metabolic mechanism under saline conditions partly depends on the ability to keep cytosolic Na^+ levels low.

For plant cells, the most important way of keeping the cytosolic Na^+ concentration at a low level is to minimize Na^+ influx into the cytosol, and to maximize the Na^+ efflux from the cytosol, either into the apoplast or into the vacuole (Nie *et al.*, 1995; Blumwald *et al.*, 2000; Zhu, 2001; Qiu *et al.*, 2004). Sodium entry into plant cells may be restricted by selective ion uptake. Several classes of cation channels including outward- and inward-rectifying K^+ -selective channels (Maathius and Sanders, 1995), and non-selective cation channels, NSCCs (Amtmann and Sanders, 1999;

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Demidchik and Tester, 2002; Demidchik *et al.*, 2002), seem to be involved in mediating the toxic influx of Na^+ . High-affinity potassium transporters have also been proposed to mediate substantial Na^+ entry into plant roots (Uozumi *et al.*, 2000; Horie *et al.*, 2001; Goldack *et al.*, 2002). However, recent findings suggest that non-selective cation channels are the dominant pathways for Na^+ influx into root cells (Roberts and Tester, 1997; Tyerman *et al.*, 1997; Buschmann *et al.*, 2000; Davenport and Tester, 2000; Demidchik and Tester, 2002; Demidchik *et al.*, 2002). The functions of these NSCCs are inhibited by Ca^{2+} at 0.5 mM or higher concentrations (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Demidchik and Tester, 2002). These channels are insensitive to most organic blockers, which inhibit different classes of cation channels, such as TEA for K^+ -selective channels (Demidchik and Tester, 2002).

Once Na^+ enters the cytosol at a toxic level, plant cells can deal with the internal Na^+ by sequestering it either in the apoplast or in the vacuole. Vacuolar compartmentalization is an efficient strategy for plant cells to cope with salinity stress (Fukuda *et al.*, 1998, 2004; Blumwald *et al.*, 2000; Chauhan *et al.*, 2000; Hamada *et al.*, 2001; Tester and Davenport, 2003). Antiporters for Na^+/H^+ in the plasma membrane and tonoplasts are expected to fulfil this function (Orlowski and Grinstein, 1997; Blumwald, 2000; Blumwald *et al.*, 2000; Hasegawa *et al.*, 2000; Fukuda *et al.*, 2004; Qiu *et al.*, 2004). Sodium extrusion through these Na^+/H^+ antiporters is driven by an inwardly directed proton gradient created by H^+ -ATPases (Blumwald *et al.*, 2000; Hamilton *et al.*, 2002).

Rice is the only major cereal crop that is grown in waterlogged conditions and, being a glycophyte, it is especially sensitive to salinity. Both the production and the planting area of rice are greatly affected by soil salinity (Akbar and Ponnamperna, 1980; Panaullah, 1993). When grown in saline conditions, rice accumulates toxic Na^+ levels in the leaves. Although toxicity from Na^+ accumulation in the important crop rice is well studied at the organ and tissue levels, the mechanism by which Na^+ enters into the cytosol, and its subsequent removal from the cytoplasm via efflux or compartmentalization or both, are still poorly understood.

The aim of this study was to investigate the mechanisms of Na^+ transport into the cytosol of both salt-sensitive and salt-tolerant rice cultivars. A second aim was to study the compartmentalization of Na^+ . The fluorescent sodium-binding benzofuran isophthalate dye (SBFI-AM), introduced by Minta and Tsien (1989), was used to determine changes in intracellular Na^+ . This probe has been shown as a tool for the investigation of Na^+ uptake into root hairs (Halperin and Lynch, 2003), and into the cytosol of plant protoplasts (D'Onofrio *et al.*, 2005), and also for the determination of apoplastic Na^+ in intact leaves (Mühling and Läuchli, 2002).

Materials and methods

Plant material

Seeds of rice (*Oryza sativa* L. *indica* cvs Pokkali and BRRI Dhan29) were provided by the Bangladesh Rice Research Institute (BRRI, Gazipur, Bangladesh). They were treated with 10% chlorine solution for 15 min and rinsed with distilled water 5–6 times. Seeds were dipped in 5 mM CaSO_4 solution for 3 h. Thereafter, seeds were soaked in water for 48 h in the darkness. They were placed on a Miracloth layer covering a metal net, which was placed on a wide beaker with culture solution. The culture solution used was based on that described by Yoshida *et al.* (1976), with modifications made by Khatun and Flowers (1995) in order to reduce the sodium concentration in the basic solution to almost zero. The beaker was covered with white polythene and placed in a Fison growth chamber (Convion, Manitoba, R3H OW9, USA). The day/night temperature was 30/25 °C under a 12 h photoperiod with $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (Philips 3629 warm white 36 W, Rosendahl, The Netherlands). Humidity was about 75%. Protoplasts were prepared from both leaf and root tips of 9–10-d-old seedlings. For some of the experiments, seedlings were pretreated for 72 h with 10 or 50 mM NaCl in the same culture solution as stated above before the isolation of protoplasts.

Protoplast isolation

The protoplasts from rice cvs Pokkali and BRRI Dhan29 were prepared as described by Shishova and Lindberg (1999) with some modifications. Leaves were sliced into 0.5 mm pieces and treated with 1% (w/v) cellulase (lyophilized powder; 10 units mg^{-1} solid) from *Trichoderma resei* (Sigma, EC 3.2.1.4) and 0.6% (w/v) macerace (lyophilized powder; 0.6 units mg^{-1} solid), Macerozyme R-10 (Serva, EC 3.2.1.4) for 2 h. Protoplasts were also isolated from root tips. The top 1 cm of root tips was sliced in 0.5 mm pieces and treated with 4% (w/v) cellulysin[®] cellulase (lyophilized powder; 1.0 units mg^{-1} solid) from *Trichoderma viride* (Calbiochem, Lab-Kemi, Sweden, EC 3.2.1.4) and 0.2% (w/v) pectolyase Y23 (lyophilized powder; 3.6 units mg^{-1} solid) from *Aspergillus japonicus* (Kemila, Sollentuna, Sweden, EC 3.2.1.15) for 3 h as described by Lindberg and Strid (1997).

Dye loading

The protoplasts were washed twice in the loading medium containing 0.5 M sorbitol (Sigma, St Louis, MO, USA), 0.1 mM CaCl_2 , 0.2% (w/v) polyvinylpyrrolidone (PVP, Sigma, St Louis, MO, USA) and a buffer (pH 5.5: medium A) containing 5 mM TRIS (Labassco, Germany) and 5 mM MES (Sigma, St Louis, MO, USA). The SBFI-AM (Molecular Probes, Eugene, OR, USA) was dissolved in dimethylsulphoxide (DMSO, Merck, Eurolab AB, Stockholm, Sweden (<0.1% water) to give a 5 mM stock solution. Two microlitres of the stock solution was diluted with 6.75 μl ethanol (Kemetyl, Stockholm, Sweden) and 1.25 μl pluronic F-127 (Molecular Probes) as described by Poenie *et al.* (1986), and added to 1 ml of protoplast suspension to get a final concentration 10 μM . Dye loading was performed in medium A for 4 h at room temperature in darkness. After loading, the samples were centrifuged and pellets were resuspended into 1 ml of a solution similar to medium A, but with TRIS-MES buffer at pH 7 (medium B). Before measurements, samples were kept in darkness at room temperature for 25 min.

Fluorescence measurements

An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochen, Germany), supplied with an electromagnetic filter-exchanger (Zeiss), Xenon lamp (Zeiss XBO 75), photometer (Zeiss 01), microprocessor (MSP 21, Zeiss), and a personal computer was used to determine fluorescence intensity after excitation at 340/380 nm. These

wavelengths represent the maximum and minimum points of the excitation spectrum of the dye SBFI (Minta and Tsien, 1989). Emission wavelengths were 530–550 nm. All measurements were performed with a Planneofluar $\times 40/0.75$ objective (Zeiss) for phase contrast. Adjustment of signals and noise were made automatically. By means of ratio microscopy (Tsien and Poenie, 1986; Bright *et al.*, 1987), the effect of different dye concentration can be eliminated. Microslides were covered with poly-L-lysine (MW 150 000–300 000, Sigma) 0.15%, to attach protoplasts to their surface.

Standard determinations of $[\text{Na}^+]$ using SBFI-AM were made *in situ* with the different types of protoplasts in TRIS-MES buffer (pH 7) containing five different concentrations of Na^+ (added as NaCl): 0, 5, 10, 50, and 100 mM. KCl was added to the solutions to give a final concentration of 100 mM $[\text{Na}^+ + \text{K}^+]$ to approximate physiological ionic strengths (Haugland, 2002). Gramicidin (Sigma) was added to the buffer with protoplasts at a final concentration of 10 μM (Negulescu and Machen, 1990). As gramicidin is a monovalent cation ionophore, forming transmembrane pores through which cations can pass, it can equilibrate extracellular and intracellular concentrations of Na^+ as well as of K^+ (Harootunian *et al.*, 1989). As salt stress also induces cytosolic acidification, nigericin (Sigma) was added at a final concentration of 5 μM to avoid pH effect (Negulescu and Machen, 1990). Protoplasts of similar size that showed fluorescence only from the cytosol and without fluorescence from the chloroplasts (in the case of leaf protoplasts) were chosen for the fluorescence measurements. Measurements were undertaken 5–10 min after the addition of gramicidin and nigericin. The fluorescence ratio 340/380 nm increased in a linear way with an extracellular concentration of Na^+ up to 100 mM (Fig. 1). It was considered that the initial cytosolic Na^+ -concentration was zero, since the rice seedlings were cultivated in a solution containing no sodium.

The fluorescence intensity ratio of the cytosol at 340/380 nm was determined in single protoplasts before, and after, the addition of

NaCl (5, 50, and 100 mM final concentrations) to the protoplast suspension. Measurements were undertaken up to 4 h after loading of the dye, because the SBFI fluorescence intensity in the cytosol may not change for at least 4 h (Halperin and Lynch, 2003). As a control for the addition of sodium, mannitol of the same osmolarity was added to determine whether the change in fluorescence intensity ratio is an artefact caused by protoplast shrinking. To investigate the interference of K^+ binding to the Na^+ -sensitive fluorescent dye SBFI-AM, measurements of the fluorescence intensity ratio were made after the addition of 5, 50, and 100 mM KCl. By means of regression analysis of the *in situ* calibration curve (Fig. 1), fluorescence intensity ratios were converted to the cytosolic Na^+ -concentration. The measurements were performed with protoplasts of similar size (approximately 10 μm).

Statistics

Each plot is a copy of printer plots and shows representative traces of a specific experiment repeated ≥ 7 times with protoplasts from independent cultivations. Each value is the average of around 100 fluorescent-ratio determinations. The tables show data from experiments repeated 5–6 times with protoplasts from independent cultivations. Data are presented as means \pm standard error (SE).

Results

Loading of leaf and root protoplasts with the SBFI-AM dye took 3 h and 4 h, respectively, at room temperature before any fluorescence was detectable. No visible difference in the efficiency of taking up the dye was found between the two cultivars. For measurements, only root protoplasts with a dense cytoplasm and no, or little, vacuolation were selected and all protoplasts from leaves were chosen from the mesophyll cells (distinguished by the presence of chloroplasts; Fig. 2a). In both the cases, protoplasts of similar size showing fluorescence only from the cytosol (Fig. 2b, c) were chosen for the measurements. Upon the addition of extracellular NaCl, the cytosolic fluorescence intensity ratio increased. On the other hand, addition of 5, 50, and 100 mM KCl or 10, 100, and 200 mM mannitol did not increase the fluorescence intensity ratio (Fig. 3a, b).

Upon the addition of 5, 50, and 100 mM NaCl to the external solution, less Na^+ was taken up into the cytosol of Pokkali, compared with that of BRRI Dhan29 (Table 1; Fig. 4). The total cytosolic concentration of Na^+ in Pokkali was approximately half of that in BRRI Dhan29 after each addition. Moreover, the increase in Na^+ concentration in the cytosol of Pokkali leaf protoplasts was transient upon the addition of 5, 50, and 100 mM NaCl, whereas the increase in cytosolic Na^+ concentration in BRRI Dhan29 was stable after the addition of 50 and 100 mM NaCl. Compared to shoot protoplasts, root protoplasts of Pokkali showed even less uptake of Na^+ upon addition of external NaCl, although the increase of the concentration was almost stable after each addition of NaCl. Both root and shoot protoplasts of BRRI Dhan29 took up Na^+ into the cytosol almost at the same concentration.

The uptake of Na^+ in the cytosol of leaf protoplasts of Pokkali and BRRI Dhan29 was different in the two

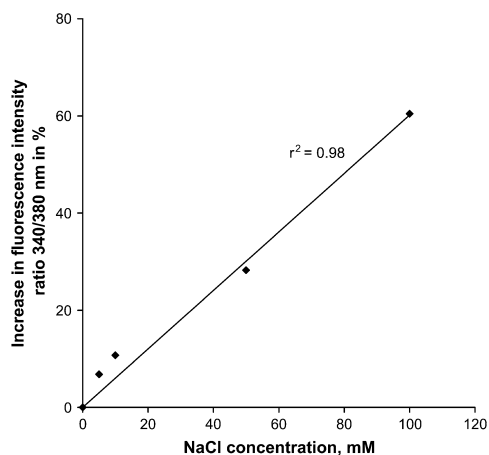


Fig. 1. Changes in fluorescence intensity ratio 340/380 nm (in %) in shoot protoplasts of Pokkali and BRRI Dhan29 at different extracellular concentrations of NaCl and in the presence of 0.1 mM Ca^{2+} . KCl was added to the solutions to get a total of 100 mM $[\text{NaCl} + \text{KCl}]$. Gramicidin at a final concentration of 10 μM was added to the buffer to equilibrate intracellular and extracellular Na^+ . Nigericin, at a final concentration of 5 μM , was also added to avoid a pH effect. Measurements were undertaken 5–10 min after the addition of gramicidin and nigericin. Each mean represents measurements from 10 protoplasts.

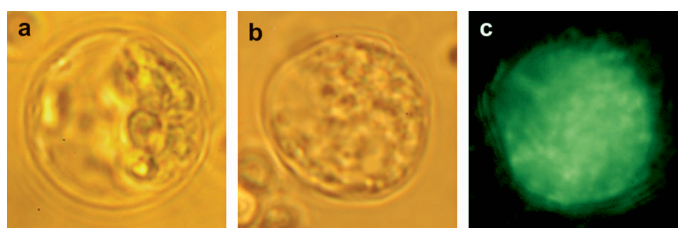


Fig. 2. Micrographs showing shoot (a) and root (b, c) protoplasts of rice. The micrographs were taken using a Planneofluar 100 \times 1.30 oil objective (Zeiss) for phase contrast. Protoplast in transmitted light (a, b) and in fluorescent light (c). Emission filter in (c) was 530–550 nm.

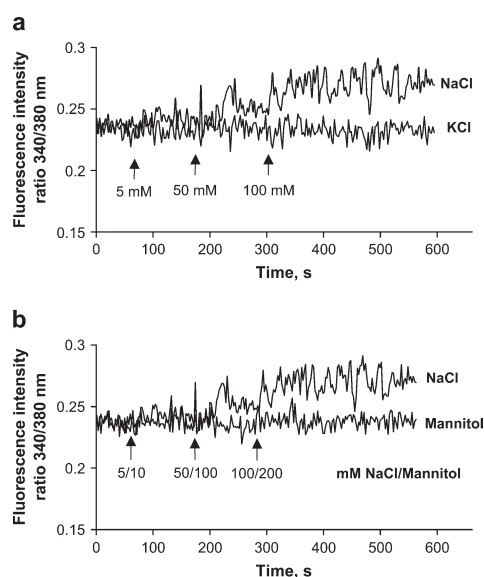


Fig. 3. Changes in fluorescence intensity ratio 340/380 nm in shoot protoplasts upon the addition of KCl (a) and mannitol (b) and upon the addition of NaCl in the presence of 0.1 mM external Ca^{2+} . Arrows indicate the addition of NaCl/KCl or NaCl/mannitol.

cultivars after pretreatment of protoplasts with some of the known inhibitors for K^+ -selective channels and NSCCs (Fig. 5a, b). When pretreated with 1.0 mM TEA, an inhibitor for K^+ -selective channels, the protoplasts from Pokkali leaf took up Na^+ almost in the same way as protoplasts from untreated controls. TEA caused, however, a significant inhibition of the uptake of Na^+ into the cytosol of BRRI Dhan29. A Hanes plot (Fig. 6a, b) shows that TEA inhibited the Na^+ uptake in BRRI Dhan29 in a non-competitive way, whereas the uptake in Pokkali was not affected by TEA. No significant inhibition of cytosolic Na^+ uptake was found in protoplasts from Pokkali leaf when they were pretreated with 10 mM Cs^+ , another

Table 1. Cytosolic Na^+ concentration of different types of protoplasts upon addition of external NaCl (means \pm SE)

The calcium concentration was 0.1 mM. After each addition of external NaCl, measurements of changes in the fluorescence intensity ratio 340/380 nm were recorded every 2.5 s. Data from measurements taken over 6 min were averaged. The changes in average fluorescence intensity ratio were then converted to cytosolic sodium concentration using the *in situ* calibration curve (Fig. 1). Each value represents data from experiments repeated 5–6 times with protoplasts from independent cultivations. Data are presented as means \pm SE. The initial cytosolic Na^+ concentration was considered zero since the seedlings were grown with culture solution containing no sodium.

NaCl addition (mM)	Concentration of Na^+ in the cytosol (mM)	
	Pokkali	BRRI Dhan29
To leaf protoplasts		
5	1.55 \pm 0.52	4.35 \pm 0.58
50	6.85 \pm 1.08	12.93 \pm 0.27
100	14.62 \pm 0.66	25.18 \pm 1.25
To root protoplasts		
5	1.62 \pm 0.67	3.96 \pm 0.78
50	4.28 \pm 1.21	13.11 \pm 2.01
100	10.28 \pm 1.54	24.43 \pm 2.31

inhibitor of K^+ -selective channels. However, treatment with 1 mM Ba^{2+} inhibited the uptake of Na^+ by about one-third of that of control protoplast. Both Cs^+ and Ba^{2+} also exerted partial inhibition in the uptake of Na^+ into the protoplasts from leaves of BRRI Dhan29 when the protoplasts were pretreated with them.

Although Pokkali protoplasts did not respond to TEA and Cs^+ regarding Na^+ uptake, the Na^+ uptake was inhibited in the presence of 1 mM Ca^{2+} , an inhibitor of NSCCs (Fig. 5b). In Pokkali, other inhibitors of NSCCs like 1 mM Zn^{2+} or 1 mM La^{3+} also showed the same type of inhibition of uptake of Na^+ as by 1 mM Ca^{2+} . On the other hand, in BRRI Dhan29 all of these inhibitors (1 mM Ca^{2+} or Zn^{2+} or La^{3+}) exerted a partial inhibition of the Na^+ uptake.

By the use of NH_4VO_3 , an inhibitor of plasma membrane H^+ -ATPase, or NH_4NO_3 , an inhibitor of tonoplast H^+ -ATPase, or both NH_4VO_3 and NH_4NO_3 , it was investigated whether the cytosolic Na^+ taken up was extruded into the vacuole or into the apoplast. When the protoplasts were

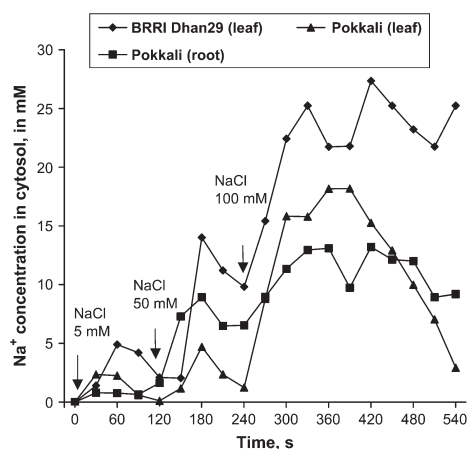


Fig. 4. Changes in cytosolic Na^+ concentration in root and shoot protoplasts from Pokkali, and shoot protoplasts from BRRI Dhan29 upon the addition of NaCl in the presence of 0.1 mM extracellular Ca^{2+} . Arrows indicate the addition of NaCl. Measurements of changes in the fluorescence intensity ratio 340/380 nm were recorded every 2.5 s and then the data were averaged for every 30 s. The change in average fluorescence intensity ratio was then converted to cytosolic sodium concentration using the *in situ* calibration curve.

pretreated with either 0.1 mM NH_4VO_3 or 1.0 mM NH_4NO_3 , or both, the cytosolic Na^+ concentration varied between these cultivars upon the addition of external NaCl (Table 2; Fig. 7a, b). Ammonium vanadate did not affect the cytosolic concentration of Na^+ in the protoplasts of Pokkali upon the addition of NaCl. However, the increase in cytosolic Na^+ concentration in the protoplasts of the same cultivar was much higher when the protoplasts were pretreated with NH_4NO_3 , compared with the untreated protoplasts. Accordingly, when the protoplasts were pretreated with both NH_4VO_3 and NH_4NO_3 , the increase in cytosolic Na^+ concentration was the same as for pretreatment with NH_4NO_3 alone. The change in cytosolic Na^+ concentration in BRRI Dhan29 was different from that of Pokkali. Sodium accumulation in the cytosol was much higher when protoplasts were pretreated with NH_4VO_3 compared with the untreated protoplasts. Cytosolic Na^+ concentration also became somewhat higher in the protoplasts pretreated with NH_4NO_3 compared with the control protoplasts. Moreover, when the protoplasts from BRRI Dhan29 were pretreated with both NH_4NO_3 and NH_4VO_3 , Na^+ absorption into the cytosol was slightly higher after the addition of 5 and 50 mM NaCl, but was similar after the addition of 100 mM NaCl compared with the protoplasts treated with NH_4VO_3 alone.

When the seedlings of both the cultivars were grown in saline conditions of 10 and 50 mM NaCl for 72 h, protoplasts from Pokkali showed less Na^+ uptake at both concentrations, whereas BRRI Dhan29 showed a similar

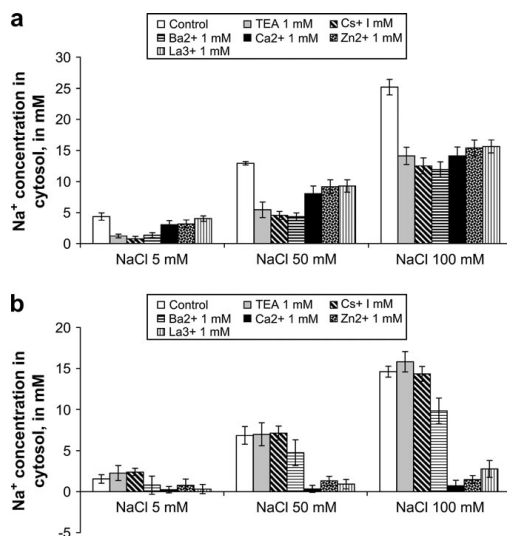


Fig. 5. Changes in cytosolic Na^+ concentration in different types of protoplasts from leaves of BRRI Dhan29 (a) and Pokkali (b) upon the addition of external NaCl after pretreatment of protoplasts with different inhibitors for K^+ -selective channels and NSCCs. Before the addition of external NaCl, protoplasts were pretreated with any of the following inhibitors for 10 min: 1 mM TEA, 10 mM Cs^+ , 1 mM Ba^{2+} , 1 mM Ca^{2+} , 1 mM Zn^{2+} , and 1 mM La^{3+} . Measurements were taken as the fluorescence intensity ratio 340/380 nm after the addition of external NaCl and converted to cytosolic Na^+ concentration using the *in situ* standard curve. Control protoplasts were not pretreated with any inhibitor, but kept for 10 min before measurement. Data represent mean values of cytosolic Na^+ concentration \pm SE.

uptake of Na^+ to that of untreated protoplasts (Fig. 8a, b). This observation was further evaluated by a growth experiment in which 10-d-old seedlings of both these cultivars were grown for 3 weeks at the same culture solution as stated in the Materials and methods, but containing 0, 5, 10, and 50 mM NaCl. Pokkali continued to increase its fresh weight up to 50 mM NaCl solution, whereas BRRI Dhan29 could survive after treatment with 10 mM NaCl (data not shown). The seedlings of BRRI Dhan29 died after treatment with 50 mM NaCl solution during the cultivation.

Discussion

Uptake of Na^+ into the cytosol

These results show that less Na^+ was taken up into the cytosol of leaf protoplast of the salt-tolerant cultivar Pokkali than in that of the salt-sensitive cultivar BRRI Dhan29 (Table 1; Fig. 4). By use of ion selective microelectrodes, Carden *et al.* (2003) also showed that the tolerant cultivar of barley after short-term Na^+ -treatment contained lower concentrations of Na^+ in the cytosol than

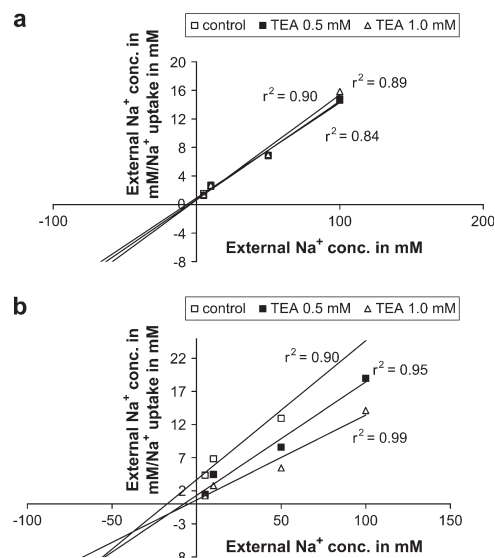


Fig. 6. Hanes plots showing cytosolic Na⁺ concentration in the protoplasts from Pokkali (a) and BRRI Dhan29 (b) where extracellular Na⁺ concentration/Na⁺ uptake is plotted against extracellular Na⁺ concentration after pretreatment with different concentrations of TEA for 10 min. Extracellular Ca²⁺ was 0.1 mM.

Table 2. Cytosolic Na⁺ concentration of different types of protoplasts from leaves upon the addition of NaCl to the protoplast medium pretreated with NH₄VO₃ or NH₄NO₃ or both for 10 min

The calcium concentration was 0.1 mM. After each addition of external NaCl, measurements of changes in fluorescence intensity ratio 340/380 nm were recorded every 2.5 s. Data from measurements taken over 6 min were averaged. The changes in average fluorescence intensity ratio were then converted to cytosolic sodium concentration using the *in situ* calibration curve (Fig. 1). Each value represents data from experiments repeated 5–6 times with protoplasts from independent cultivations. Data are presented as means \pm SE. The initial cytosolic Na⁺ concentration was considered zero since the seedlings were grown with culture solution containing no sodium.

NaCl addition (mM)	Concentration of Na ⁺ in the cytosol (mM)	
	Pokkali	BRRI Dhan29
Protoplasts treated with NH ₄ VO ₃		
5	3.40 \pm 1.01	5.58 \pm 0.50
50	7.75 \pm 1.38	19.08 \pm 0.75
100	16.17 \pm 1.41	39.31 \pm 1.85
Protoplasts treated with NH ₄ NO ₃		
5	3.12 \pm 0.71	4.68 \pm 0.84
50	13.22 \pm 1.76	14.69 \pm 1.12
100	25.44 \pm 1.79	27.08 \pm 1.79
Protoplasts treated with NH ₄ VO ₃ +NH ₄ NO ₃		
5	3.08 \pm 0.61	7.60 \pm 1.43
50	11.52 \pm 1.23	21.46 \pm 2.01
100	26.32 \pm 2.02	39.21 \pm 2.48

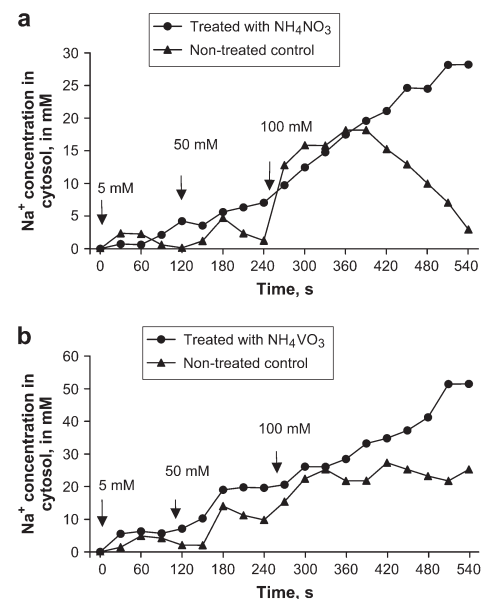


Fig. 7. Changes in cytosolic sodium concentration in shoot protoplasts from Pokkali pretreated with 1 mM NH₄NO₃ for 10 min (a) and from BRRI Dhan29 pretreated with 0.1 mM NH₄VO₃ for 10 min (b) and upon the addition of NaCl in the presence of 0.1 mM extracellular Ca²⁺. Arrows indicate the addition of NaCl. Measurements of changes in fluorescence intensity ratio 340/380 nm were recorded every 2.5 s and then the data were averaged for every 30 s. The change in average fluorescence intensity ratio was then converted to cytosolic sodium concentration using the *in situ* calibration curve.

Table 3. Concentration of Na⁺ in the cytosol upon addition of NaCl to different types of leaf protoplasts from seedlings pretreated with NaCl for 72 h during cultivation

The calcium concentration was 0.1 mM. After each addition of external NaCl, measurements of changes in fluorescence intensity ratio 340/380 nm were recorded every 2.5 s. Data from measurements taken over 6 min were averaged. The changes in average fluorescence intensity ratio were then converted to cytosolic sodium concentration using the *in situ* calibration curve (Fig. 1). Each value represents data from experiments repeated 5–6 times with protoplasts from independent cultivations. Data are presented as means \pm SE.

NaCl addition (mM)	Concentration of Na ⁺ in the cytosol (mM)	
	Pokkali	BRRI Dhan29
Leaf protoplasts from seedlings pretreated with 10 mM NaCl		
5	0.62 \pm 0.29	5.18 \pm 1.38
50	4.52 \pm 0.71	14.35 \pm 2.28
100	11.86 \pm 1.20	25.52 \pm 2.87
Leaf protoplasts from seedlings pretreated with 50 mM NaCl		
5	0.75 \pm 0.41	6.14 \pm 0.71
50	4.56 \pm 1.14	16.49 \pm 1.76
100	11.02 \pm 1.12	26.92 \pm 2.01

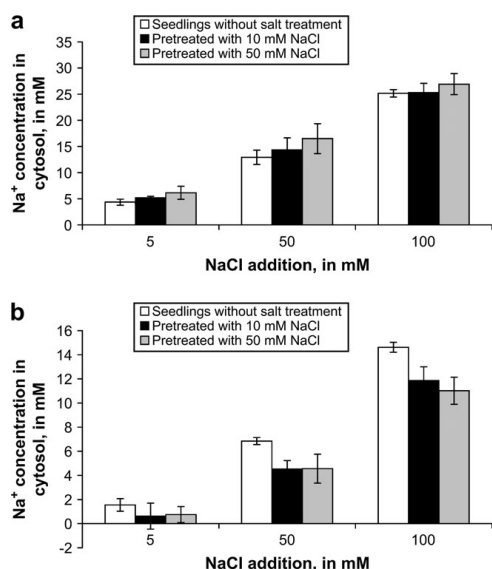


Fig. 8. Cytosolic Na⁺ concentration in shoot protoplasts from BRRI Dhan29 (a) and Pokkali (b) upon the addition of external NaCl at 0.1 mM extracellular Ca²⁺. Before isolating the protoplasts, seedlings were pretreated with 10 or 50 mM NaCl for 72 h during cultivation. Data represent the mean values \pm SE.

the sensitive cultivar. The addition of membrane-permeable cyclic nucleotides to *Arabidopsis* during growth assays improved plant salinity tolerance, which corresponded to lower levels of Na⁺ accumulation in plants (Maathuis and Sanders, 2001). Golldack *et al.* (2003) showed a similar difference in Na⁺ uptake between Pokkali and a salt-sensitive cultivar of rice, IR29.

Possible channels for the uptake of Na⁺

By use of inhibitors for K⁺-selective channels such as TEA, Cs⁺, and Ba²⁺, and for non-selective cation channels (NSCCs) such as Ca²⁺, Zn²⁺, and La³⁺, it was found that Na⁺ influx into the cytosol was mediated by different channels or transporters in these cultivars (Fig. 5a, b). The inhibition of Na⁺ uptake in BRRI Dhan29 by all of these inhibitors indicates that both K⁺-selective channels and NSCCs are involved in mediating Na⁺ uptake in this cultivar. The non-competitive inhibition of Na⁺ uptake in protoplasts of BRRI Dhan29 (Fig. 6a) suggests that TEA binds to the K⁺-channel protein. This result is consistent with other studies, suggesting that K⁺-selective channels contribute to Na⁺ influx in rice. Horie *et al.* (2001) isolated two isoforms of HKT transporters from rice and suggested that they are a Na⁺ transporter (OsHKT1) and a Na⁺- and K⁺-coupled transporter (OsHKT2). Rice homologues to

the wheat HKT1 are also known to contribute to substantial Na⁺ influx (Uozumi *et al.*, 2000). In addition to the HKT type transporters, other proteins for non-specific Na⁺ uptake include HAK/KT/KUP-type transporters, inward-rectifying potassium channels, and low-affinity cation transporters (Schachtman *et al.*, 1997). Although inward rectifying potassium channels are highly selective for K⁺, they could also mediate comparable uptake of Na⁺ at high salinity (Ammann and Sanders, 1999). Garciadeblas *et al.* (2003) suggested that a member of the HKT family of transporters could mediate Na⁺ uptake in rice. Recent studies of *Arabidopsis* mutants with extreme NaCl sensitivity have shown that suppression of this phenotype by a defect in AtHKT1 leads to lowered Na⁺ influx. Therefore, AtHKT1 could be the major protein for Na⁺ uptake, at least in some species (Rus *et al.*, 2001).

Except for Ba²⁺, the inhibitors for K⁺-selective channels of Na⁺ uptake did not significantly affect the uptake of Na⁺ in cv. Pokkali (Fig. 5b). It is, however, shown that Ba²⁺ may also block NSCCs (Demidchik and Tester, 2002). Therefore, the inhibition of Na⁺ uptake by Ba²⁺ in this cultivar might be the result of blocking of NSCCs, since other K⁺-selective channels did not inhibit the uptake. The Hanes plot (Fig. 6b) showing a lack of inhibition also indicates that TEA does not bind to K⁺-selective channels in cv. Pokkali. Thus, K⁺-selective channels are probably not involved in Na⁺ uptake in this cultivar. Instead the inhibitor analyses indicate that NSCCs are the main pathways for Na⁺ influx in cv. Pokkali, since inhibitors for NSCCs almost totally blocked the uptake of Na⁺. The NSCCs have been shown to be the major pathways for Na⁺ influx for many species (Roberts and Tester, 1997; Tyerman *et al.*, 1997; Buschmann *et al.*, 2000; Davenport and Tester, 2000; Demidchik and Tester, 2002).

Uptake in root and shoot protoplasts

Upon the addition of NaCl to protoplasts, the concentration of cytosolic Na⁺ in root protoplasts of Pokkali was less than that of leaf protoplasts (Table 1; Fig. 4). On the other hand, the uptake of Na⁺ was the same in both root and shoot protoplasts of BRRI Dhan29. Golldack *et al.* (2003) found a clear correlation of OsAKT expression in differentiated roots with whole-plant Na⁺-selectivity in the salt-sensitive and salt-tolerant rice cultivars IR29 and Pokkali, respectively. In response to salinity, a down-regulation occurred in cells of Pokkali. Therefore, the differences in cytosolic Na⁺ concentration in leaf and root protoplasts of Pokkali obtained in this study might depend on a different expression of NSCCs in root and leaf protoplasts.

In the present study, it was found that the sequestration process in the salt-tolerant cultivar Pokkali is less efficient in root protoplasts, compared with that in shoot protoplasts (Fig. 4). The result corroborates the study of Fukuda *et al.* (2004), which indicated that the transcript levels of

OsNHX1 expression were higher in shoots, than those in roots. Thus, it is likely that rice OsNHX1 may play an important role in the salt tolerance of shoots, rather than in roots.

Compartmentalization of Na^+

A distinct difference was found in the increase in cytosolic Na^+ concentration for both the cultivars when the protoplasts were pretreated with inhibitors for either plasma membrane H^+ -ATPase or tonoplast H^+ -ATPase. The tonoplast H^+ -ATPase energizes the Na^+/H^+ antiporter at the same membrane for compartmentalization of cytosolic Na^+ into the vacuole, and plasma membrane H^+ -ATPase does the same for the sequestration of cytosolic Na^+ into the apoplast (Blumwald *et al.*, 2000; Hamilton *et al.*, 2002). In Pokkali, an increase in cytosolic Na^+ concentration was obtained in the presence of the tonoplast H^+ -ATPase inhibitor (Table 2; Fig. 7a) compared with the control. This indicates an involvement of tonoplast Na^+/H^+ antiporter in the compartmentalization of cytosolic Na^+ into the vacuole. On the other hand, apoplastic Na^+ sequestration is probably absent in this cultivar, since inhibition of plasma membrane H^+ -ATPase did not increase the cytosolic Na^+ concentration compared with the untreated control protoplasts. Experiments with inhibitors for both tonoplast and plasma membrane H^+ -ATPase also suggest a vacuolar compartmentalization of cytosolic Na^+ in this cultivar, rather than apoplastic Na^+ sequestration.

In the sensitive cultivar BRRI Dhan29, the mechanisms for keeping cytosolic Na^+ concentration low was quite opposite to that of Pokkali. Inhibition of plasma membrane H^+ -ATPase in this cultivar resulted in a higher cytosolic Na^+ concentration. This indicates the involvement of the plasma membrane Na^+/H^+ antiporter for apoplastic Na^+ sequestration in this cultivar. However, the inhibitor analyses of the tonoplast H^+ -ATPase shows that the vacuolar compartmentalization is also involved to some extent, especially at a high external Na^+ concentration. From these studies it can be suggested that there is a fast efflux (vacuolar compartmentalization) of cytosolic Na^+ from leaf protoplasts of the salt-tolerant Pokkali, and some sequestration of Na^+ into the apoplast along with some vacuolar compartmentalization from the sensitive cultivar BRRI Dhan29. The salt-tolerant cultivar Pokkali does not use plasma membrane Na^+/H^+ antiporters for Na^+ extrusion into the apoplast, whereas the sensitive cultivar does. This might make the latter cultivar sensitive to salt stress, since sodium transported from cells by plasma membrane Na^+/H^+ antiporters would cause a problem for the neighbouring cells. Yeo *et al.* (1999) showed that Na^+ leakage into the transpiration stream through the apoplast may account for a major part of Na^+ entry into rice plants. On the other hand, Fukuda *et al.* (2004) found that an OsNHX1 (Na^+/H^+ antiporter) is localized in the tonoplast of rice and, at high salt concentrations, plays an important role in the compartmentalization of Na^+ into the vacuoles. Therefore, a large

amount of the tonoplast antiporter is one of the most important salt tolerance factors in rice.

Salt stress also induces a Na^+/H^+ antiport mechanism at the tonoplast of the salt-tolerant *Plantago* species, but not in the salt-sensitive species (Prins, 1995). For the salt-tolerant species quince, it was suggested that the main Na^+ extrusion operates at internal membranes and not at the plasmalemma (D'Onofrio *et al.*, 2005).

In some of the protoplasts, a subcellular compartmentalization of the dye, as in chloroplasts, can be found. The AM ester loading may cause intracellular compartmentalization of SBFI, especially if the loading is done at a higher temperature than room temperature (Haugland, 2002). It is reported that dye movement from the cytosol to other cell compartments does not necessarily affect the validity of measurements (Gryniewicz *et al.*, 1985; Brauer *et al.*, 1995). Moreover, as only protoplasts loaded in the cytosol were considered for measurements, and not those in the chloroplasts or vacuoles, there can be confidence that these results are free from any retranslocation of the dye from the cytosol.

K^+ - Na^+ and pH interferences with SBFI

At a low Na^+ concentration and a high K^+ concentration there might be some competition between Na^+ and K^+ for binding to the dye (Minta and Tsien, 1989; Haugland, 2002; Halperin and Lynch, 2003). Similar to Halperin and Lynch (2003), it was found here that the SBFI fluorescence is very weak compared with Fura. In the present study, addition of KCl up to 100 mM did not change the SBFI fluorescence ratio (Fig. 3a), although extracellular KCl addition might change cytosolic or vacuolar K^+ concentration. Therefore, the interference of cytosolic or vacuolar K^+ on SBFI- Na^+ fluorescence is unlikely. Mühling and Läuchli (2002) also reported that SBFI fluorescence was not significantly affected by 40 mM K^+ , but significantly affected by pH changes from 5.0 to 6.5. Another report, however, shows that SBFI fluorescence is unaffected by pH changes between 6.5 and 7.5 (Haugland, 2002). NaCl stress produces pH changes in cells involved in cell signalling (Gao *et al.*, 2004), and the changes in pH in the cytosol under NaCl stress are between 6.5 and 7.5 (Gao *et al.*, 2004; MA Kader and S Lindberg, unpublished results). Therefore, it is unlikely that the fluorescence ratio in the present study was affected by changes in the cytosolic pH.

Effects by viscosity and osmolarity

The fluorescence of SBFI is affected by changes in viscosity (Minta and Tsien, 1989). In response to osmotic stress caused by salinity, cells accumulate compatible osmolytes like glycinebetaine, proline, and sugars, which could change the viscosity if they are present at high concentration. Several reports showed that the fluorescence of SBFI is significantly affected only at a very high concentration of these osmolytes (Harootunian *et al.*, 1989; Mühling and Läuchli, 2002). In the present study,

however, no change in viscosity can be expected since measurements were taken only for 10 min after the addition of NaCl. Moreover, the SBFI ratio may also be affected by ionic strength (Negulescu and Machen, 1990; Haugland, 2002). Using KCl during the *in situ* calibration approximated the effect of ionic strength in the present study.

There was no increase in the fluorescence intensity ratio upon the addition of mannitol up to 200 mM (Fig. 3b), indicating that the increase in fluorescence intensity ratio upon the addition of extracellular NaCl was not an artefact of protoplast shrinking.

Effects of pretreatment with NaCl during cultivation

Pretreatment of seedlings with NaCl (10 and 50 mM) for 72 h before the uptake experiments resulted in less Na⁺ uptake into the protoplasts of the tolerant cultivar, but no significant difference in the sensitive cultivar (Fig. 8a, b). The growth experiments indicated that 10 mM NaCl caused some toxicity in the sensitive cultivar BRRI Dhan29, but not in Pokkali (data not shown). Also at 50 mM NaCl concentration, all seedlings of Pokkali survived with a positive growth rate (but with a significant reduction compared with the control). Therefore, the lower uptake of Na⁺ in Pokkali, after pretreatment with NaCl, might depend on induction of some tolerance mechanisms to Na⁺. On the other hand, the somewhat higher uptake of Na⁺ in BRRI Dhan29 might be due to some toxic effect of an endogenous high concentration of Na⁺. The present results are in agreement with the results of Golladack *et al.* (2002, 2003), which showed that OsHKT1 transcription was down-regulated in roots and leaves of Pokkali as a response to salt stress. These authors also showed that rice OsAKT1-type potassium channels are regulated in different ways under salt stress in the salt-tolerant and salt-sensitive cultivars. In Pokkali the OsAKT1-type transcripts disappeared in plants treated with 150 mM NaCl for 48 h, but this transcript in cv. IR29 was not repressed.

Summary

From this investigation it can be concluded that Na⁺ uptake in the cytosol is much higher (approximately double) in the salt-sensitive cultivar BRRI Dhan29, than in the salt-tolerant cultivar Pokkali. The NSCCs seem to be the main pathway for Na⁺ uptake in Pokkali, whereas K⁺-selective channels and NSCCs both contribute to the Na⁺ influx in BRRI Dhan29. Under salt stress, root protoplasts of Pokkali prevent Na⁺ uptake more efficiently than shoot protoplasts, whereas both root and shoot protoplasts of BRRI Dhan29 take up Na⁺ at almost the same rate. Pokkali compartmentalizes the internal Na⁺ into the vacuole more efficiently in shoots than in roots. Therefore, it is likely that the tolerance to salt in Pokkali depends on preventing Na⁺-influx into the roots, and sequestering of Na⁺ from cytosol into the vacuole of the shoots.

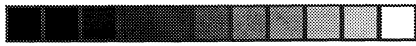
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III



Sodium sensing induces different changes in free cytosolic calcium concentration and pH in salt-tolerant and -sensitive rice cultivars

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Abstract

Perception of salt stress in plant cells induces changes in the free cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_{\text{cyt}}$, which transduces downstream reactions towards salt tolerance. Changes in cytosolic H^+ concentration, $[\text{H}^+]_{\text{cyt}}$, are closely linked to the $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics under various stress signals. In this study salt-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$, and $[\text{H}^+]_{\text{cyt}}$ and vacuolar $[\text{H}^+]$ concentrations were monitored in single protoplasts of rice (*Oryza sativa* L. indica cvs. Pokkali and BRRI Dhan29) by fluorescence microscopy. NaCl addition induced a higher increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the salt-tolerant cv. Pokkali than in the salt-sensitive cv. BRRI Dhan29. From inhibitor studies we conclude that the internal stores appear to be the major source for $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in Pokkali, although the apoplast is more important in BRRI Dhan29. The $[\text{Ca}^{2+}]_{\text{cyt}}$ -measurements in rice also suggest that Na^+ should be sensed inside the cytosol, before any increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ occurs. Moreover, our results with individual mesophyll protoplasts suggest that ionic stress causes an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and that osmotic stress sharply decreases $[\text{Ca}^{2+}]_{\text{cyt}}$ in rice. The $[\text{H}^+]_{\text{cyt}}$ was differently shifted in the two rice cultivars in response to salt stress and was coupled to different H^+ -ATPases.

Key-words: Cytosolic Ca^{2+} and pH; Vacuolar pH; Fluorescence microscopy; Protoplasts; Rice.

Introduction

Salt stress, mostly caused by excess of NaCl, elicits two primary effects on plants: osmotic stress and ionic toxicity due to high levels of Na⁺ and/or Cl⁻ ions in the cytoplasm (Greenway & Munns 1980; Hasegawa et al. 2000; Zhu 2001). However, Na⁺ is the primary cause of the ion-specific damage of many plants such as graminaceous plants (Amtmann & Sanders 1999; Tester & Davenport 2003). One of the early responses of plant cells to many stresses, including salt stress, is the generation of a cytosolic Ca²⁺ increase. In plant cells, Ca²⁺ serves as a second messenger during stress signaling (Sanders, Brownlee & Harper 1999; Knight 2000).

The perception of Na⁺ and its signal transduction to switch on adaptive responses are critical steps for plants exposed to salt environments. Under salinity both osmotic stress and ionic stress are sensed at the cellular level and, consequently, elicit a transient change in [Ca²⁺]_{cyt} levels. The changes in [Ca²⁺]_{cyt} either can be attributed to the apoplast, or to the internal stores like ER, golgi bodies, mitochondria or vacuole (Sanders et al. 2002). Until now, however, very little information is given about the sensors in plants for ionic toxicity and osmotic stress under high salt. So far several osmosensors are reported to be involved in osmotic stress signal perception (Urao et al. 1999; Reiser, Raitt & Saito 2003; Tamura et al. 2003; Boudsocq & Lauriere 2005). In recent years, a substantial progress has been made regarding plant response under salt stress including the SOS (Salt-Overly-Sensitive) pathway in *Arabidopsis* (Zhu 2002). But, how crop plants sense Na⁺ toxicity in cells, and whether they sense Na⁺ inside or outside the plasma membrane are still unknown. However, it is speculated that SOS1, a plasma membrane Na⁺/H⁺ antiporter, senses Na⁺ by its long C-terminal tail, predicted to reside in the cytoplasm (Zhu 2003; Zhang, Creelman & Zhu 2004; Shabala et al. 2005). It is also speculated, that Na⁺ sensors control the [Ca²⁺]_{cyt} level that exerts at least two roles in salt tolerance; a pivotal signaling function (the SOS signaling pathway) for the regulation of Na⁺ homeostasis leading to plant adaptation, and a direct inhibitory effect on the Na⁺ entry system.

In many studies high concentration of NaCl has been reported to cause an increase in [Ca²⁺]_{cyt} (Bittisnich, Robinson & Whitecross 1989; Lynch, Polito & Läuchli 1989; Knight, Trewavas & Knight 1997; Halfter, Ishitani & Zhu 2000; Zhu 2001; Gao et al. 2004; Henriksson & Henriksson 2005). Conversely, in some studies high salinity has been shown to reduce cytosolic Ca²⁺ concentration (Lynch & Läuchli 1988; Cramer & Jones 1996; Halperin, Gilroy & Lynch 2003). The results are also contrasting in studies whether osmotic stress increases or decreases [Ca²⁺]_{cyt} (Cramer & Jones, 1996; Knight et al. 1997; Kiegle et al. 2000). From the results so far, it can be concluded that change in [Ca²⁺]_{cyt} is not uniform and varies with species, cell type or tissue type (Cramer & Jones, 1996). Specific Ca²⁺ signatures are also important for plant cells to follow the subsequent events in the signaling process. Such processes may change with the particular stress (Kiegle et al. 2000), the rate of stress development (Plieth et al. 1999), pre-exposure to the stress (Knight et al. 1997) and the tissue type (Kiegle et al. 2000).

Both the apoplast and intracellular organelles are stores of Ca^{2+} . Calcium-permeable channels in the plasma membrane, which are activated by membrane depolarization, are thought to lead to elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in many species after the perception of a range of stimuli (Gelli & Blumwald 1993; Gelli, Higgins & Blumwald 1997; Hamilton et al. 2000; Véry & Davis 2000; White 2000; Sanders et al. 2002). The generation of Ca^{2+} in the cytosol further modulates other messengers like inositol phosphate, which induces a further Ca^{2+} elevation in the cytosol through the opening of Inositol-(1, 4, 5)- triphosphate (IP_3)- regulated Ca^{2+} channels (Sanders et al. 2002). There are some studies showing that salt stress induces a rapid increase in IP_3 concentration in the cytosol (Takahashi et al. 2001; DeWald et al. 2001). By use of the inhibitors verapamil and nifedipine for plasma membrane Ca^{2+} -permeable channels (Polevoi et al. 1996; Babourina, Shabala & Newman 2000; White et al. 2002) and LiCl for inhibition of Ca^{2+} release from internal stores, like vacuole or ER, through IP_3 -regulated Ca^{2+} -permeable channels (Gillaspy et al. 1995; Knight, Trewavas & Knight 1996; Liang, Shen & Theologis 1996; Knight et al. 1997), the major sources for $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics could be suggested.

The $[\text{Ca}^{2+}]_{\text{cyt}}$ and pH homeostasis in cells are closely linked (Bush 1993). Upon shifting of $[\text{Ca}^{2+}]_{\text{cyt}}$ under salt stress, cells are challenged with the excess of other monovalent ions like H^+ in the cytosol (Plieth, Sattelmacher & Hansen 1997; Plieth et al, 1999; Gao et al. 2004). Moreover, transient shifts in intracellular and apoplastic pH are essential steps in several signal transduction processes, and pH is involved in cell signaling, either directly, or in cross talk with plant hormones, or Ca^{2+} (Gilroy & Trewavas 1994; Ward, Pei & Schroeder 1995; Blatt & Grabov 1997; Roos 2000; Felle 2001; Gao et al. 2004).

The aim of this study was to monitor the sensing of Na^+ in the cell and the subsequent dynamics of $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{H}^+]_{\text{cyt}}$ under salt stress in rice. The specific signatures of shifts in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{H}^+]_{\text{cyt}}$ were compared in salt-sensitive indica rice cv. BRRI Dhan29 and salt-tolerant indica rice cv. Pokkali. By use of inhibitors for calcium channels and ATPases, the sources for changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{H}^+]_{\text{cyt}}$ could be investigated.

Materials and Methods

Plant material

Seeds of rice (*Oryza sativa* L. *indica* cvs. Pokkali and BRRI Dhan29) were provided by the Bangladesh Rice Research Institute (BRRI, Gazipur, Bangladesh). They were treated with 10% chlorine solution for 15 min and rinsed with distilled water 5 to 6 times. Seeds were kept in 5 mM CaSO_4 solution for 3 h. Thereafter, seeds were soaked with water for 48 h in the darkness. They were placed on a miracloth layer covering a metal net, which was placed on a wide beaker with culture solution. The culture solution used was based on that described by Yoshida

et al. (1976), with modifications made by Khatun & Flowers (1995) to reduce the sodium concentration in the basic solution (to almost zero). The beaker was covered with white polyethene and placed in a Fison growth chamber (Convion, Manitoba, R3H OW9, USA). The day/night temperature was 30⁰C/25⁰C under a 12-h-photoperiod with 100 E $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (Philips 3629 warm white 36W, Rosendahl, The Netherlands). Humidity was about 75%. Protoplasts were prepared from leaves of 9- to 10-d-old seedlings.

Protoplast isolation

The protoplasts from rice, cvs. Pokkali and BRRI Dhan29, were prepared as described by Shishova and Lindberg (1999) with some modifications. Leaves were sliced in 0.5 mm pieces and treated with 1% (w/v) cellulase (lyophilized powder; 10.0 units mg^{-1} solid) from *Trichoderma resei* (Sigma, EC 3.2.1.4) and 0.6% (w/v) macerage (lyophilized powder; 0.6 units mg^{-1} solid), Maceroenzyme R-10 (Serva, EC 3.2.1.4) for 2 h.

Dye loading

The protoplasts were washed twice in the loading medium containing 0.5 M sorbitol (Sigma, St. Louis, MO, USA), 0.1 mM CaCl_2 , 0.2% (w/v) polyvinylpyrrolidone (PVP, Sigma St. Louis, MO, USA) and a buffer (pH 5.5: medium A) containing 5 mM Tris (Labassco, Germany)] and 5 mM MES (Sigma, St. Louis, MO, USA).

For Ca^{2+} measurement the protoplasts were loaded with Fura 2 in the acetoxymethyl ester form (Fura 2-AM, Molecular Probes, Leiden, the Netherlands). The Fura 2-AM dye solution was prepared by mixing 2 μL of Fura 2-AM stock solution (5mg / ml) in dry (<0.1% v/v water) DMSO (dimethyl sulfoxide), 1.25 μL of pluronic F-127 (Molecular Probes) solution (20% w/v) in DMSO and 6.75 μL of ethanol (99.5% v/v) (Sebastiani, Lindberg & Vitagliano 1999). From the above mixed Fura 2-AM dye solution, 5 μL was added to 1 ml of protoplast suspension. Loading was performed at 22⁰C for 2 to 3 h.

For the measurement of cytosolic pH, protoplasts were loaded with BCECF-AM in the same way as for Fura 2-AM, but without any pluronic, and with a loading time 1 h at 4⁰C (Lindberg & Strid 1997). After loading the protoplasts were washed in a solution similar to medium A, but with Tris-MES buffer at pH 7 (medium B). Afterwards, the protoplast samples were kept in darkness at room temperature for about half an hour before starting the measurements.

For the measurement of pH in the vacuoles, protoplasts were stained with 3 μL of 5 mM 6-carboxyfluorescein diacetate (6-CFDA, Sigma-Aldrich) in DMSO through PEG-mediated osmotic shock. Non-specific esterase-activity in the vacuolar lumen cleaves the non-fluorescent 6-CFDA to the fluorescent, pH dependant dye 6-carboxyfluorescein (Preston, Murphy & Jones 1989).

Fluorescence measurements

An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochen, Germany), supplied with an electromagnetic filter-exchanger (Zeiss), Xenon lamp (Zeiss XBO 75), photometer (Zeiss 01), microprocessor (MSP 21, Zeiss) and a personal computer was used to determine fluorescence intensity after excitation at 340/380 nm for Fura 2 measurements and at 485/436 nm for BCECF measurements. Emission wavelengths were 500 to 530 nm and 510 to 550 nm for Fura 2 and BCECF, respectively. All measurements were performed with a Planneofluar x 40/0.75 objective (Zeiss) for phase contrast. Adjustment of signals and noise were made automatically. By means of ratio microscopy (Tsien & Poenie 1986; Bright et al. 1987), the effect of different dye concentration could be eliminated. Microslides were covered with poly-L-lysine (MW 150000-300000, Sigma) 0.15%, to attach protoplasts to their surface.

The 6-CF fluorescence in the vacuoles was measured by confocal microscopy (Leica SP2). The dye was excited sequentially using the 458 nm and 488 nm lines of an argon-ion laser. The signal was detected in the range of 500 to 530 nm after passing the short pass filter RSP500 (Leica). The ratio of the emission intensities of both the excitation wavelengths depends on the vacuolar pH (Preston et al. 1989). The 6-CF fluorescence was measured in the protoplasts before and after salt addition. Reference measurements were performed to calibrate the observed emission ratios to specific pH values (Seidel, Golldack & Dietz 2005).

Standard determinations of $[Ca^{2+}]_{cyt}$ in single protoplasts using Fura 2-AM were made from an *in situ* calibration (Fig. 1a) of the fluorescence ratio (340/380 nm) as described by Sebastiani *et al.* (1999). The calibration curve was calculated according to Grynkiewicz et al. (1985). The mean values of R_{min} , R_{max} , Sf_2 and Sb_2 were determined from *in situ* calibration experiments as described in the protocol of Thomas & Delaville (1991). An *in situ* calibration of BCECF fluorescence ratio 485/436 nm at pH values 4.0, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0 was performed for the standard determinations of pH in the cytosol of protoplasts (Fig 1b). To equilibrate intracellular and extra cellular pH, nigericin was used at a final concentration 5 μ M (Negulescu & Machen 1990). Measurements were undertaken 5 to 10 min after addition of nigericin.

Mesophyll protoplasts of similar size, that showed fluorescence only from the cytosol, were chosen for the fluorescence measurements. The fluorescence intensity ratio of the cytosol was determined in single protoplasts before, and after, the addition of NaCl 100 mM final concentrations to the protoplast suspension. Our previous study indicated that addition of 100 mM NaCl to rice protoplasts does not make any protoplast shrinkage and thus, not any artifact of fluorescence change because of protoplast shrinking (Kader & Lindberg 2005). By means of regression analysis of the *in situ* calibration curve (Fig. 1a, b), fluorescence intensity ratios were converted to the cytosolic Ca^{2+} and pH. The measurements were performed with protoplasts of similar size (approximately 10 μ m).

RNA extraction and qRT-PCR of rice vacuolar H⁺ATPase (OsVHA)

Three-weeks-old rice plants cultivated in the same way as stated before were used for the extraction of RNA. Prior to harvesting, the plants were stressed by NaCl for 1 and 24 h at the final concentration of 150 mM, by addition to the nutrient solution. Non-stressed control plants were grown in parallel and harvested at the same time. For qRT-PCR the total RNA was isolated by the guanidinium thiocyanate method. Synthesis of cDNA was performed as described by Golldack et al. (2002). For PCR amplification of OsVHA the following sequence-specific forward and reverse oligonucleotide primers were used: 5'-CTTCTGGCAATCTTGGAG-3' and 5'-CAGTGTAGACGAAGTGCA-3'. The following conditions in PCR reactions were used: 1 cycle 94 °C for 1 min 30 s, 1 min 94 °C, 1 min 55 °C, 2 min 72 °C, and a final extension at 72 °C for 10 min. The PCR amplification was done with 28 cycles and the products were separated on 1.7% (w/v) agarose gels and stained with ethidium bromide. Photographic documentation was performed with a gel documentation system (INTAS, Göttingen).

Statistics

Each plot is a copy of printer plots and shows representative traces of a specific experiment repeated ≥ 7 times with protoplasts from independent cultivations. Each value before salt addition is the average of around 25 fluorescent-ratio determinations, and after salt addition, 240 fluorescent-ratio determinations. The figures with bar diagrams show data from experiments repeated 5 to 6 times with protoplasts from independent cultivations. Data are presented as means \pm standard error (SE).

Results

Standard determinations of $[Ca^{2+}]_{cyt}$ (Fig. 1a) show the manipulation of $[Ca^{2+}]_{cyt}$ in a single protoplast in response to the addition of EGTA (1), Ca^{2+} ionophore (2), extra cellular Ca^{2+} (3) and extra cellular Mn^{2+} (4). Addition of EGTA decreased the extra cellular free Ca^{2+} . This could be seen as a decrease in the fluorescence ratio 340/380 nm, after the addition of the Ca^{2+} ionophore, which equilibrated intracellular and extra cellular free Ca^{2+} concentration. The addition of extra cellular Ca^{2+} then increased the fluorescence ratio 340/380 nm and reached a peak within 4 min. The ratio started to decrease after the addition of Mn^{2+} , due to the binding of Mn^{2+} to Fura-2. The *in situ* calibration of BCECF fluorescence ratio 485/436 nm at pH values between 4.0 and 8.0 shows a linear relationship between different pH values and their respective fluorescence ratio (Fig. 1b).

In the present study we observed changes in the $[Ca^{2+}]_{cyt}$ and $[H^+]_{cyt}$ dynamics in the mesophyll cells under salt stress. We chose mesophyll protoplasts with the dye

loaded into the cytosol (Fig. 2b, protoplast shown by green arrow) for the measurements. In both cases of Fura 2-AM and BCECF-AM dye loading, we noticed in a few cases compartmentalization of the dye within the vacuole and chloroplast (Fig. 2b, protoplast shown by red arrow) that was also found in some other studies (Read et al. 1992; Sebastiani et al. 1999). However, since we used a high concentration of protoplasts for loading, it was not difficult to get correctly loaded mesophyll protoplasts for measurements. The viability of the cells was always checked before, and after, making the fluorescence measurements upon salt addition. It could be assayed by the presence of the fluorescence inside the cells, because the hydrolysis of the dye is a good viability indicator (Gualtieri 1992). After every measurement upon salt addition protoplast viability was also confirmed by checking the protoplasmic streaming and by any visible change in size and shape of the protoplasts. Very seldom (less than 5%) the protoplasts changed shape after salt addition, and the measurements from those protoplasts were ignored.

[Ca²⁺]_{cyt} dynamics upon addition of NaCl to rice protoplasts

Addition of 100 mM NaCl induced an increase in free cytosolic Ca²⁺ both in cvs. BRRI Dhan29 and Pokkali in the presence of 0.1 mM extra cellular Ca²⁺ (Figs. 3a, c). In both cultivars the increase in [Ca²⁺]_{cyt} was transient. The total increase of [Ca²⁺]_{cyt}, and the duration of the Ca²⁺-signal, were higher in cv. Pokkali than in cv. BRRI Dhan29. The average and maximal [Ca²⁺]_{cyt} increases were approximately 22 nM and 77 nM, respectively in BRRI Dhan29, whereas the average and maximal [Ca²⁺]_{cyt} increases were about 29 nM and 111 nM, respectively in Pokkali (Fig. 5a, b). The increase in free cytosolic Ca²⁺ concentration in Pokkali showed a clear two-phase increase, first one within few seconds after salt addition, and the second one around 3 min after salt addition (Fig. 3c). This two-phase increase was absent in BRRI Dhan29 (Fig 3a). In the latter case, the one-phase increase started a few seconds after the salt addition.

We also investigated whether the increase in [Ca²⁺]_{cyt} was specific to ion (Na⁺ and/or Cl⁻), or just an osmotic effect. When 200 mM sorbitol was added to protoplasts, it induced a significant decrease in [Ca²⁺]_{cyt} in both the cultivars, quite different from the ionic effect (Figs. 4a-b).

To investigate the influx of Ca²⁺ into the cytosol from external sources like the apoplast, we added salt in the presence of higher extra cellular Ca²⁺, e.g. 1.0 mM. In the salt sensitive cultivar BRRI Dhan29 the free cytosolic Ca²⁺ responses became more pronounced when NaCl was added in the presence of 1.0 mM extra cellular Ca²⁺ (Figs. 3b & 5a). Upon 100 mM NaCl addition the [Ca²⁺]_{cyt} maximally increased approximately by 139 nM and an average by about 39 nM, in the presence 1.0 mM extra cellular Ca²⁺. Surprisingly, however, there was no increase in free cytosolic Ca²⁺ at all in the salt-tolerant cultivar Pokkali when NaCl was added in the presence of 1.0 mM extra cellular Ca²⁺ (Figs. 3d & 5b). Rather, the free cytosolic Ca²⁺ concentration decreased a little from the resting [Ca²⁺]_{cyt} concentration, similar to that of osmotic stress (mimicked by sorbitol of the same osmolarity; Fig. 4b).

We pretreated the protoplasts with 1.0 mM Zn^{2+} that also blocks NSCCs and thus, the influx of Na^+ into the cytosol in Pokkali (Kader and Lindberg 2005). There was no increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon salt addition when protoplasts of Pokkali were pretreated with 1.0 mM Zn^{2+} for 5 to 10 min.

Pretreatment of rice protoplasts with different blockers of Ca^{2+} -channels resulted in a considerable variation in the increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ in both the cultivars, compared to non-treated protoplasts (Fig. 5a-b). In BRRI Dhan29, the average increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ concentration was lowered compared to the non-treated protoplasts by approximately 56% and 51% in the protoplasts pretreated with verapamil and nifedipine, respectively (believed to block Ca^{2+} influx from the apoplasts; Polevoi et al. 1996; Babourina et al. 2000; White et al. 2002). The increase in maximal amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ was even more lowered (75% and 72% in the protoplasts pretreated with verapamil and nifedipine, respectively, compared to the non-treated protoplasts). On the other hand, LiCl at the concentration 1.0 mM or higher, believed to block Ca^{2+} influx from intracellular stores (Gillaspy et al. 1995; Knight et al. 1996; Liang et al. 1996; Knight et al. 1997), did not block substantially $[\text{Ca}^{2+}]_{\text{cyt}}$ increase both at the maximal amplitudes and average values. The increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in Pokkali could also be blocked by use of inhibitors both for plasma membrane Ca^{2+} -channels and IP_3 -regulated Ca^{2+} -channels in the internal organelles. However, pretreatment of protoplasts with 5 mM LiCl (inhibitor of IP_3 -regulated Ca^{2+} -channels in the internal organelles) blocked the maximum amplitude of $[\text{Ca}^{2+}]_{\text{cyt}}$ increase and average $[\text{Ca}^{2+}]_{\text{cyt}}$ increase by 77% and 72%, respectively, compared to the non-treated control protoplasts. On the other hand, the blocking of average $[\text{Ca}^{2+}]_{\text{cyt}}$ increase by inhibitors of plasma membrane Ca^{2+} -channels were only 28% and 30% for verapamil and nifedipine, respectively, compared to non-treated control protoplasts.

$[\text{H}^+]_{\text{cyt}}$ dynamics in rice protoplasts upon NaCl addition

Measurements of the cytosolic pH, detected by fluorescence intensity ratio F485/436 nm of BCECF-AM loaded protoplasts, revealed that salt stress differently influenced the cytosolic pH of Pokkali and BRRI Dhan29 (Fig 6a-b). Osmotic stress (sorbitol), however, did not cause any significant alterations in cytosolic pH (data not shown). Moreover, salt addition caused a significant decrease in pH in the vacuole of Pokkali protoplasts, both within 5-10 min after salt addition, and 24 h after salt addition (Fig. 7). The change in vacuolar pH in BRRI Dhan29 upon salt stress was not significant immediately after salt addition (5 to 10 min), but significantly lower, compared to the initial value, 24 h after salt addition. After 24 h the protoplasts were still viable with the intact shape. The changes in vacuolar pH in these two rice cultivars were consistent with the expression of vacuolar H^+ ATPase (OsVHA) 1 and 24 h after NaCl stress. The expression of OsVHA was induced by NaCl stress in Pokkali 1 h after NaCl stress, but in BRRI Dhan29 only 24 h after the stress (Fig. 8).

Involvement of plasma membrane H^+ -ATPase and tonoplast H^+ -ATPase in shifting cytosolic pH was investigated by pretreatment of protoplast with NH_4VO_3 , an

inhibitor of plasma membrane H^+ -ATPase, or NH_4NO_3 , an inhibitor of tonoplast H^+ -ATPase (Fig. 9). Without pretreatment of the protoplasts the cytosolic pH decreased by approximately 0.56 units in BRRI Dhan29 upon addition of 100 mM NaCl, whereas it increased by 0.57 units in Pokkali. In BRRI Dhan29, the shifts in cytosolic pH were similar to the control, and when the protoplasts were pretreated with 1.0 mM NH_4NO_3 , but significantly lower, when the protoplasts were pretreated with 0.1 mM NH_4VO_3 . Pretreatment of protoplasts from Pokkali with NH_4NO_3 or NH_4VO_3 both resulted in a lower shift in pH compared to the non-treated ones. However, in the case of pretreated Pokkali protoplasts, the shift of cytosolic pH was much lower when protoplasts were pretreated with NH_4NO_3 , than with NH_4VO_3 .

Discussion

Alterations in free cytosolic Ca^{2+} occurred in rice mesophyll protoplasts both upon salt stress and osmotic stress (mimicked by sorbitol). Salt stress (100 mM NaCl) transiently increased $[Ca^{2+}]_{cyt}$ in both the rice cultivars in the presence of 0.1 mM Ca^{2+} (Fig. 3a, c). However, the sharp spikes of elevated $[Ca^{2+}]_{cyt}$, as obtained in many studies using intact plants like Knight et al. (1997), Kiegle et al. (2000); Gao et al. (2004) and Henriksson & Henriksson (2005) were absent in our measurements. Since we were measuring the reaction in only one cell, the total increase in $[Ca^{2+}]_{cyt}$ under salt stress was probably not so distinct as in some other studies (Knight et al. 1997; Henriksson & Henriksson 2005). The changes of $[Ca^{2+}]_{cyt}$ in our study in nM range, were also found in response to salt stress in quince (*Cydonia oblonga* (Mill), D'Onofrio C and Lindberg S, unpublished result) and in response to cold stress (Sebastiani et al. 1999). Besides, a decrease in $[Ca^{2+}]_{cyt}$ to a new steady-state level within 5 min after salt addition, was obtained in other studies (Knight et al. 1997; Henriksson & Henriksson 2005). The decrease in $[Ca^{2+}]_{cyt}$ upon osmotic stress is consistent with the studies of Lynch & Läuchli (1988), who showed a decrease in cytosolic Ca^{2+} in corn root protoplast upon osmotic stress. It is likely that Ca^{2+} upon osmotic stress is transported from the cytosol into the vacuole (that is substantially involved in the adaptation process with osmotic stress) or into some other intracellular organelles. We also assume that use of individual protoplast could be the reason for obtaining the decrease in $[Ca^{2+}]_{cyt}$ upon osmotic treatment, also obtained by Lynch & Läuchli (1988). One concern for this osmotic study could be the use of wall-less protoplasts that might not react in the same way as in the intact plants. However, we found in our earlier study with rice protoplasts that 100 mM NaCl or 200 mM mannitol do not cause any protoplast shrinkage (Kader & Lindberg 2005) and, therefore, could be used for this type of study. In experiments with intact plants, the increase in $[Ca^{2+}]_{cyt}$ obtained shows a mean value of many cells. Then not all events may be detected. Therefore, it is also important to show what happens in one single cell upon osmotic stress within a certain time.

Extra cellular Ca^{2+} at 0.5 mM or higher concentration blocks NSCCs mediated Na^+ influx (Amtmann & Sanders 1999; Schachtman & Liu 1999; Demidchik &

Tester 2002). In an earlier study, we found that NSCCs are the major pathways for cytosolic Na^+ influx in Pokkali, and 1.0 mM extra cellular Ca^{2+} almost totally blocked the cytosolic Na^+ influx in this cultivar (Kader & Lindberg 2005). Pretreatment of protoplasts of Pokkali with 1.0 mM Zn^{2+} also showed an almost total blockage of Na^+ influx (Kader & Lindberg 2005). In the present study pretreatment of Pokkali protoplasts either with 1.0 mM extra cellular Ca^{2+} or 1.0 mM extra cellular Zn^{2+} did not result in any increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 3d, 5b). Therefore, it is likely, that addition of 100 mM NaCl to Pokkali protoplasts, in the presence of 1.0 mM Ca^{2+} , or 1.0 mM Zn^{2+} , is not sensed by the cells as a Na^+ -stress, since Na^+ is not entering the cytosol. This result supports the idea that Na^+ stress is sensed into the cytosol internally. This is consistent with the current idea about Arabidopsis SOS1 protein, which might sense Na^+ by its long C-terminal tail predicted to reside in the cytoplasm (Zhu 2003; Zhang et al. 2004; Shabala et al. 2005).

Verapamil and nifedepine are thought to block plasma membrane Ca^{2+} -channels (Babourina et al. 2000; White et al. 2002), although at concentration higher than 1 μM , these inhibitors could also block potential-dependant Ca^{2+} -channels in the tonoplast (Gelli & Blumwald 1993). It seems that Ca^{2+} enters into the cytosol both from apoplast and internal stores in the rice cultivar Pokkali, because the inhibitors for plasma membrane Ca^{2+} -permeable channels, as well as inhibitors for Ca^{2+} -permeable channels in the tonoplast, significantly blocked influx of Ca^{2+} into the cytosol. The contribution from the internal stores seems less important in the salt-sensitive cultivar BRRI Dhan29. The latter cultivar produced one-phase increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, whereas the salt-tolerant Pokkali showed a two-phase increase. The two-phase increase was supported by the fact that LiCl substantially blocked the Ca^{2+} influx into the cytosol (Fig. 3c, 5b). In the sensitive cultivar BRRI Dhan29, a further increase in cytosolic Ca^{2+} , in the presence of 1.0 mM extra cellular Ca^{2+} , means that the influx of more Ca^{2+} occurs through the plasma membrane (Fig. 3a, b). This was further evidenced by the blockage of influx of the extra cellular Ca^{2+} by use of the plasma membrane Ca^{2+} -channel blockers (Fig. 5a).

Upon salt addition, the cytosolic pH *increases* in the salt-tolerant cultivar Pokkali, whereas it *decreases* in the salt-sensitive cultivar BRRI Dhan29 (Fig. 6). On the other hand, salt addition significantly *decreases* the vacuolar pH in Pokkali and a little increases (not significant) in BRRI Dhan29, within 5 to 10 min after addition (Fig. 7). Therefore, it is likely that salt addition to cv. Pokkali causes proton transport from the cytosol into the vacuole, most likely by the activation of the induced vacuolar H^+ -ATPase (Fig. 8). When the protoplasts of Pokkali were pretreated with NH_4NO_3 , an inhibitor of vacuolar H^+ -ATPase, a significantly lower increase in cytosolic pH was obtained, probably as a result of blockage of the vacuolar H^+ -ATPase (Fig. 9). On the other hand, in BRRI Dhan29 the vacuolar pH little increased (Fig. 7). It is likely that proton movement occurs mainly within the apoplast and cytosol in this cultivar, since NH_4VO_3 , but not NH_4NO_3 , caused an inhibition of shifting cytosolic pH (Fig. 9). However, 24 h after the stress the significant decrease in the vacuolar pH is consistent with the induction of the vacuolar H^+ -ATPase in this cultivar. Therefore, 24 h after the stress proton movement occurs from the cytosol into the vacuole as well. Upon salt stress

Halperin et al. (2003) did not find any change in cytosolic pH in *Arabidopsis* roots, whereas Gao et al. (2004) found a decrease in cytosolic pH. The latter result is consistent with our result with the salt-sensitive cultivar. Therefore, a fast increase in the cytosolic pH under salt stress could be the trait for salt-tolerant plants.

From this study we propose the model (Fig 10) concluding that Na^+ is sensed inside the cytosol, and subsequently induces the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase. This increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ upon NaCl addition is supposed to be induced by ionic stress (Na^+ stress in particular), since either blockage of Na^+ into the cytosol or addition of sorbitol does not increase the cytosolic free Ca^{2+} . Both the apoplast and intracellular parts of the cell contribute towards the shift in cytosolic- Ca^{2+} concentration in cv. Pokkali, although the apoplast part seems to be the major source for $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics in BRRI Dhan29. It is likely, that the cytosolic H^+ -dynamics are connected with $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in the stress signaling. In Pokkali, mainly the vacuolar H^+ -ATPase early contributes towards the $[\text{H}^+]_{\text{cyt}}$ dynamics, whereas its activity is induced much later in cv. BRRI Dhan29.

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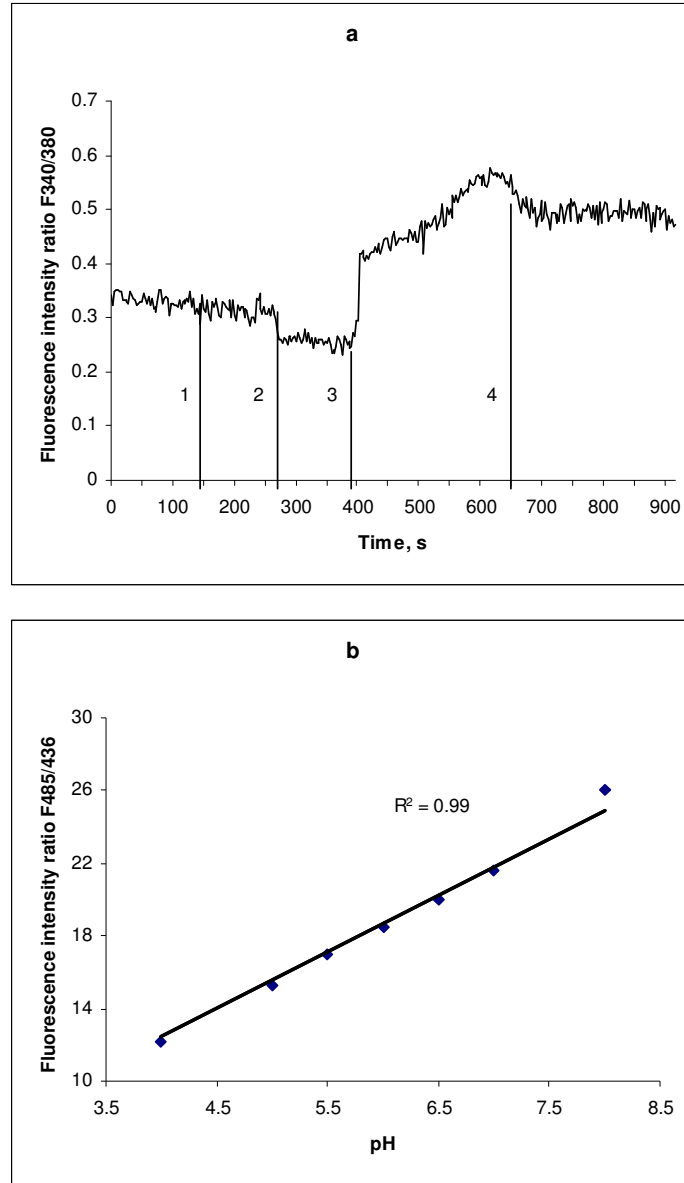


Fig. 1 In situ calibration of the cytosolic fluorescence ratio 340/380 nm in protoplasts loaded with Fura 2-AM (a) and of the cytosolic fluorescence ratio 485/436 nm in protoplasts loaded with BCECF-AM (b) and suspended in buffers with different pH. In (a) the following solutions were added: (1) 1 μ l of 400 mM EGTA, 3 M Tris-HCl, pH 8.7; (2) 2 μ l of 160 μ M 4-bromo calcium ionophore Br-A23187; (3) 2 μ l of 500 mM CaCl_2 ; and (4) 4 μ l of 125 mM MnCl_2 , to 50 μ l of protoplast suspension. In (b), nigericin was used at a final concentration of 5 μ M to equilibrate intracellular and extra cellular pH. Measurements were taken 5-10 min after the addition of nigericin. Each mean represents measurements from 10 protoplasts.

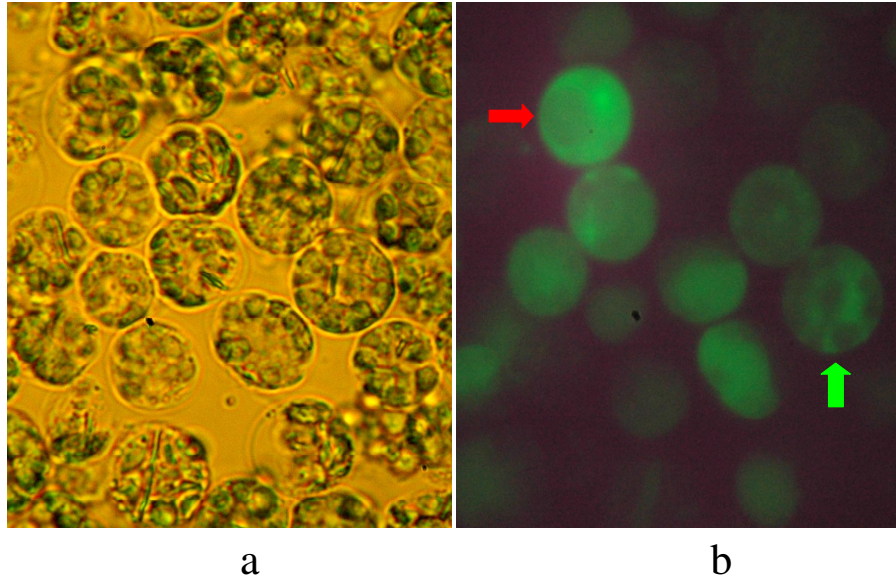


Fig. 2 Micrographs of rice mesophyll protoplasts loaded with Fura 2-AM. The micrographs were taken using a Planneofluar 100 x 1.30 oil objective (Zeiss) for phase contrast. Protoplasts in transmitted light (a) and in fluorescent light (b) showing both correctly loaded into the cytosol (green arrow) and protoplasts showing dye loading also into vacuole and chloroplasts (red arrow).

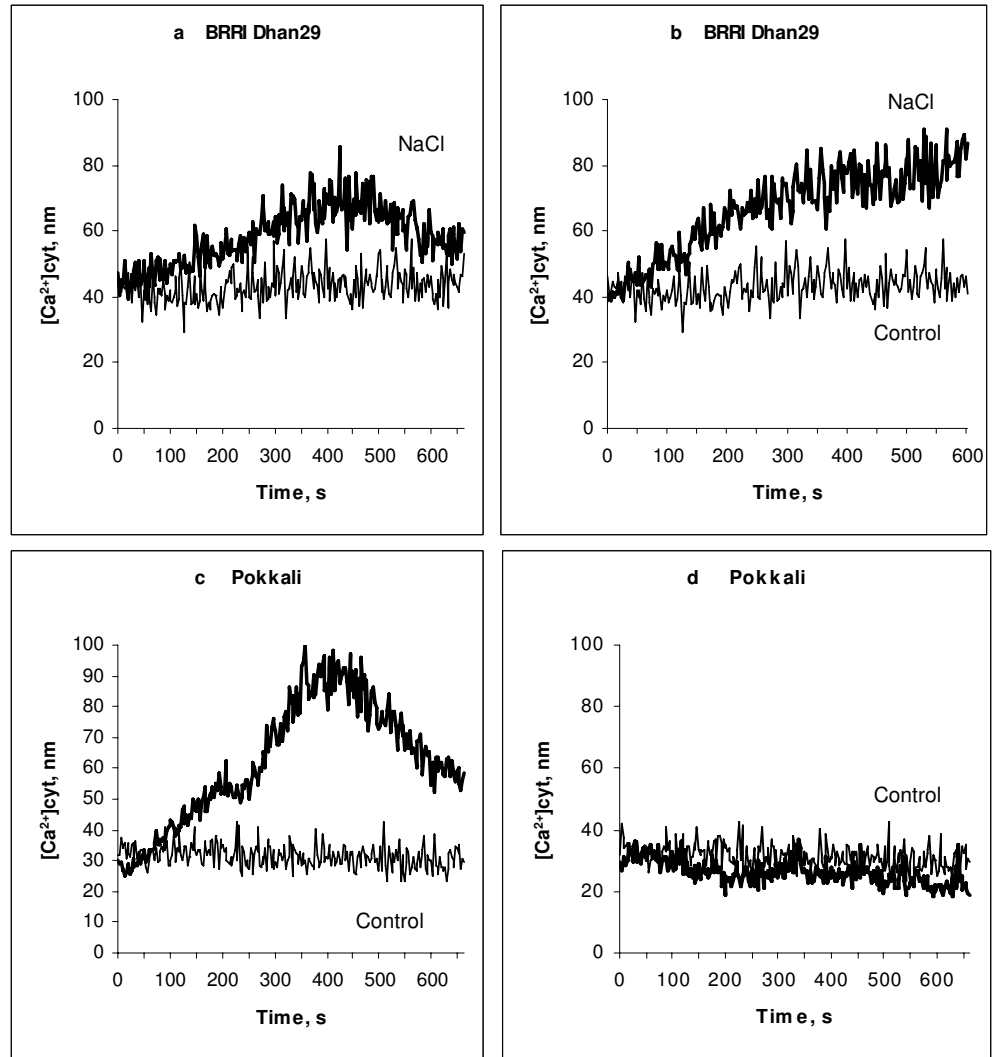


Fig. 3 Changes in free cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, upon addition of 100 mM NaCl to protoplast of BRRI Dhan29 (a, b) and Pokkali (c, d) in the presence of 0.1 mM external Ca^{2+} (a, c) and 1.0 mM external Ca^{2+} (b, d). Arrows indicate the addition of 100 mM NaCl. In case of control, measurements were undertaken without addition of NaCl. Measurements of changes in the Fura 2-AM fluorescence intensity ratio 340/380 nm were recorded and converted to free cytosolic Ca^{2+} concentration using the *in situ* calibration curve.

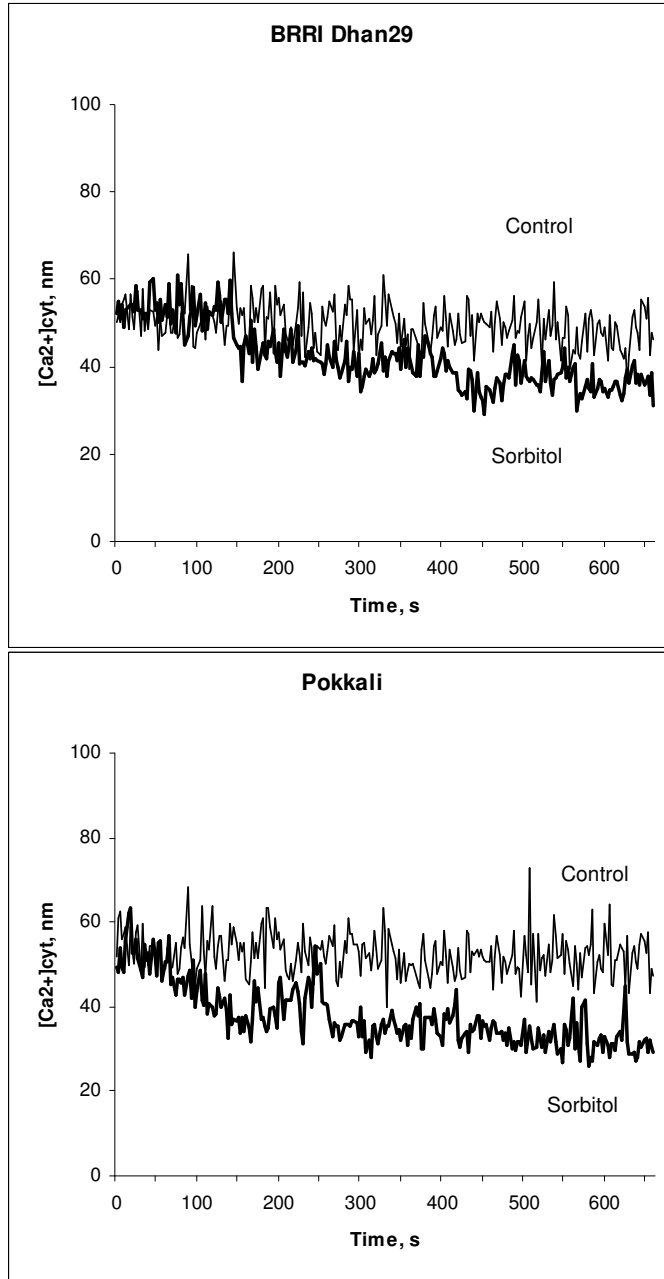


Fig. 4 Changes in free cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, in response to osmotic stress (addition of 200 mM sorbitol) to protoplast of BRRi Dhan29 (a) and Pokkali (b) in the presence of 0.1 mM external Ca^{2+} . Arrows indicate the addition of 200 mM sorbitol. Measurements of changes in the Fura 2-AM fluorescence intensity ratio 340/380 nm were recorded and converted to free cytosolic Ca^{2+} concentration using the *in situ* calibration curve.

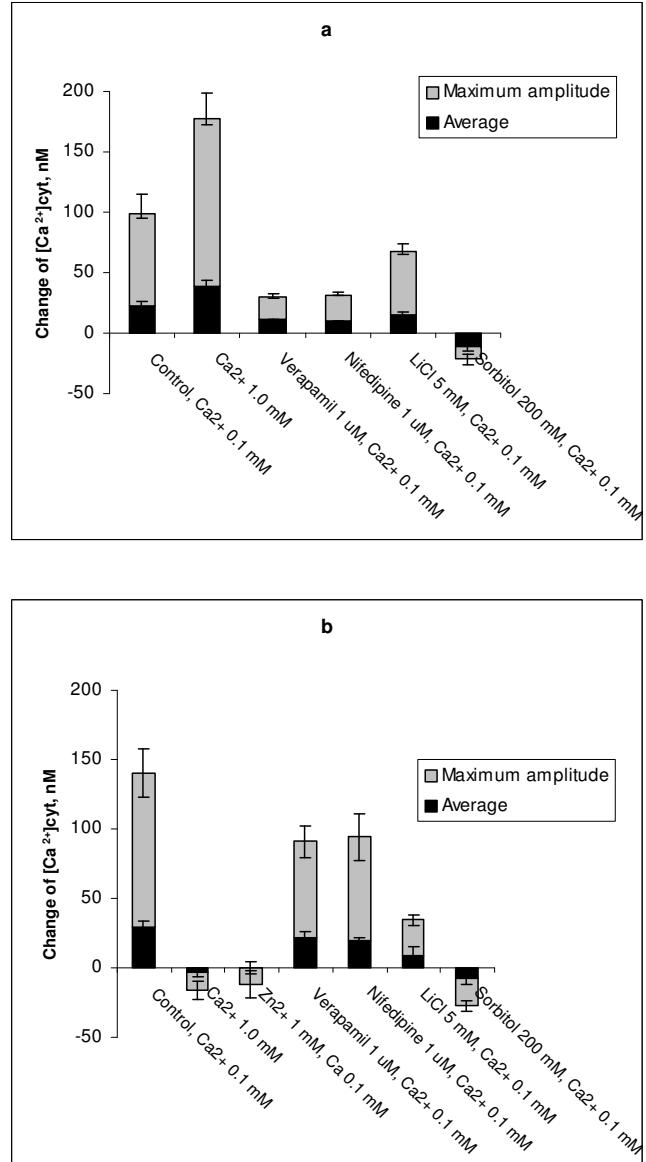


Fig. 5 Effects of inhibitors of ion channels and sorbitol on changes in the free cytosolic Ca^{2+} concentration in protoplasts of BRRI Dhan29 (a) and Pokkali (b) in response to salt stress. Control, extra cellular Ca^{2+} 0.1 mM; extra cellular Ca^{2+} 1.0 mM; extra cellular Zn^{2+} 1.0 mM (only in case of Pokkali); verapamil 1 μM ; nifedipine 1 μM and LiCl 5 mM (pre-incubation 1h, 0.1 mM external Ca^{2+}). The columns indicate average changes of $[\text{Ca}^{2+}]_{\text{cyt}}$ and also the maximal amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes for 10 min after the addition of 100 mM NaCl. Measurements of the Fura 2-AM fluorescence intensity ratio 340/380 nm were recorded every 2.5 s for 1 min before, and for 10 min after, NaCl addition. The fluorescence intensity ratio were then converted to $[\text{Ca}^{2+}]_{\text{cyt}}$ by using the *in situ* calibration curve. Mean values \pm SE.

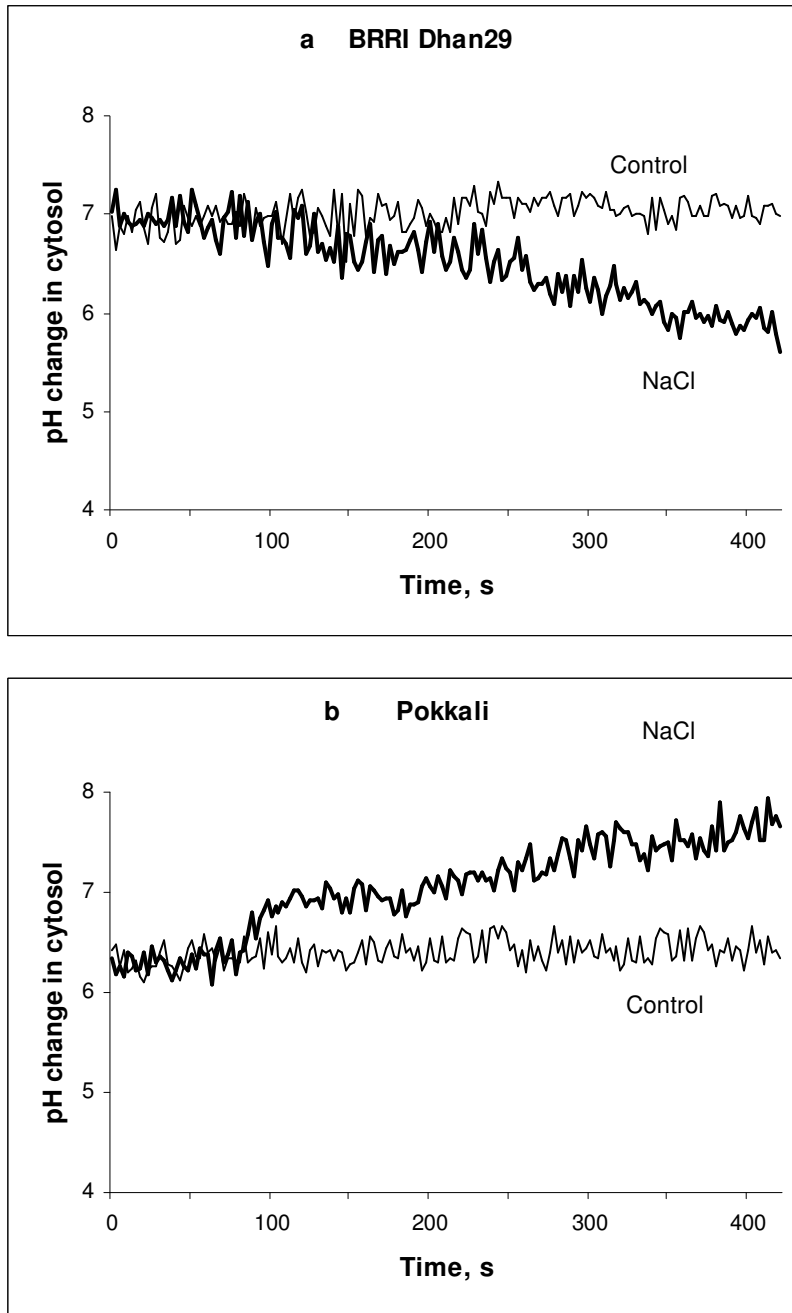


Fig. 6 Changes in cytosolic pH upon addition of 100 mM NaCl to protoplast of BRRI Dhan29 (a) and Pokkali (b) in the presence of 0.1 mM external Ca^{2+} . Arrows indicate the addition of 100 mM NaCl. In case of control, measurements were undertaken without addition of NaCl. Measurements of changes in the BCECF-AM fluorescence intensity ratio 486/435 nm were recorded and converted to cytosolic pH using the *in situ* calibration curve.

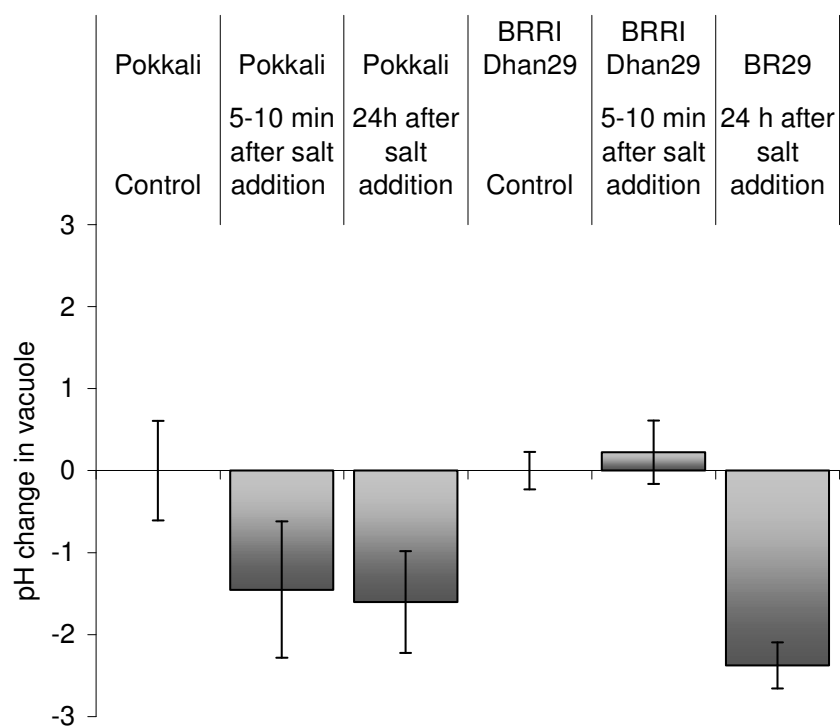


Fig. 7 Changes in the vacuolar pH upon addition of 100 mM NaCl to protoplasts of Pokkali and BRRi Dhan29 in the presence of 0.1 mM external Ca^{2+} , within 5-10 min after salt addition, and 24 h after salt addition. Protoplasts were loaded with the vacuole specific dye 6-CFDA. The dye was excited sequentially using the 458 nm and 488 nm lines of an argon-ion laser and the signal was detected in the range of 500-530 nm after passing the short pass filter RSP500 (Leica). The 6-CF fluorescence was measured in the protoplasts before, and after, salt addition and converted to vacuolar pH using the calibration curve. The columns indicate average changes of vacuolar pH measured from 40 protoplasts for each after the addition of 100 mM NaCl. Mean values \pm SE.

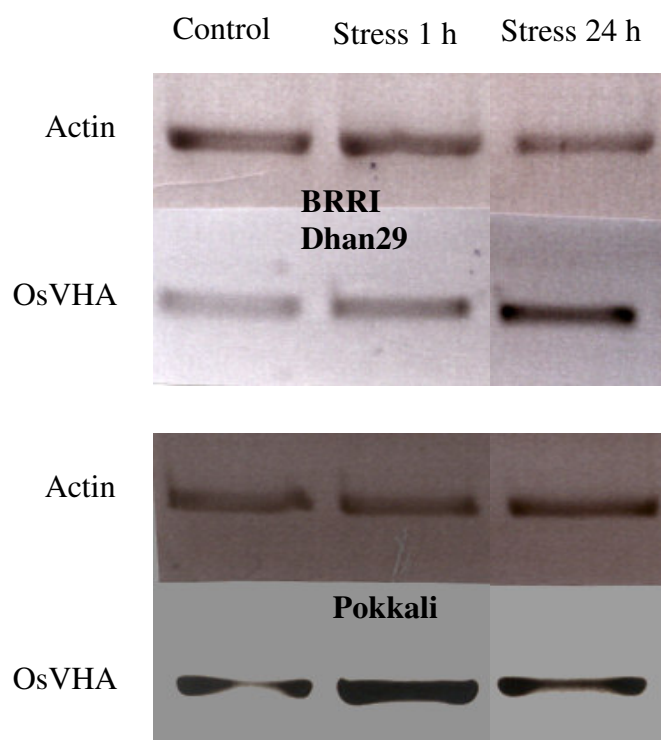


Fig. 8 Expression of rice vacuolar H^+ ATPase (OsVHA) analyzed using qRT-PCR in Pokkali and BRR1 Dhan29. The number of hours (1 and 24 h) elapsed after growing plants under salinity stress (150 mM NaCl) condition is indicated. Non-stressed control plants were grown in parallel and harvested at the same time. Three-weeks-old rice plants were used for the extraction of total RNA. Synthesis of cDNA was performed as described by Gollmack et al. (2002). The PCR products from RT-PCR amplifications were separated on 1.7% agarose gels and stained with ethidium bromide. Actin was used as a loading control.

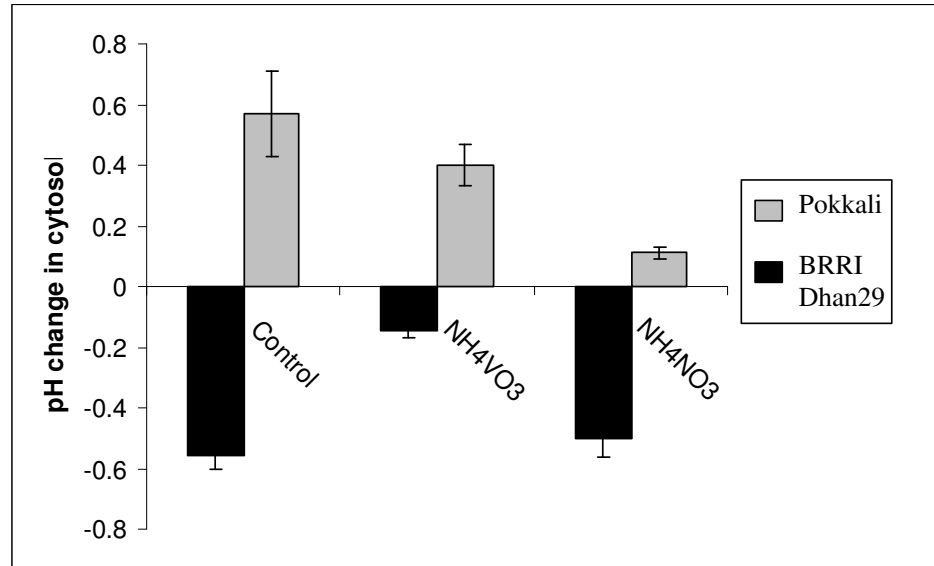


Fig. 9 Changes in cytosolic pH in response to salt stress in protoplasts of Pokkali and BRRI Dhan29 pretreated with NH_4NO_3 and NH_4VO_3 along with non-treated control protoplasts. The columns indicate average changes of $[\text{pH}]_{\text{cyt}}$ changes for 10 min after the addition of 100 mM NaCl. Measurements of the BCECF-AM fluorescence intensity ratio 486/435 nm were recorded every 2.5 s for 1 min before, and for 10 min after, NaCl addition. Measurements of changes in the BCECF-AM fluorescence intensity ratio 486/435 nm were recorded and converted to cytosolic pH using the *in situ* calibration curve. Mean values \pm SE.

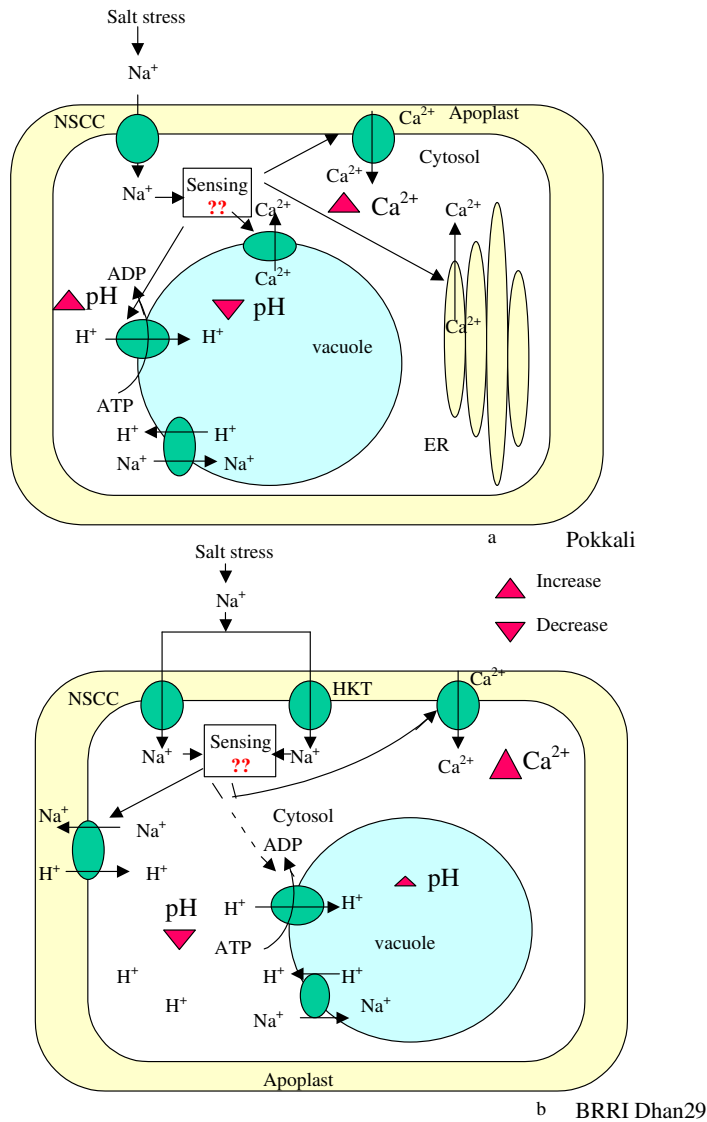


Fig. 10 A proposed model for the immediate changes in cytosolic Ca^{2+} and pH and of vacuolar pH in rice cultivars Pokkali (a) and BRR1 Dhan29 (b) upon NaCl stress. Sodium enters into the cytosol mainly through non-selective cation channels (NSCC) in Pokkali and through NSCC and K^+ -selective channels like HKT in BRR1 Dhan29 (Kader and Lindberg, 2005). Once Na^+ enters into the cytosol, it is sensed internally, and induces transient increase in cytosolic Ca^{2+} . Calcium is transported into the cytosol both from the apoplast and internal stores like vacuole and ER in Pokkali, whereas mainly from the apoplast in BRR1 Dhan29. In Pokkali, the activation of vacuolar H^+ -ATPase (VHA) causes movement of H^+ from the cytosol into the vacuole and thus, decreases vacuolar pH rather immediately with the increase in cytosolic pH. Since VHA energizes tonoplast Na^+/H^+ antiporter (NHX) for compartmentalizing Na^+ into the vacuole, with the activity of this antiporter there is a movement of H^+ from vacuole to cytosol later on as well. On the other hand, in BRR1 Dhan29 Na^+ is transported out by the Na^+/H^+ antiporter at the plasma membrane making an acidification of cytosolic pH and no change in vacuolar pH. Later on, however, after 24 h the VHA is activated (as indicated by dotted line) as shown in Fig. 8 and starts acidification of the vacuole.



IV



Expressions of OsHKT1, OsHKT2 and OsVHA are differentially regulated under NaCl stress in salt-sensitive and salt-tolerant rice (*Oryza sativa* L.) cultivars

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Abstract

Under NaCl-dominated salt stress the key step for plant survival is to maintain a low cytosolic Na^+ or Na^+/K^+ ratio. The transporter genes OsHKT1, OsHKT2 and OsVHA might play important roles in keeping the cytosolic Na^+ homeostasis in rice (*Oryza sativa* L. cvs. Pokkali and BRRI Dhan29). Upon NaCl stress the transcript of OsHKT1 significantly down-regulated in salt-tolerant cv. Pokkali. On the other hand, NaCl stress induced the expression of OsHKT1 in salt-sensitive cv. BRRI Dhan29. The expression of OsHKT1 was detected in the epidermis, exodermis and vascular tissue of the root, and mostly in mesophyll cells in leaves. A down-regulation in cv. Pokkali upon NaCl stress occurred in the same cells as mentioned above. The expressions of OsHKT2 and OsVHA were induced by NaCl stress both in cv. Pokkali and cv. BRRI Dhan29. But in cv. Pokkali, the transcript levels of OsHKT2 and OsVHA were induced immediately after NaCl stress both in roots and shoots. Compared with cv. Pokkali, the induction of OsHKT2 in cv. BRRI Dhan29 was quite low and that of OsVHA was also low and delayed. The induction of OsHKT2 was mostly pertained in the phloem, the transition from phloem to mesophyll cells, and mesophyll cells in leaves and in epidermis, exodermis and vascular cylinder in the roots. The induction of OsVHA was found in the same cells as OsHKT2. The vacuolar area in cv. Pokkali neither changed at short term (5-10 min) nor at long-term (24 h) salt stress, but significantly increased 24 h after the stress in cv. BRRI Dhan29. Expressional constructs of VHA-c and VHA-a with YFP and CFP were introduced into isolated protoplasts of cv. Pokkali and cv. BRRI Dhan29. The FRET efficiency between VHA-c and VHA-a upon salt stress decreased a little in cv. Pokkali, but increased significantly in cv. BRRI Dhan29. The results suggest that the mechanisms for cytosolic Na^+ homeostasis in the salt-tolerant rice cv. Pokkali are likely different from that in the salt-sensitive cv. BRRI Dhan29.

Key words: BRRI Dhan29; cytosolic Na^+/K^+ homeostasis; FRET efficiency; NaCl stress; Na^+ uptake; OsHKT1; OsHKT2; OsVHA; Pokkali; vacuolar compartmentalization of Na^+ ; vacuole volumes.

Introduction

Salt stress is one of the most important abiotic stresses for natural productivity and causes a significant crop loss worldwide. For plants, the sodium ion (Na^+) is harmful whereas potassium ion (K^+) is one of the essential ions. The cytosol of plant cells normally contains 100-200 mM K^+ and 1-10 mM Na^+ (Taiz & Zeiger, 2002). This Na^+/K^+ ratio is optimal for many metabolic functions in cells. Physico-chemically Na^+ and K^+ are similar cations. Therefore, under the typical NaCl dominated salt environment in nature, accumulation of high Na^+ into the cytosol, and thus, high Na^+/K^+ ratios, disrupts the enzymatic functions that are normally activated by K^+ in cells (Bhandal & Malik, 1988; Tester & Davenport, 2003; Munns, James & Läuchli, 2006). Therefore, it is very important for cells to keep a low concentration of cytosolic Na^+ or to maintain a low Na^+/K^+ ratio in the cytosol under NaCl stress (Maathuis & Amtmann, 1999).

The most important way to keep a low cytosolic Na^+ concentration is to minimize the influx of Na^+ into the cytosol. Na^+ -influx can be restricted by selective ion uptake. Non-selective cation channels (NSCCs) are proposed to be the dominant pathways for Na^+ -influx in many plant species (Roberts & Tester, 1997; Tyerman *et al.*, 1997; Buschman *et al.*, 2000; Davenport & Tester, 2000; Demidchik & Tester, 2002; Demidchik, Davenport & Tester, 2002; Kader & Lindberg, 2005). However, the molecular identity of these NSCCs is still unknown. High-affinity potassium transporters (HKTs) have also been suggested to mediate a substantial Na^+ -influx in some species including rice (Uozumi *et al.*, 2000; Horie *et al.*, 2001; Golldack *et al.*, 2002). In a recent study, we showed that K^+ -selective channels play a significant role in Na^+ uptake in a salt-sensitive rice cv. BRRI Dhan29 (Kader & Lindberg, 2005). With some exceptions plant species have multiple HKT transporters. In rice, eight functional HKT homologs (OsHKT1-4 and 6-9) have been identified (Garcia-deblás *et al.*, 2003). All of these functional genes encode proteins with distinct transport activities, which might be expressed in various tissues and/or organs. OsHKT1 has been suggested as a Na^+ transporter (Horie *et al.*, 2001; Mäser *et al.*, 2002; Garcia-deblás *et al.*, 2003) and OsHKT2 as a Na^+/K^+ co-transporter (Horie *et al.*, 2001; Mäser *et al.*, 2002). OsHKT2 is believed to be the sole HKT gene in rice involved in K^+ transport (http://www.ausbiotech.org/pdf/2006_Honours_booklet.pdf#search=Identifying%20expression%20patterns%20of%20the%20HKT; 30-June-2006). Very recently, OsHKT8 was shown to be a Na^+ transporter, and contributes to the increased ability of salt tolerance by maintaining K^+ homeostasis in the shoot under salt stress (Rus, Bressan & Hasegawa, 2005; Ren *et al.*, 2005). This transporter is thought to be analogous to the function of AtHKT1 gene in *Arabidopsis*, which is a Na^+ -transporter, and interestingly, plays a very important role in controlling the cytosolic Na^+ detoxification (Berthomieu *et al.*, 2003; Rus *et al.*, 2004; Sunarpi *et al.*, 2005). Therefore, it is likely that the HKT gene family has an important role in Na^+/K^+ homeostasis in rice, even though some of the members are evidently Na^+ -transporters.

Another way, which the plant has to deal with high cytosolic Na^+ , is to transport it out of the cytosol, either into the vacuole or into the apoplast. Apoplastic sequestration of Na^+ , however, does not play a role in salt tolerance in rice, since most Na^+ in the rice shoot comes through apoplastic streaming (Yeo *et al.*, 1999). Conversely, vacuolar compartmentalization is an efficient strategy for plant cells to cope with salt stress (Fukuda *et al.*, 1998, 2004; Apse *et al.*, 1999; Blumwald, 2000; Chauhan *et al.*, 2000; Hamada *et al.*, 2001; Tester & Davenport, 2003). When compartmentalized into the vacuole, Na^+ is no more toxic for cells (Flowers & Läuchli, 1983; Subbarao *et al.*, 2003) and also an advantage for growth and osmotic adjustment (Zhu, 2003; Rodriguez-Navarro & Rubio, 2006), particularly since the vacuole may occupy more than 90% of the volume of mature cells. In a recent study, we also showed that vacuolar compartmentalization is evident under salt stress in the salt-tolerant rice cv. Pokkali, whereas apoplastic sequestration of cytosolic Na^+ is dominant in the salt-sensitive cv. BRRI Dhan29 (Kader & Lindberg, 2005). The candidate protein for compartmentalization of Na^+ into the vacuole is the tonoplast Na^+/H^+ -antiporter, which is energized by the vacuolar H^+ -ATPase (VATPase or VHA) to do so. VHA as well as pyrophosphatase is in general required for the maintenance of intracellular pH and ion homeostasis (Padmanaban *et al.*, 2004). Through the hydrolysis of ATP, VHA generates a proton motive force that energizes secondary transports across the vacuolar membrane, like that of Na^+ , under high salt. VHA was shown to be important for salt tolerance in *Saccharomyces cerevisiae* (Hamilton, Taylor & Good, 2002) and in many plant species (Golldack & Dietz 2001; Kluge *et al.*, 2003a; Senthilkumar *et al.*, 2005; Vera-Estrella *et al.*, 2005).

Like other salt-tolerant species, salt-tolerant rice cv. Pokkali contains less Na^+ both in root and shoot under salt stress compared to salt-sensitive rice cultivars (Golldack *et al.*, 2003; Kader & Lindberg, 2005). Several Na^+/K^+ -transporters could be involved in conferring the ability to maintain a low cytosolic Na^+ in Pokkali. With the view to clarify the regulatory mechanisms for maintaining cytosolic Na^+ homeostasis in rice, we compared the expressions of OsHKT1, OsHKT2 and OsVHA in a salt-sensitive cv. BRRI Dhan29 and a salt-tolerant cv. Pokkali at different time points under NaCl stress. The transcripts of these transporter genes were quantified by real-time RT-PCR. The cell-specific expressions of the transporter genes were performed by *in situ* PCR to investigate their specific role in cytosolic Na^+ accumulation/detoxification.

Materials and Methods

Plant Materials

Seeds of rice (*Oryza sativa* L. *indica* cvs. Pokkali and BRRI Dhan29) were provided by the Bangladesh Rice Research Institute (BRRI, Gazipur, Bangladesh). The seeds were soaked with water for 48 h in the darkness. Afterwards, they were germinated on vermiculite soaked with a half-strength Hoagland's solution. After a week the seedlings were transferred to hydroponic tanks with half-strength Hoag-

land's solution. Seedlings were grown for another 2 weeks in a controlled environment chamber with the day/night temperature 25⁰/21⁰C under a 14-h light with 300 $\mu\text{E m}^{-2}\text{s}^{-1}$. Humidity was about 50%. Afterwards, the plants were stressed by NaCl at the final concentration of 150 mM added to the nutrient solution for 1 to 72 h. Non-stressed control plants were grown in parallel and harvested at the same time.

RNA extraction and cDNA synthesis for qRT-PCR

RNA was isolated from shoots and roots of rice cvs. Pokkali and BRRI Dhan29. For qRT-PCR the total RNA was isolated by the guanidinium thioisocyanate method. Synthesis of cDNA was performed as described by Golldack, Popova & Dietz (2002). For PCR amplification the following sequence-specific forward and reverse oligonucleotide primers were used: 5'-GAGTCGTCTCAGAAATGA-3' and 5'-TGAACCTTTCAGGCAGAAC-3' (OsHKT1), 5'-GAGTCGTCTCAGAAATGA-3' and 5'-TTCTACGATTCAAAAGGC-3' (OsHKT2) and 5'-CTTCTGGCAATCTTGGAG-3' and 5'-CAGTGTAGACGAAGTGCA-3' (OsVHA). The following conditions in PCR reactions were used: 1 cycle 94 °C for 1 min 30 s, 1 min 94 °C, 1 min 55 °C, 2 min 72 °C, and a final extension at 72 °C for 10 min. OsHKT1 was amplified with 30 cycles, OsHKT2 with 45 cycles and OsVHA with 28 cycles both from the root and shoot of rice. The PCR products from RT-PCR amplifications were separated on 1.7% (w/v) agarose gels and stained with ethidium bromide. Photographic documentation was performed with a gel documentation system (INTAS, Göttingen).

RNA extraction and cDNA synthesis for real-time RT-PCR

For real-time RT-PCR the total RNA was extracted using the RNeasy plant mini kit (QIAGEN, Valencia, CA) according to manufacturers instructions. Total RNA of 5 μg was reverse transcribed to cDNA with oligo (dT) 20 mer with a T7 promoter in 5' position and two randomized nucleotides in 3' position, using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was diluted to 2 ng/ μl and 5 μl of the diluted cDNA (10 ng cDNA) was used as a template in each well for quantitative real-time PCR analysis. The cDNA was amplified using the SYBR Green PCR Master Mix (FINNZYMES, Finland) on the ABI 7000 thermocycler (Applied Biosystems, Foster City, CA). Primers for the actin gene were used as an internal control to normalize the expression data for each gene. Following primer sequences were used: 5'-ACACCCAATATTATTCCTCTTAA-3' and 5'-CGGGAATACGCTAAAGG-3' (OsHKT1), 5'-CAAAGGCAGGTGAATCAAG-3' and 5'-CGATTCAAAAGGCCCTAA-3' (OsHKT2), 5'-TTTCTTTTGCTACTGCCTTTATATTGC-3' and 5'-AGTGTAGACGAAGTGCATGAAGGA-3' (OsVHA).

The PCR conditions were as follows: 50⁰C for 2 min and 95⁰C for 10 min, followed by 40 cycles of 95⁰C for 15 s and 60⁰C for 1 min. A dissociation kinetic analysis was performed at the end of the experiment to check the specificity of

annealing. Three replications were performed for each sample in one experiment. The experiments independently were repeated twice. Standard determination curves were generated using serial dilutions of 50, 10, 2 and 0.4 ng cDNA in each well for every experiment. Results were analyzed according to Muller *et al.* (2002).

***In situ* PCR**

Sections from leaves and roots were fixed with FAA, dehydrated and embedded with Paraplast Plus (Fisher Scientific) as described by Golldack, Popova & Dietz (2002). Microtome sections of 12 μm were mounted on microscopic slides coated with aminoalkylsilane (silane-prep slides, Sigma, Germany). The tissue sections were de-parafinized and re-hydrated, treated with proteinase K and RNase-free DNase I. Synthesis of cDNA was performed with oligo-dT primers and Superscript Reverse Transcriptase II (Invitrogen, the Netherlands). PCR reactions were performed with the same gene-specific oligonucleotide primers used for qRT-PCR reactions stated earlier. The PCR conditions were as follows: 94°C for 1 min 30 s in the first cycle followed by 1 min 94°C, 1 min 55°C, 2 min 72°C with the same number of cycles for each gene as qRT-PCR, and a final extension at 72°C for 10 min. For signal detection alexa fluor 488-5-dUTP (Molecular Probes, Netherlands) was used as a label. Negative control reactions were performed without adding the gene-specific oligonucleotide primers to the PCR reactions. Subsequently to PCR amplifications, the tissue sections were stained with 10 μM propidium iodide for 10 min. Microscopic images were taken with a cooled CCD-Camera coupled to an Axioskop fluorescence microscope (Zeiss, Germany). In the microscopic images the specific signals were shown by green to yellow fluorescence and the negative background signals by red.

FRET analysis

Protoplast isolation

The protoplasts from leaves of both Pokkali and BRRI Dhan29 were prepared as described by Shishova & Lindberg (1999) with some modifications. Leaves from about 10-day-old seedlings were sliced in 0.5 mm pieces and treated with 1% (w/v) cellulase (lyophilized powder; 10.0 units mg^{-1} solid) from *Trichoderma reesei* (Sigma, EC 3.2.1.4) and 0.6% (w/v) macerascase (lyophilized powder; 0.6 units mg^{-1} solid), Maceroenzyme R-10 (Serva, EC 3.2.1.4) for 2 h. The protoplasts were washed twice in the loading medium containing 0.5 M sorbitol (Sigma, St. Louis, MO, USA), 0.1 mM CaCl_2 , 0.2% (w/v) polyvinylpyrrolidone (PVP, Sigma St. Louis, MO, USA) and a buffer (pH 5.5) containing 5 mM Tris (Labassco, Germany)] and 5 mM MES (Sigma, St. Louis, MO, USA).

Protoplast transfection

Constructs of the V-ATPase subunits VHA-a, VHA-c, VHA-E and VHA-B of *Mesembryanthemum crystallinum* fused to fluorescent proteins (Seidel *et al.*, 2004, Seidel, Golldack & Deitz, 2005) were used for the transient transfection of rice

protoplasts. The transfection was performed as described before for *Arabidopsis* protoplasts (Seidel *et al.*, 2004).

FRET measurement

Fluorescence resonance energy transfer was measured by confocal microscopy (Leica SP2, Heidelberg). Cyan fluorescence protein (CFP) was excited by the 458 nm line of an argon-ion-laser and detected in the range of 470-510 nm as a control for the CFP-crosstalk into the FRET channel (530-600 nm detection bandwidth; 458 nm excitation). Yellow fluorescence protein (YFP) was detected in the range of 530-600 nm using the 514 nm line of the argon-ion-laser for excitation. The YFP-signal was required for the estimation of YFP direct excitation by the 458 nm laser line (Seidel, Gollack & Dietz, 2005).

The FRET-efficiency was calculated by relating the corrected emission intensity in the FRET channel to the sum of the CFP emission and the FRET emission (Seidel, Gollack & Dietz, 2005):

$$E = (Em_{FRET} - (0.61 \times Em_{CFP})) / (Em_{FRET} + Em_{CFP})$$

For each FRET-pair the FRET-effect of 40 protoplasts was measured in two independent experiments.

Measurement of vacuolar area

Protoplasts from shoots of cvs. Pokkali and BRRI Dhan29 were isolated in the same way as mentioned above. Protoplasts were stained with 3 μ L of 5 mM 6-carboxyfluorescein diacetate (6-CFDA, Sigma-Aldrich) in DMSO through PEG-mediated osmotic shock. Non-specific esterase-activity in the vacuolar lumen cleaves the non-fluorescent 6-CFDA to the fluorescent, pH dependant dye 6-carboxyfluorescein (CF; Preston, Murphy & Jones, 1989). The 6-CF fluorescence in the vacuoles was imaged by a laser scanning confocal microscopy (Leica SP2, Heidelberg). The dye was excited sequentially using the 458 nm and 488 nm lines of an argon-ion laser. The images of the vacuoles were taken showing the signal in the range of 500 to 530 nm after passing the short pass filter RSP500 (Leica). The areas of the vacuolar images were measured using the Leica Confocal Software LCS Lite (<ftp://ftp.llt.de/pub/softlib/LCSLite/>).

Results

The transcripts levels of OsHKT1 (a Na^+ -transporter), OsHKT2 (a K^+ - Na^+ co-transporter) and OsVHA (an energizer for tonoplast Na^+/H^+ antiporter under salt stress) were quantified by real-time RT-PCR in the salt-sensitive rice cv. BRRI Dhan29 and salt-tolerant rice cv. Pokkali at the time points of 0, 1, 6, 24, 48 and 72 h after salt stress of 150 mM NaCl. Cell-specificity of these transcripts was also

studied by *in situ* PCR. The conformational change and energy transfer efficiency of OsVHA upon NaCl stress was studied by measurement of FRET efficiencies between the peripheral stalk subunit VHA-E and the catalytic head subunit VHA-B and between the proteolipid VHA-c and the C-terminal transmembrane domain of VHA-a, respectively. The changes in vacuolar area in leaf cells (protoplasts) upon salt stress were also investigated.

Expression of OsHKT1

The transcripts of OsHKT1 showed a variable expression with time up to 72 h after 150 mM NaCl stress both in root and shoot tissues of rice cvs. Pokkali and BRRI Dhan29 (Fig. 1). There was a clear upregulation of the transcript in BRRI Dhan29 in the root, 1 h after the stress, and in the shoot 6 h after the stress. In the root the upregulation further increased substantially 6 h after the stress. However, 6 h after the stress, when the transcript showed the highest induction both in root and shoot, the induction in root was higher (about 4-times higher) than that in the shoot (about 2-times higher) compared to their respective control levels. Both in the root and shoot the upregulation of the transcript compared to the control expression continued up to 72 h after the stress, but with some variation in terms of degree of upregulation. On the other hand, there was a significant upregulation of the transcript in cv. Pokkali only 24 h after stress both in shoots and roots and it was less than 1-fold higher expression in both the cases compared to the respective control levels. Though the transcript showed an upregulation compared to the control expression until 72 h after the stress in the salt-sensitive cv. BRRI Dhan29, there was a significant down-regulation of this transcript in the salt-tolerant cv. Pokkali from 48 h after the stress both in shoot and root tissues. The down-regulation of this transcript in Pokkali shoot, at 72 h after the stress, was about 62% and that in Pokkali root was about 72% compared to the control expression. *In situ* expression of OsHKT1 transcript confirmed its expression both in root and shoot of BRRI Dhan29 and Pokkali (Fig. 4 & 5). Under NaCl stress condition, in root of BRRI Dhan29 (Fig. 4), a strong signal was detected from the epidermis and vascular cylinder, but only a weak signal from the cortex. In the root of Pokkali, the signal was weaker upon stress compared to the control signal. Under control condition the strong signal in BRRI Dhan29 leaves came from the mesophyll cells and the phloem and a weaker signal from the epidermal cells and no signal from the xylem. In Pokkali leaves, a strong signal was found from the mesophyll cells, phloem and xylem. After 48 h of NaCl stress the OsHKT1 transcript in BRRI Dhan29 disappeared from epidermis and notably also from the phloem, while a somewhat higher signal came from the mesophyll cells. However, in Pokkali down-regulation of the transcript was noticed from all types of cells, but most notably from phloem, xylem and mesophyll cells.

Expression of OsHKT2

The real-time analysis showed a substantial increase of OsHKT2 transcript immediately after 150 mM NaCl stress in Pokkali, but not in BRRI Dhan29 (Fig. 2). There was about 15-fold higher expression of OsHKT2 in Pokkali shoot 1 h

after the stress compared to control expression. Although the upregulation of OsHKT2 in Pokkali leaf gradually decreased in the following time points, it was still a 158% increased expression at 72 h after stress over control. In the shoot of BRRI Dhan29, we found a significant upregulation of the transcript at 6 h after stress, which was nearly a 2-fold higher expression compared to the control. The upregulation decreased slightly afterwards up to 72 h after the stress. There was also an upregulation of OsHKT2 in root tissue of Pokkali, and the upregulation continued up to 72 h after the stress. However, the upregulation in Pokkali root was not as high as in the shoot. It was less than 50% higher compared with control until 48 h after the stress, and then about 177% higher 72 h after the stress than the control. On the other hand, OsHKT2 was down-regulated in the root of BRRI Dhan29 until 24 h after the stress (nearly 50% of control expression) and then a slight upregulation occurred 48 and 72 h after the stress.

As shown by *in situ* analysis (Fig. 4) OsHKT2 under control condition was mainly expressed in epidermis and vascular cylinder in the root with some low expression in the cortex. After 150 mM NaCl stress, a weaker signal was detected from all of these root cell types in BRRI Dhan29, whereas a stronger signal was obtained in Pokkali, in correspondence with the real-time quantification of this transcript. Under control condition the expression of OsHKT2 in leaves was confined mainly in the mesophyll cells (Fig. 5). But after the stress (24 h), a very strong signal appeared from phloem and the connecting area between phloem and mesophyll cells. The signal in mesophyll cells was also somewhat stronger in stressed cells than in control cells.

Expression of OsVHA

As shown in Figure 3, there was an induction of OsVHA in both roots and shoots of Pokkali 1 h after the stress, which further increased 6 h after the stress. The induction was also noticed both in the root and shoot tissues of BRRI Dhan29, but not until 6 h after the stress, and also to a lesser amount compared to cv. Pokkali. The induction of the transcript 6 h after the stress was around 4-times higher in Pokkali shoot and nearly 2-folds higher expression in BRRI Dhan29 shoot, compared to their respective control expressions. Afterwards, the induction in both the cultivars started to decrease with time. In shoot of both the cultivars, the lowest induction occurred 48 h after the stress, which then gave some induction again at 72 h after the stress. On the other hand, in comparison with the shoots, the initial induction of OsVHA in roots was lower both in Pokkali and BRRI Dhan29, though its induction later (72 h after the stress) was close to the shoot.

Under control condition, a strong signal from OsVHA in roots was found from epidermis and vascular cylinder, but a very weak signal from the cortex region in both the cultivars (Fig. 4). Moreover, after 24 h stress of 150 mM NaCl the signal from the vascular cylinder in BRRI Dhan29 became weaker, whereas the signal from epidermis in Pokkali was stronger. In leaves of BRRI Dhan29 there was an OsVHA expression under control condition in phloem, and at the transition from phloem to mesophyll cells, as well as an expression in mesophyll cells (Fig. 5). On

the other hand, in leaves of Pokkali no signal was seen from phloem and the transition from phloem to mesophyll cells. The expression was rather just confined within the mesophyll cells. After 1 h stress with 150 mM NaCl, a very strong signal was detected in the Pokkali leaves from phloem and the transition from phloem to mesophyll cells, along with the comparatively stronger expression in the mesophyll cells. A stronger signal in the aforesaid cells was also found in BRRI Dhan29 leaf, but not to the same extent as in Pokkali.

Effect of salt stress on vacuolar area

Vacuoles, comprising up to 95% of cell volume in mature cells (Epimashko *et al.* 2004), are thought to be a good sink for cytosolic Na⁺ to be compartmentalized. As a result, changes in the area of vacuole could be evident under salt stress. However, we did not find any significant change in the vacuolar area in the salt-tolerant cv. Pokkali upon salt stress, both at a short-term (5-10 min) and a long-term (24 h) treatment (Fig. 6). On the other hand, compared to the control one (91.1 ± 16.06), the vacuolar area in the salt-sensitive cv. BRRI Dhan29 decreased (62.62 ± 10.5) after 5-10 min NaCl stress and then increased (115.16 ± 0.81) after 24 h stress. The vacuolar area without stress was, however, larger in Pokkali ($102.4 \pm 6.26 \mu\text{m}^2$) than that of BRRI Dhan29 ($91.1 \pm 16.06 \mu\text{m}^2$).

Effect of salt stress on the structure of the VHA

The VHA is a protein complex of 13 different subunits with a bipartite structure similar to F-ATP-synthases (Fig. 7). The membrane integral sector V₀ is composed of subunits VHA-a, c, d and e and the cytoplasmically exposed V₁ sector contains the subunits VHA-A to VHA-H. The sector V₀ catalyses the proton transport whereas V₁ is involved in ATP binding and hydrolysis. To test whether salt has an effect on the stator structure of the VHA, the FRET-efficiency between the peripheral stalk subunit E and the catalytic head subunit VHA-B was measured. No changes were identified due to NaCl stress in cv. Pokkali (48.34 ± 1.73 and 46.76 ± 1.32) as well as in cv. BRRI Dhan29 (38.57 ± 1.86 and 40.81 ± 2.26). The VHA of cv. Pokkali showed a significant (t-test; $p=0.000128$) higher FRET efficiency between VHA-E and VHA-B than BRRI Dhan29, indicating structural differences between these two enzymes. To investigate the structural flexibility of the transmembrane sector V₀ of the V-ATPase we measured the FRET efficiency between the proteolipid VHA-c and the C-terminal transmembrane domain of VHA-a. As shown in Fig. 8, under control conditions the FRET efficiency was similar in Pokkali (46.16 ± 1.84) and BRRI Dhan29 (44.6 ± 1.82), but the efficiency of energy transfer increased significantly (t-test, $p=0.00154$) due to NaCl stress in cv. BRRI Dhan29 (51.54 ± 1.45) and decreased a little in the case of cv. Pokkali (43.43 ± 1.76).

Discussion

In the present study there was an induction of OsHKT1 both in roots and shoots of BRRI Dhan29 under high NaCl, although the degree of induction compared to the control expression varied with time. On the other hand, in Pokkali the transcript of OsHKT1 remained unaffected up to 6 h after the stress both in roots and shoots and showed a down-regulation 48 and 72 h after the stress. There was, however, some induction of OsHKT1 both in roots and shoots of Pokkali 24 h after the stress. As shown before, OsHKT1 mediates Na⁺ influx, but not K⁺ influx (Horie *et al.*, 2001). It has also been shown that OsHKT1 specifically mediates Na⁺ uptake in rice roots when the plants are K⁺ deficient (Garcia-deblás *et al.*, 2003), and is induced by low-K⁺ conditions (Horie *et al.*, 2001). In the present study, the induction of OsHKT1 in root epidermis and vascular cylinder, as well as shoot mesophyll cells of salt-sensitive BRRI Dhan29, might indicate its involvement of Na⁺ uptake by the root and then circulating Na⁺ in the leaf mesophyll cells, where Na⁺ causes a damage. Since the experimental plants were grown with an optimal K⁺ concentration in the growth medium, there should not be any deficiency of K⁺ in cells under control condition. However, at high NaCl condition, Na⁺ competition at binding sites for K⁺ may result in K⁺ deficiency (Maathuis & Amtmann, 1999), and thus, might cause the induction of OsHKT1 in both the cultivars, as we observed. Another possibility could be that under high NaCl, when excess Na⁺ enters the cytosol, it increases the optimal ratio of cytosolic Na⁺/K⁺ that cells might realize as K⁺ deficiency, and thus, induces the OsHKT1 as suggested by Horie *et al.* (2001) for K⁺ deficiency. The higher uptake of Na⁺ into the cytosol of cv. BRRI Dhan29 compared to that in cv. Pokkali (Kader & Lindberg, 2005) rationally induces OsHKT1 faster in BRRI Dhan29 (1 and 6 h after the stress) than that in Pokkali (24 h after the stress).

With an increasing stress period, the salt-tolerant cv. Pokkali starts to down-regulate OsHKT1 expression both in roots and shoots as found here. A down-regulation of OsHKT1 in leaf mesophyll cells in Pokkali justifies the salt-tolerance of this cultivar, possibly by hindering Na⁺-influx into these metabolically very important cells. A down-regulation of OsHKT1 in response to salt stress was also shown by Horie *et al.* (2001) and Golldack *et al.* (2002). However, the initial induction of OsHKT1 in the salt-tolerant cv. Pokkali, whatever the reason, is against its salt-tolerance ability. One possibility is that there could be some post-transcriptional changes, or any conformational changes of the protein, that hinders Na⁺ transport into Pokkali by this Na⁺ transporter. In a recent study, we showed that the uptake of Na⁺ by K⁺-selective channels/transporters was not found in this cultivar (Kader & Lindberg, 2005).

This study also suggests the involvement of OsHKT2 in salt-stress response, especially in the salt-tolerant cv. Pokkali. There was a substantial induction (some 15-folds higher compared to control) of OsHKT2 in the shoot of Pokkali and to a lesser extent in the root of the same cultivar, but not in the salt-sensitive cv. BRRI Dhan29. Although OsHKT2 (K⁺-Na⁺ coupled transporter) does not mediate K⁺ influx from a high K⁺ solution in the absence of Na⁺, it confers tolerance to salt

stress under high Na^+ probably by increased ability of K^+ uptake, as shown in *Saccharomyces cerevisiae* (Horie *et al.*, 2001). In the present study, the induction of OsHKT2 in epidermis, exodermis and xylem tissue in roots might indicate its involvement in K^+ uptake and transport through xylem. The induction at the mesophyll cells and the transition from phloem to mesophyll cells may indicate its involvement in the recirculation of K^+ within the mesophyll cells through the phloem. In addition to the metabolites such as sugars, minerals and salts also can use the phloem pathway to be redistributed from the old source leaves towards young and expanding sink leaves (Sondergaard, Schulz & Palmgren, 2004). Thus, the induction of OsHKT2 in the salt-tolerant cv. Pokkali might confer salt tolerance by increasing its expression in leaf, through contributing towards a low cytosolic Na^+/K^+ ratio, as suggested by Horie *et al.* (2001). Recently Maathuis (2006) reported a more than 3-fold change of Na^+/K^+ symport in response to salt stress. Several other K^+ -transporter genes have also been shown to be upregulated under high NaCl, which possibly reflects the plants ability to maintain certain cytosolic K^+ levels at various capacities. Salt stress increases the transcript of K^+ -transporter genes AtKC1 (Pilot *et al.*, 2003), KMT1 and various HAK/KUP (Su *et al.*, 2001, 2002). The ability to maintain ionic homeostasis was shown to be an important salt-tolerance determinant in barley when it was compared with a moderate salt-sensitive rice cultivar IR64 (Ueda *et al.*, 2006). Our study also revealed that the regulatory mechanism for controlling K^+/Na^+ homeostasis in cells of the salt-tolerant cv. Pokkali seemed to be working by increasing the K^+ uptake (by inducing the expression of OsHKT2), and then also by reducing Na^+ influx (by decreasing the expression of OsHKT1). These mechanisms were not as efficient in BRRI Dhan29 as in Pokkali. Since K^+ , at a high concentration, also is inhibitory for enzymatic functions (Greenway & Osmond, 1972), the induction of OsHKT2 in Pokkali leaves decreased with the course of stress period.

In addition to the possible increased uptake of K^+ and decreased Na^+ -influx, Pokkali might maintain K^+/Na^+ homeostasis in the cytosol also by compartmentalizing cytosolic Na^+ into the vacuole. Upon NaCl stress in Pokkali, we found immediate (1 h after the stress) induction of OsVHA in this cultivar. Although the expression of OsVHA was induced in the salt-sensitive cv. BRRI Dhan29, the induction compared to the salt-tolerant cv. Pokkali was much delayed (until 6 h after the stress) and to a lesser extent. This result is consistent with our earlier study, where we showed a compartmentalization of Na^+ into the vacuole in few minutes of NaCl stress in Pokkali, but not in BRRI Dhan29 (Kader & Lindberg, 2005). The induced expression of OsVHA upon salt stress was found mainly in the epidermis of the roots. This is quite rational, since these cells first encounter the excess of cytosolic Na^+ , before it enters into the xylem through symplastic pathway to be transported into the shoot. The induction in phloem and the transition from phloem to mesophyll cells in leaves might also indicate the effort of these cells to keep the harmful Na^+ away from the mesophyll cells. In addition to maintaining cytosolic ionic homeostasis and pH, VHA was also shown to be important for salt tolerance in *Saccharomyces cerevisiae* (Hamilton, Taylor & Good, 2002) and in many plant species (Golldack & Dietz 2001; Kluge *et al.*, 2003a; Senthilkumar *et al.*, 2005; Vera-Estrella *et al.*, 2005). We, however, observed some variable expression of OsVHA in course of time under salt stress in

both the cultivars (Fig. 3). This increase-decrease-increase induction of OsVHA in both the cultivars might indicate certain limitations of the vacuole as a sink for cytosolic Na^+ . Immediately after the stress the salt-tolerant cv. Pokkali increased the expression of VHA and the induction continued to increase until 6 h after the stress and thus, possibly compartmentalized cytosolic Na^+ into the vacuole. Afterwards, with time a high amount of Na^+ into the vacuole may give a signal to the cell to reduce the induction of OsVHA, which decreased near to the control level at 48 h after the stress. At 72 h after the stress there is again an induction of OsVHA, which might be correlated with the increased volume of vacuole upon NaCl stress, as was found in BRRI Dhan29 after 24 h of stress.

The FRET-measurements between two different subunits of a particular protein fused with fluorophores like CFP and YFP facilitate the quantitative estimates of distance in terms of the efficiency of resonance energy transfer (Seidel, Gollack & Dietz, 2005). Thus, the changes in FRET efficiency between two different subunits of VHA upon salt stress might be a good indication of any structural change of the protein due to salt stress, if there is any. In general, salinity affects the expression of VHA-genes in plants. One important mechanism of salt-tolerance in plants seems to be the adaptation of transport capacities via changes in the amount of VHA and therefore VHA activity (Ratajczak, 2000; Wang, Luttge & Ratajczak, 2001; Kluge *et al.*, 2003b). However, so far there is no report yet showing any structural changes of VHA due to salt stress, but some data of ATP-hydrolysis and proton transport revealed changes in the coupling ratio of the plant VHA upon salinity (Ratajczak, 2000). The FRET efficiency between the proteolipid VHA-c and the C-terminal transmembrane domain of VHA-a decreased a little in cv. Pokkali, but increased significantly cv. BRRI Dhan29 upon NaCl stress. The signaling for regulation of the VHA is transduced from the V_1 domain to V_0 via conformational changes in VHA-a (Landolt-Marticorena *et al.*, 1999). On the other hand, flexibility in the stoichiometry of the proteolipid VHA-c within the complex might be an explanation for the observed structural changes, too. Either due to an increase of the diameter of the proteolipid-ring (Klink *et al.*, 1990, Ratajczak, 2000) or by a replacement of VHA-c by isoforms as indicated by differential immunological cross reactions (FischerSchliebs *et al.*, 1997). Changes in the coupling ratio of the V-ATPase from *Mesembryanthemum crystallinum* (Rockel *et al.*, 1994) support this hypothesis. Although the analysed VHA subunits are derived from *M. crystallinum* artificial effects can be excluded, because the increase of FRET efficiency was specific for cv. BRRI Dhan29 upon salinity. Nevertheless, this differential changes of FRET efficiency between VHA-a and VHA-c in Pokkali and BRRI Dhan29 might be correlated with their differential Na^+ -compartmentalization into the vacuole.

Under salt stress both the increased ability of vacuolar compartmentalization of Na^+ (by inducing the expression of VHA) and decreased uptake of Na^+ into the cytosol (by decreasing the expression of OsHKT1), seems to work more efficiently in the salt-tolerant cv. Pokkali than salt-sensitive rice cv. BRRI Dhan29. Maathuis (2006) suggested that both the downregulation of HKT1 and an upregulation of NHX isoform (tonoplast Na^+/H^+ antiporter) could contribute greatly to limiting Na^+ loading in plant tissue, particularly where cytosolic Na^+ contents are concerned.

Conclusion

With respect to the expression data of OsHKT1, OsHKT2 and OsVHA, the regulatory mechanism for cytosolic Na^+/K^+ homeostasis seems to be an important salt-tolerance determinant in the salt-tolerant rice cv. Pokkali. This mechanism is less efficient in the salt-sensitive cv. BRRI Dhan29. At the onset of NaCl stress, Pokkali increases the expression of OsHKT2 both in the root and shoot. Since the induction is very strong in the shoot, and in particular in the mesophyll cells, the OsHKT2 possibly enhances the re-circulation of K^+ in metabolically active leaf mesophyll cells under high NaCl condition for maintaining an adequate cytosolic Na^+/K^+ ratio. Although the cells also take up Na^+ along with K^+ through this transporter, cells might transport back Na^+ from mesophyll cells by OsHKT8, as suggested by Rus *et al.* (2005) and as delineated in figure 9. Similar to OsHKT2, Pokkali also induces the expression of OsVHA at the onset of high NaCl, most likely to compartmentalize cytosolic Na^+ into the vacuole. The induction of OsVHA decreases near to the control expression levels in root 24 h after the stress and in shoot 48 h after the stress, although the expression of OsVHA again increases 72 h after the stress. Moreover, Pokkali induces the expression of Na^+ transporter OsHKT1 24 h after NaCl stress. This might occur either because of K^+ deficiency in cells (caused by Na^+ competition at transport sites), or by interruption of the cytosolic Na^+/K^+ ratio that cells might sense as a K^+ -deficiency. However, at a certain stage later on, Pokkali down-regulates the expression of OsHKT1. We conclude that at the onset of high NaCl, Pokkali maintains cytosolic Na^+/K^+ homeostasis by increasing the Na^+/K^+ coupled uptake through induction of OsHKT2, as well as by increasing the compartmentalization of cytosolic Na^+ into the vacuole. The latter is facilitated by induced expression of OsVHA. There is however, no structural change of OsVHA, as estimated in terms of the FRET efficiency between VHA-a and VHA-c, in cv. Pokkali. Pokkali might also keep a low-influx of cytosolic Na^+ , either by a conformational change of the OsHKT1 protein, and/or any post-transcriptional changes of the OsHKT1 gene. On the other hand, 48 h after the stress, Pokkali maintains cytosolic K^+/Na^+ homeostasis by down-regulating the OsHKT1. It again induces the expression of OsVHA, probably by increasing the volume of the vacuole, as found in cv. BRRI Dhan29. However, to fully understand the mechanism for K^+/Na^+ homeostasis in rice, cell- and tissue-specific expression pattern of other members of the HKT family needs to be investigated under NaCl stress.

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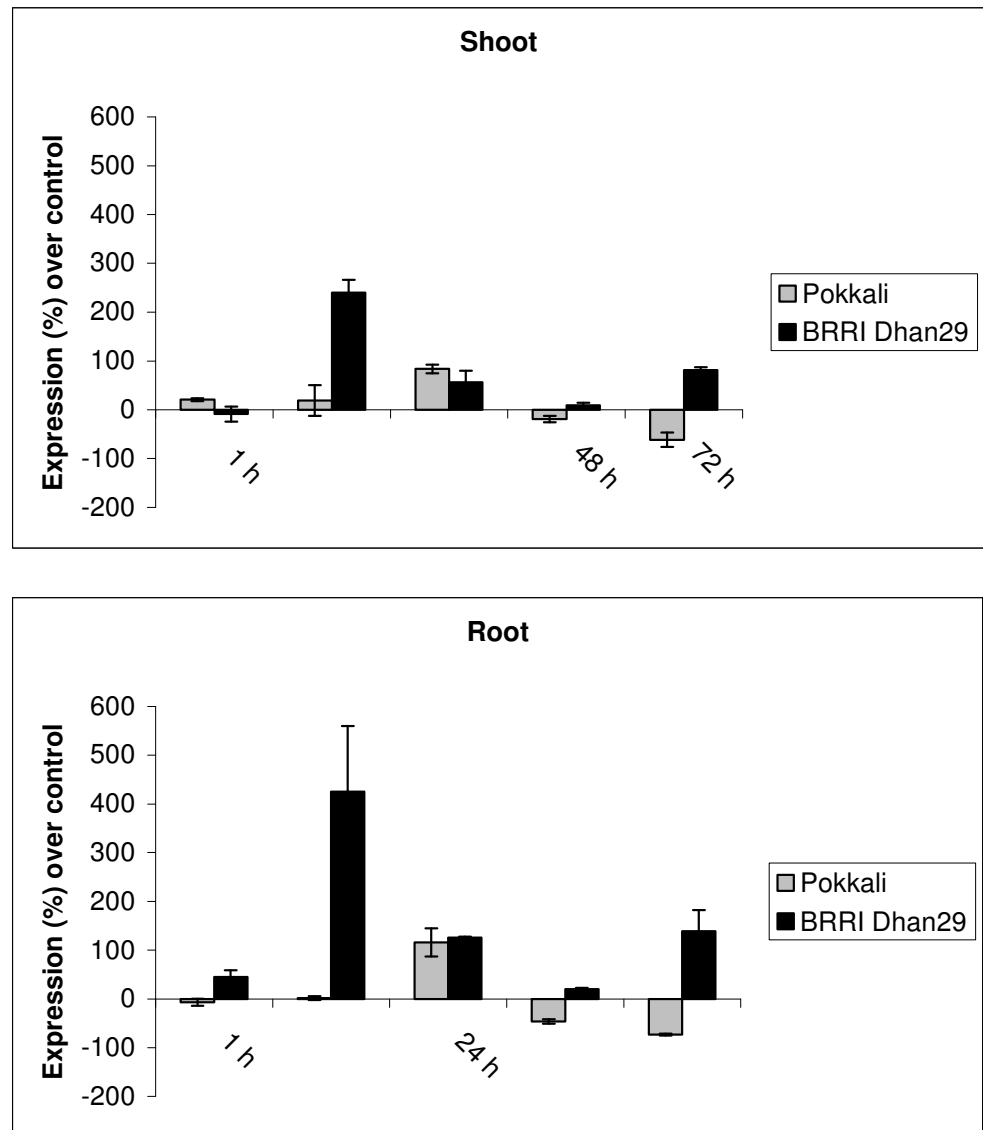


Fig. 1 Expression analysis of OsHKT1 by real-time RT-PCR amplification of RNA from root and shoot tissues of rice cvs. Pokkali and BRRI Dhan29. The number of hours (1, 6, 24, 48 and 72 h) elapsed after growing plants under salt stress (150 mM NaCl) condition is indicated. Amplifications were performed with specific primers for OsHKT1 and compared with the expression under control condition (no stress).

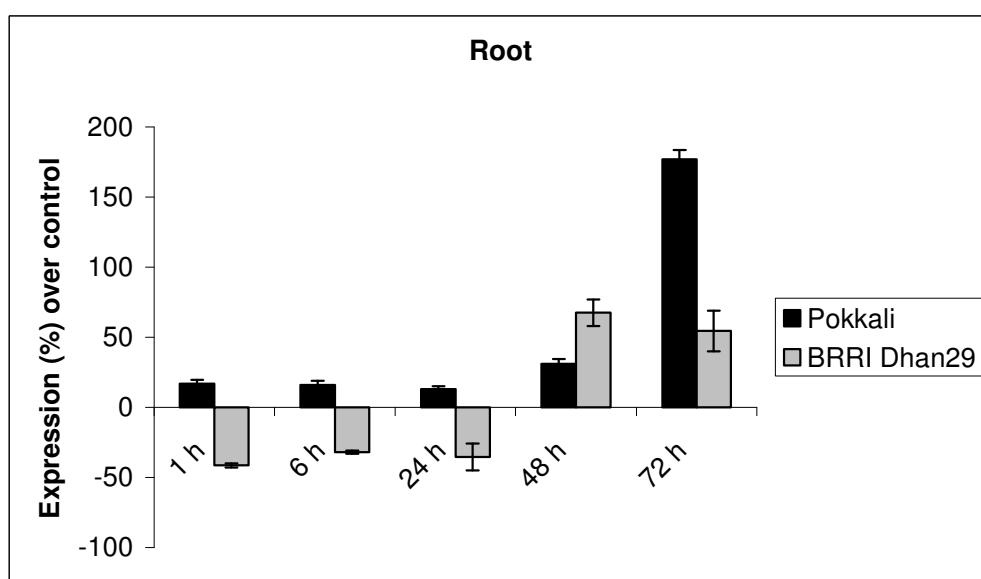
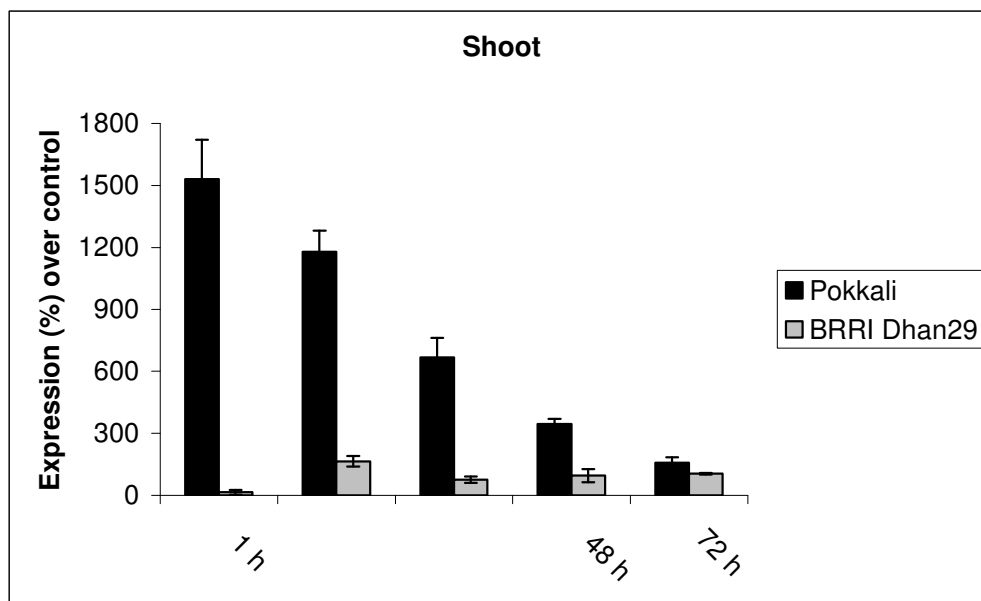


Fig. 2 Expression analysis of OsHKT2 by real-time RT-PCR amplification of RNA from root and shoot tissues of rice cvs. Pokkali and BRRI Dhan29. The number of hours (1, 6, 24, 48 and 72 h) elapsed after growing plants under salt stress (150 mM NaCl) condition is indicated. Amplifications were performed with specific primers for OsHKT2 and compared with the expression under control condition (no stress).

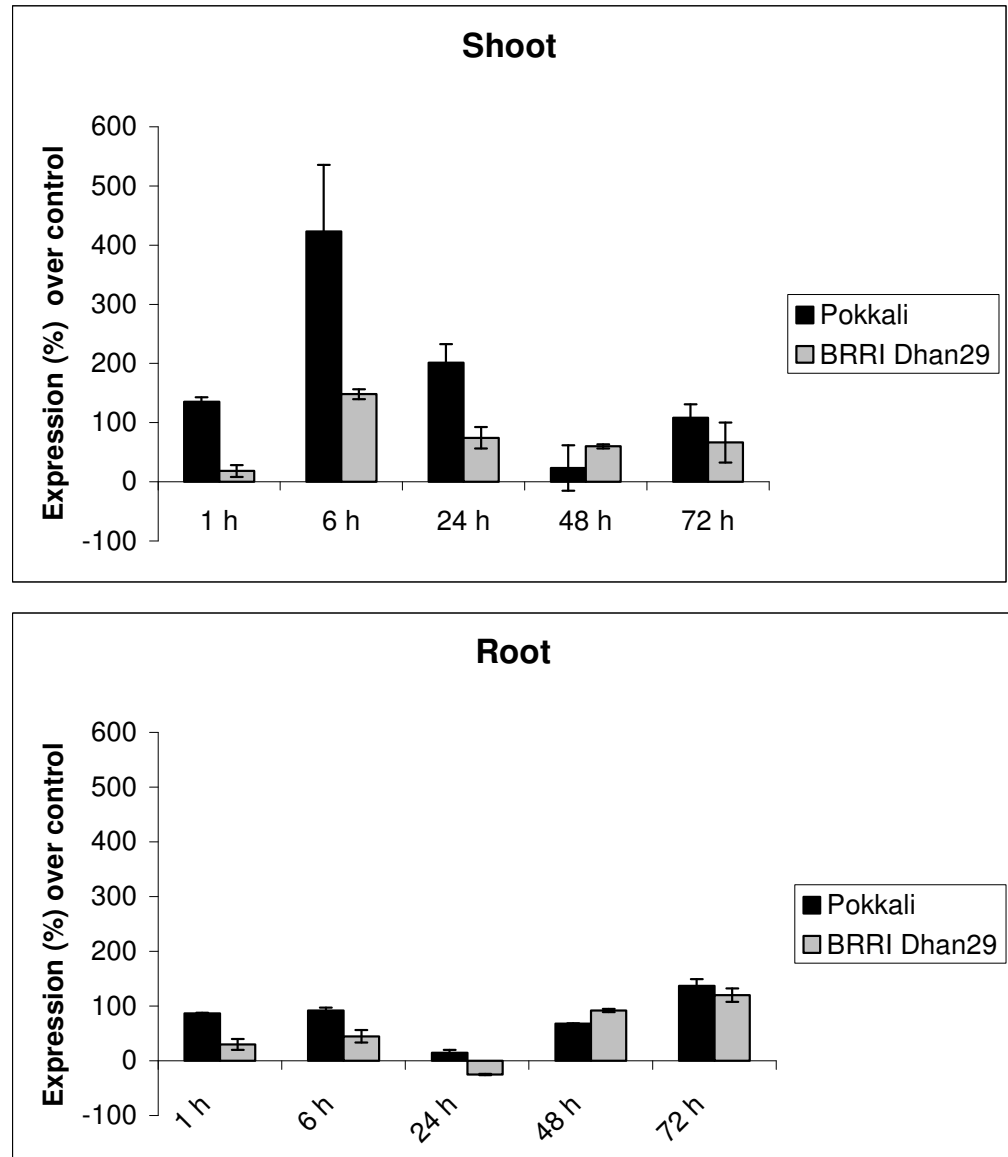


Fig. 3 Expression analysis of OsVHA by real-time RT-PCR amplification of RNA from root and shoot tissues of rice cvs. Pokkali and BRRI Dhan29. The number of hours (1, 6, 24, 48 and 72 h) elapsed after growing plants under salt stress (150 mM NaCl) condition is indicated. Amplifications were performed with specific primers for OsVHA and compared with the expression under control condition (no stress).

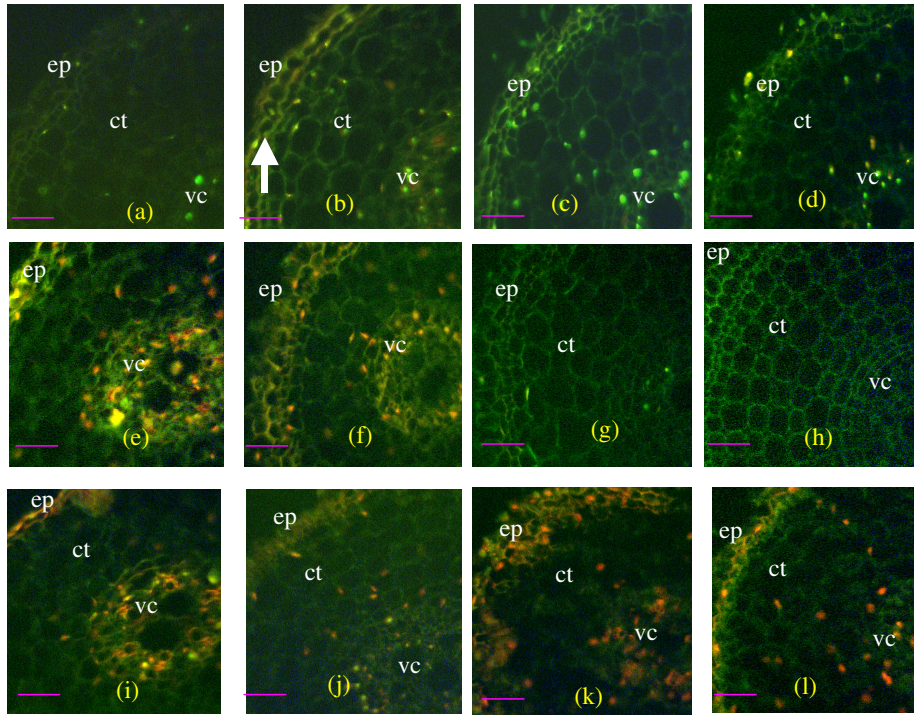


Fig. 4 Cell-specific expression analysis through *in situ* PCR of (i) OsHKT1 in rice cv. BRR1 Dhan root (a: control; b: 150 mM NaCl stressed 24 h) and Pokkali root (c: control; d: 150 mM NaCl stressed 24 h), (ii) OsHKT2 in rice cv. BRR1 Dhan root (e: control; f: 150 mM NaCl stressed 48 h) and Pokkali root (g: control; h: 150 mM NaCl stressed 48 h) and (iii) OsVHA in rice cv. BRR1 Dhan root (i: control; j: 150 mM NaCl stressed 1 h) and Pokkali root (k: control; l: 150 mM NaCl stressed 1 h)

Abbreviations: ep, epidermis; ct, cortex; vc, vascular cylinder. The bar in all prints represents 50 μ m. The signal is shown by green fluorescence of alexa fluor 488-5-dUTP (Molecular Probes, Netherlands) as indicated by white arrow.

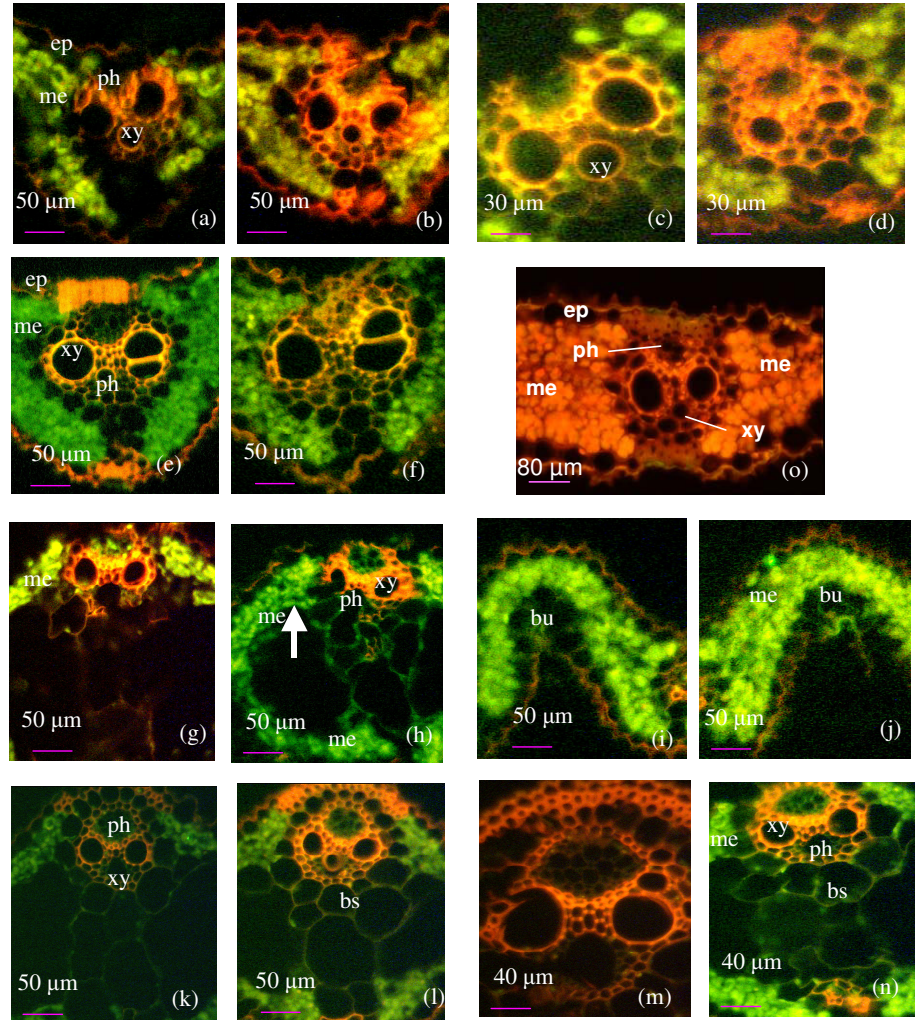


Fig. 5 Cell-specific expression analysis through *in situ* PCR of (i) OsHKT1 in rice cv. BRRI Dhan leaf (a,c: control; b,d: 150 mM NaCl stressed 24 h) and Pokkali leaf (e: control; f: 150 mM NaCl stressed 24 h), (ii) OsHKT2 in rice cv. BRRI Dhan leaf (g: control; h: 150 mM NaCl stressed 48 h) and Pokkali leaf (i: control; j: 150 mM NaCl stressed 48 h) and (iii) OsVHA in rice cv. BRRI Dhan leaf (k: control; l: 150 mM NaCl stressed 6 h) and Pokkali leaf (m: control; n: 150 mM NaCl stressed 1 h).

Abbreviations: bs, bundle sheath; bu, bulliform cell; ep, epidermis; me, mesophyll; ph, phloem; xy, xylem. The specific signal is shown by green to yellow fluorescence of alexa fluor 488-5-dUTP (Molecular Probes, Netherlands) as indicated by white arrow. The negative background and the negative control (o) are shown in red.

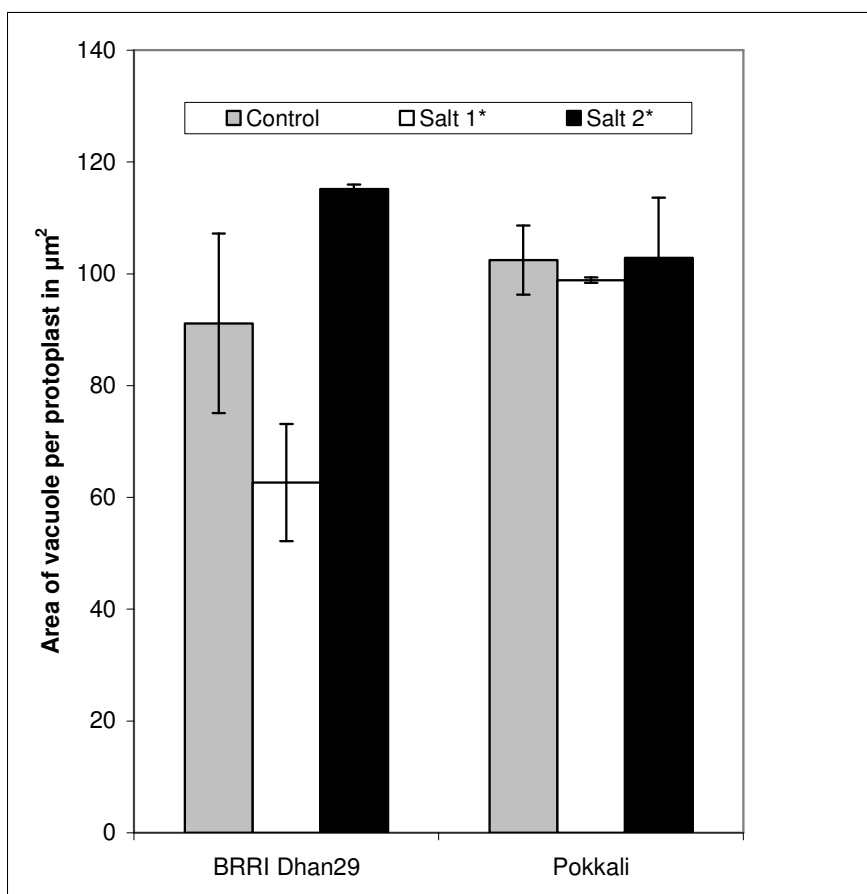


Fig. 6 Changes in vacuolar area of shoot protoplasts of rice cvs. BRRI Dhan29 and Pokkali upon salt stress (150 mM NaCl). Protoplasts were loaded with the vacuole specific dye 6-CFDA. The dye within the vacuole was excited sequentially using the 458 nm and 488 nm lines of an argon-ion laser and the images of the vacuoles with the signal from the dye was detected in the range of 500-530 nm after passing the short pass filter RSP500 (Leica). The images of the vacuoles were taken before, and after, salt addition. The area of the vacuole was measured by using the Leica Confocal Software LCS Lite. Each column indicates average vacuolar area measured from 40 protoplasts. Mean values \pm SE. Salt 1; protoplasts were stressed with NaCl 100 mM for 5-10 min. Salt 2; protoplasts were stressed with 100 mM NaCl for 24 h.

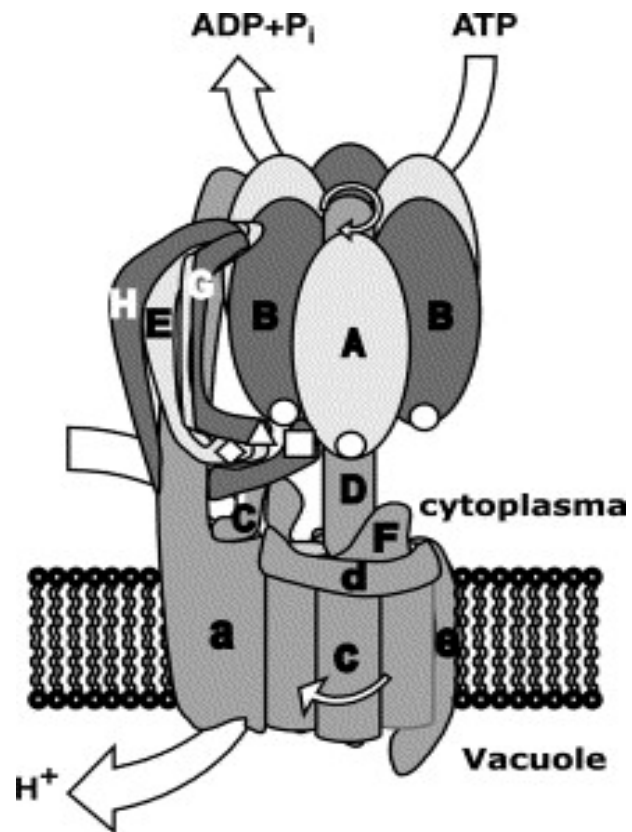


Fig. 7 Structural model of the vacuolar H^+ -ATPase VHA (Seidel, Golldack & Deitz, 2005). The membrane integral sector V_0 composing with subunits VHA-a, c, d and e is involved in the catalyses of the proton transport. On the other hand, the cytoplasmically exposed V_1 sector composed of the subunits VHA-A to VHA-H is involved in ATP binding and hydrolysis.

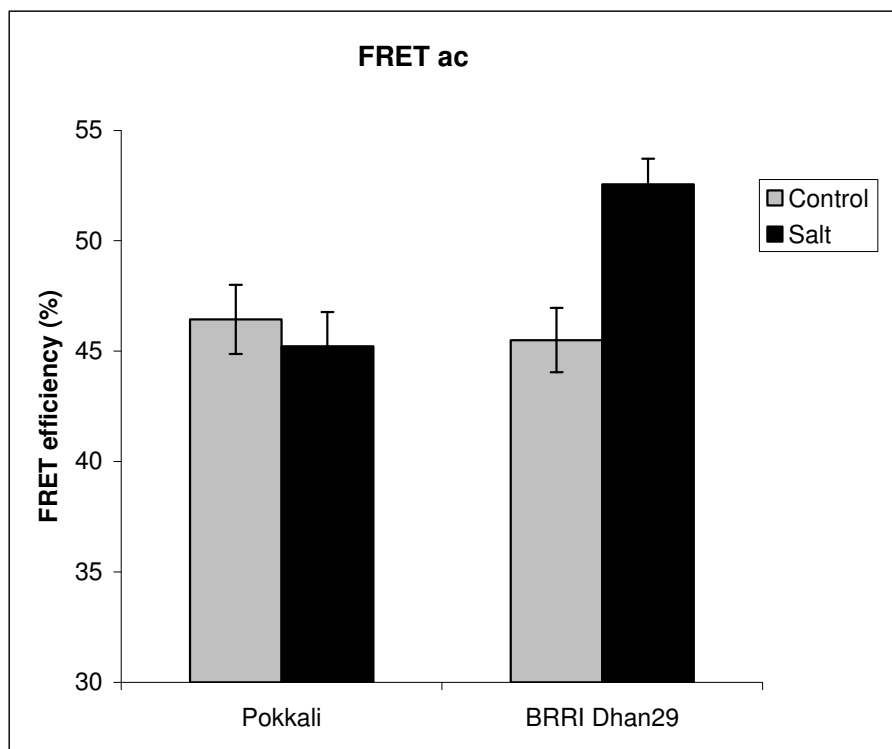


Fig. 8 Calculated FRET-efficiencies of VHA-subunits. The FRET effect of the FRET-pairs VHA-a-YFP/VHA-c-CFP was measured in more than 60 protoplasts. The FRET efficiency was calculated for each measurement. The data represent the mean values \pm SE.

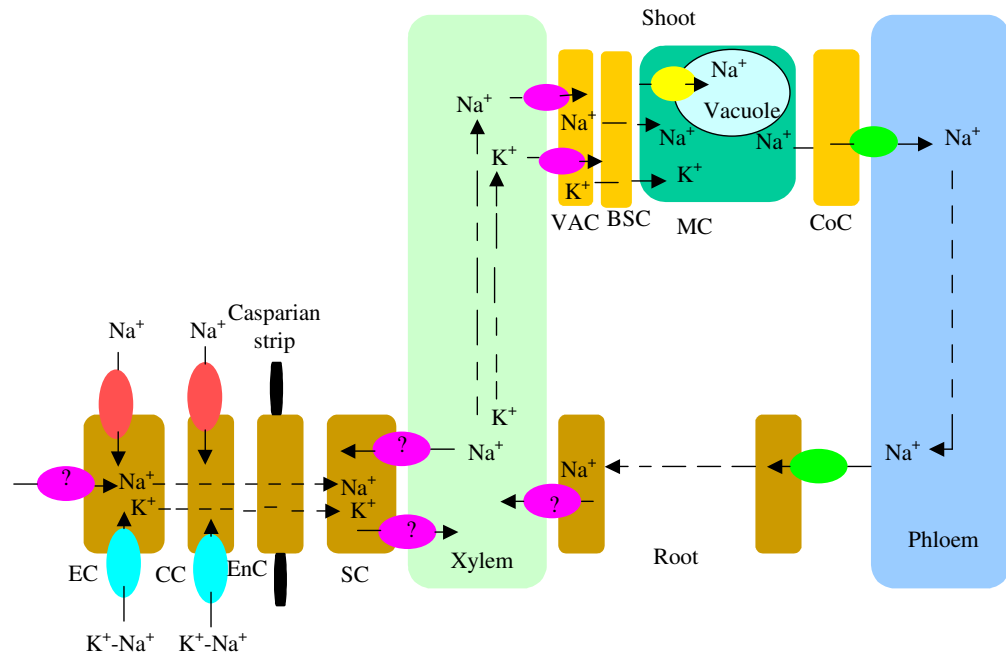


Fig. 9 A model describing possible functions of OsHKT1 & 2 and OsVHA in regulating cytosolic Na^+/K^+ homeostasis. OsHKT1 (red ovals) functions as Na^+ uptake system (Garcia-deblás *et al.*, 2003), OsHKT2 (blue ovals) functions as K^+-Na^+ coupled uptake system (Horie *et al.*, 2001), OsHKT8 (green ovals) functions as Na^+ transporter, but plays important role in maintaining K^+ homeostasis in shoot (Rus, Bressan & Hasegawa, 2005), OsVHA (yellow oval) energizes tonoplast Na^+/H^+ antiporter to compartmentalize cytosolic Na^+ into vacuole (Golldack & Dietz, 2001). Abbreviations: EC, epidermal cell; CC, cortical cell; EnC; endodermal cell; SC, stellar cell, VAC, Vessel associated cell; BSC, bundle sheath cell; MC, mesophyll cell; CoC, companion cell.

