Mechanisms of Salt Tolerance: Sodium, Chloride and Potassium Homeostasis in two Rice Lines with Different Tolerance to Salinity Stress

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Rice marks second among the agricultural crop plants in the world (FAO, 2004). This work aimed at identifying the molecular mechanisms implicated in tolerance to salt. Salinity is a major environmental threat for agricultural production that affects ionic and osmotic as well as nutritional relation of plants. Ion channels are key players in maintaining ion homeostasis also under salinity. Cl^- content was very low in control conditions but under 150 mM NaCl, Cl^- was abundantly accumulated in leaves of the salt sensitive rice line IR29, whereas the salt tolerant line Pokkali excluded it from the leaves. Transcript of *OsCLC1* i.e. voltage-dependent Cl^- channels was found in both lines in roots and leaves under normal growth conditions and was repressed in IR29 and induced transiently in Pokkali upon salt treatment. Simultaneous, transcript amounts of the Na^+/H^+ antiporter *OsNHX1* and the vacuolar H^+-ATPase subunit *OsVHA-B* decreased in IR29, whereas Pokkali showed transient increase of *OsVHA-B*. Subsequent analysis of the water channel aquaporin *OsPIP2;1* and the cell-specificity of *OsCLC1* transcript distribution by *in situ* PCR showed coordinated regulation of *OsCLC1*, *OsVHA-B*, *OsNHX1* and *OsPIP2;1* on the one hand and suggest that *OsCLC1* functions in osmotic adjustment at high salinity on the second hand.

Transcript of the K^+ transporter *OsHAK7* that belongs to the HAK/KT/KUP family were also analysed in relation to K^+ homeostasis. K^+ content was high in plant tissues under normal conditions, however salt stress decreased root levels and strongly increased its accumulation in leaf cells in both IR29 and Pokkali. *OsHAK7* showed high transcript abundance only during the first 6 h of the salt treatment in leaves, whereas in roots the induction was maintained up to 48 h in both lines. Tissue and cell-specificity distribution of *OsHAK7* transcript by *in situ* PCR revealed expression in plant tissues under normal conditions. Strong signals in the mesophyll of both rice lines were detected in leaves, whereas expression in the vasculature cells was specific to Pokkali. In response to salt stress, transcript amounts were reduced in the mesophyll and were detectable in phloem and xylem parenchyma cells of both lines. Analyses of these results demonstrated transcriptional regulation of *OsHAK7* under salinity stress and suggest that the K^+ transporter functions in salt-dependent K^+ homeostasis in rice.

A comparative analysis of salt stress responses in the monocotyledonous halophyte *Festuca rubra* ssp *littoralis* and the salt sensitive crop species wheat (*Triticum aestivum*) were investigated for better understanding strategies of salt tolerance. Ion accumulation was similar in both species except for Ca, Mg, Fe and Na, whose contents were higher in *Festuca*

than in wheat in control conditions. In response to 125 mM NaCl (which characterised severe stress for wheat), the crop species (*Triticum aestivum)* limited the uptake of Na^+ in leaves whereas *Festuca* significantly accumulated it in root and leaves. In addition, Mg and Fe content increased in *Festuca*. At 500 mM NaCl, *Festuca* accumulated Na^+ in both tissues. Expression of genes with important function in the regulation of ion homeostasis was also analysed. In root tissue treatment of 125 mM NaCl improved the transcript level of *Festuca FrPIP2;1*, *FrVHA-B* and *FrNHX*, whereas in wheat the expression of *TaPIP2;1* and *TaVHA-B* was down regulated. *FrPIP2;1*, *FrVHA-B* and *FrNHX* cell-specificity analysis indicated expression in root epidermis, cortex cells, endodermis and in the vasculature tissue. Treatment of 500 mM NaCl showed repression in the epidermis and the outer cortex cells whereas strong signals were observed in the endodermis and the vasculature. These results indicated divergent transcriptional regulation of the aquaporin *PIP2;1*, V-ATPase and the Na^+/H^+ antiporter *NHX* and seems to be correlated with salt tolerance and salt sensitivity in *Festuca*, in the rice lines Pokkali, IR29 and wheat and suggested coordinated control of ion homeostasis and water status at high salinity in plants.

As reported in many studies, salinity is a complex constraint that induced the regulation of many of other genes with significant function in the mechanism of salt tolerance. Identification of probable salt induced genes was investigated by using rice and *Festuca* cDNA-arrays to identify 192 and 480 salt responsive expressed sequence tags (ESTs) from a rice and *Festuca* salt stress-cDNA-library. The rice cDNA-array hybridizations compared between the salt sensitive line IR29 and the salt tolerant line Pokkali showed no significant difference. Considering the number of salt regulated genes, more induced genes could be showed in Pokkali leaf than in IR29 under 150 mM NaCl 6 h. IR29 recovered slowly according to the duration of the treatment and at 48 h, more genes were regulated in IR29 than in Pokkali. While more genes were up and down-regulated under NaCl and LiCl stress, salt stress under K^+ starvation induced more regulated genes in Pokkali than in IR29. Salt-induced gene expression was compared between the salt sensitive line IR29 and the halotolerant *Festuca* using *Festuca* cDNA-arrays. Treatment of 125mM NaCl during 6 h indicated no significant difference in the number of upregulated genes in both species, however, several genes were repressed in *Festuca*. *Festuca* showed only a high rate of upregulated genes at high salt concentration (500 mM NaCl). Functional classification of salt-induced genes identified gene products related to metabolism such as the NADP-dependent oxidoreductase that is a component of the antioxidative system. Second large

group corresponded to genes with unknown function. In these groups as well as in the group of defence, many of the induced genes were only observed at 500 mM NaCl. These results suggest a small rate of genes were needed to maintain normal growth under low salinity in the halophyte *Festuca*. This number increased and reached the maximum at 500 mM NaCl, whereas in the salt sensitive rice line IR29 the maximum was reached at low salt concentration. Transcription factors, translation and signal transduction constituted a small group with a slight increase in *Festuca* treated for 6 h with 125 mM NaCl and 500 mM NaCl. The expression of the translation initiation factor *SUI1* as well as the signalling tanscduction element protein kinase *SPK3* seemed to be moderate in the *Festuca*-cDNA-array. However Northern blot expression of the rice translation initiation factor *OsTIF* (*SUI1*) and the rice serine-theonine proteine kinase *OsSPK3* showed clear improvement in the halophythe Festuca at 500 mM NaCl. In IR29, Northern blot analysis showed a decrease in the transcript abundance of the genes. According to their induced expression in *Festuca* to high salinity, sequences of *OsTIF* as well as the sequence of *OsSPK3* inserted and analyzed in the salt sensitive rice IR29. Under salt stress conditions, transgenic plants overexpressing *OsTIF* or *OsSPK3* increased the transcript level of both genes and improved the tolerance to salinity compared to the wild-type. In addition, expression of the V-ATPase in transgenic plants was significantly induced under salt stress. These results suggest that the translation initiation factor *OsTIF* and the protein Kinase *OsSPK3* are useful for improvement of salt tolerance in rice.

Liste of publications:

Diedhiou CJ and Golldack D (2005) Salt-dependent regulation of chloride channel transcripts in rice. Plant Science, in press

Diedhiou CJ and Golldack D (2006) Wheat and a salt-tolerant relative, *Festuca rubra* ssp. *litoralis*, regulate a plasma membrane aquaporin, the vacuolar H^+- ATPase and Na^+/H^+ antiporter differently. Physiologia Plantarium, in revision

Diedhiou CJ and Golldack D (2006) Salt stress regulates expression of the HAK-type K^+-transporter *OsHAK7* in rice, Submitted

1- Introduction

Plant productivity is severely threatened by enhanced salinity. Actually 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated conditions of sodicity (434 million ha) (FAO, 2005). Salinity is generally defined as the presence of excessive amounts of soluble salt that hinders or affects the normal functions needs for plant growth. It is measured in terms of electric conductivity (ECe), or of the exchangeable Na^+ percentage (ESP) or with the Na^+ absorption ratio (SAR) and pH of saturated soil paste extract. Therefore saline soils are those with ECe more than 4 dSm^{-1} equivalent to 40 mM NaCl, ESP less than 15 % and a pH below 8.5 (Waisel, 1972; Abrol, 1986; Szabolcs, 1994).

Table 1 Definition of saline and sodic soils, classed according to the USDA Salinity Laboratory (USSL, 2005).

Term	Description	Effect on plant growth	Definition	Comments
Salinity	Saline soils have a high concentration of soluble salts. They are classed as saline when the ECe = 4 dS m^{-1}.	This definition of salinity derives from the ECe that would reduce yield of most crops. However, many crops are affected by an ECe< 4 dS m-1	Osmotic and salt specific components inhibit root and shoot growth	ECe is the electrical conductivity of the saturated the paste extract, and reflects concentration of salts in saturated soil. A conductivity of 4 dS m^{-1} is equivalent to 40 mM NaCl.
sodicity	Sodic soils have a low concentration of soluble salts, but a high exchangeable Na+ percentage (ESP). They are classed as sodic when the ESP is= 15%.	This definition of sodicity Derives from the ES P that causes degradation of the structure of clay soils, caused by Na+ displacing divalent cations bound to negative charges on the clay particles.	Poor soil structure inhibits root growth	At high ESP, the clay particles separate. The soil drains poorly and becomes waterlogged when wet. It also becomes very hard when dry.
Alkalinity	Alkaline soils are a type of sodic soil with a high pH. They are defined as having an ESP = 15% with a pH of 8.5–10.	The high pH is caused by carbonate salts in parent material.	High pH affects nutrient uptake	

Salinity is an environmental factor that greatly affects plant growth and development and is a major constraint for crop production. This stress is complex and causes a number of determinant effects. Among them ionic and water constraints constitute the most important. The water constraint even called osmotic pressure is characterised by difficulties to absorb

water. The ionic constraint interferes with the uptake of nutrients, and causes direct toxicity due to the ions Na^+ and Cl^-. Salinity also interferes with the structure of the soil, causes an indirect stress and increases the sensitivity to diverse biotic stresses (Araya et al., 1991). To limit the effect of salt in plant productivity, amelioration and utilisation of salt-affected soils are needed. Two approaches are used to solve the problem: by using technical approaches (water and soil management) and biological approaches. Financial difficulties as well as the environmental injuries caused by desalinization programms oriented many research programs to the biological approach, a posteriori in this direction to screen and develop novel plants with increased salt tolerance and better ability to grow in saline areas. Among species, rice (*Oryza sativa*) may play a major role because of its role as 2nd most consumed cereal in the world, and on the other hand its capacity to survive a long submerging time. Since the genome of rice became completely sequenced, rice is increasingly becoming the model plant for cereals. In addition, the establishment of the rice-mutant database with at present approximately 40.000 independent lines (IR64-Database) is another important reason for the selection of rice as model plant. To create rice tolerant lines capable to grow and minimize the toxicity effects induced by salt stress and capable to improve the productivity, it is necessary to identify the molecular mechanisms involved in the tolerance or the sensitivity of plants to salt.

1.1 Mechanisms of plants adaptation to salt

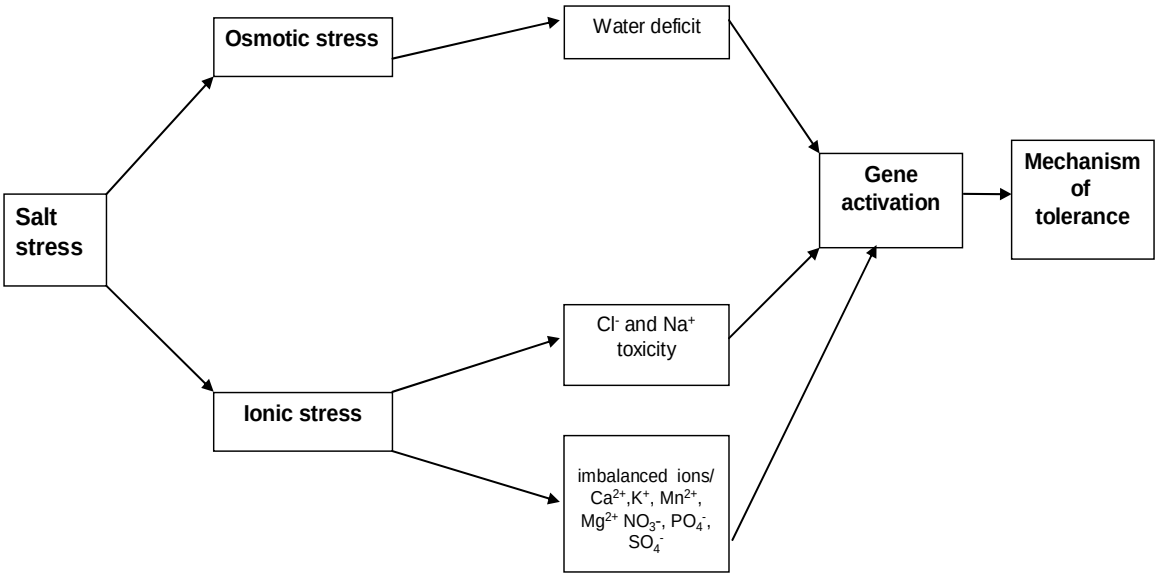

Fig. 1.1 Salt stress constraints addressed in this study.

Salt stress is a complex trait which involves two major parameters. The osmotic resultant induces water deficit, while the ionic resultant induces on the one hand an ionic toxicity due to Cl^- and Na^+ accumulation and on the other hand indirect toxicity due to the difficulty of essential nutrient elements uptake. All these constraints are perceived and send to the genome which activates appropriated mechanisms to re-establish water transport, limit Na^+ and Cl^- uptake or lowers their concentration in cytoplasm and allowing the absorption of ions indispensable for growth. Tolerance depends on a range of physiological, biochemical and molecular adaptations activated by the genome to survive in salt medium.

1.1.1 Osmotic stress

When submitted to salt stress, plants perceive first the constraint as drought stress. Due the low water potential in the medium, plants cannot absorb water and tend to lose its water. This constitutes the osmotic constraint or water deficit of salinity. As consequence, plant growth is inhibited because the plant ability to take up water is reduced, and growth becomes slower. According to Song et al. (2005), the reduction of germination observed by the three species of the halophyte *Sueda* is due to the osmotic and ionic toxicity. Germination of the species is improved, when the salinity is alleviated. Therefore to survive in conditions of osmotic potential, plants must either limit water loss by regulation of its transpiration or proceed to the readjustment of its osmotic potential.

1.1.2 Regulation of osmotic potential: synthesis of compatible solutes

Osmotic adjustment is one of the vital cellular responses to water deficit generated by drought, salinity or freezing temperature conserved by halophytes and glycophytes (Chinnusamy et al., 2004). This adjustment may contribute to maintain turgor despite low water potentials and proceed to the uptake of K^+, compartmentalization of Na^+ and Cl^- into the vacuole or synthesis of compatible solutes such as proline, glycinebetaine, polyol, sugar etc. (Asraf, 1994). These solutes are called compatible because they are non-toxic at high concentration, they have low weight, are highly soluble and protect plants from stress by turgor maintenance, detoxification of radical oxygen species (ROS), and by stabilisation of quaternary structure of proteins (Yancey et al., 1982; Bohnert and Jensen, 1996). Different compatible solutes are synthesised according to the species. Glycinebetaine was found to be the major organic substrate accumulating under hypersaline growth conditions in the halotolerant cyanobacterium *Spirulina subsalsa*. In this species, it is shown that this osmolyte specifically protects enzymatic activity such as glucose-6-phosphate dehydrogenase which

remained fully activated in the presence of NaCl (Gabbay-Azaria et al., 1988). Accumulation of glycinebetaine has been also reported under conditions of salt stress for example barley and *Atriplex* (Jagendorf and Takabe, 2001**;** Shen et al. 2002). In cereal species (wheat, barley, rice and maize), Ayliffe et al. (2005) noted the presence of proline and demonstrated the importance of accumulation and degradation of proline in the mechanisms of tolerance to abiotic stress namely drought and salt. In the halotolerant *M. crystallinum*, Popova et al., (2002; 2003) reported under salt stress an increased expression of NADP-specific isocitrate deshydrogenase that is involved in proline biosynthesis.

1.1.3. **Reduction of transpiration**

One of the most important criteria of stress tolerance is correlated with the early perception of the stress. The first developmental interference of salt stress is linked to the growth inhibition induced by water deficit. Signals of water stress could be detected by ABA accumulation. Thus, in the Na^+ excluder *Phaseolus vulgaris*, ABA mediates both the short- and long-term responses to Na^+ toxicity and the signalling of the salt-induced water deficit (Montero et al., 1998; Sibole et al., 1998, 2000). Signal perception induces mechanisms of adaptation or tolerance to salt tress. For example certain species living in an environment rich in salt survive by limiting the transpiration through closure of stomata (Sibole et al., 2003). Carbon assimilation is central to leaf growth and productivity. Under saline conditions, photosynthetic carbon assimilation is severely restricted by reduced leaf expansion. This reduction instead of being disastrous can be advantageous for tolerant varieties which positively correlated the decrease of CO_2 assimilation with chlorophyll a accumulation and soluble protein contents improving the regulation of photosynthesis in presence of NaCl (Sibole et al., 2000). Another strategy used by *M. crystallinum* consists in closing during the day its stomata at lower temperatures and increased relative humidity and open them at night. CO_2 will be taken up in the night when the stomata are open, and the carboxylation processes continue in the day. These plants pass from C3 to CAM (Crassulacean acid metabolism) metabolism (Flowers et al., 1977).

In addition to the ABA effect, K^+ plays determinant role in stomata closure. Thus the presence of Na^+ in the apoplastic space of guard cells could disturb the K^+ channels that participate in stomata movement (Schroeder et al., 2001). Recent results indicated that most of the conductance of water is realized by aquaporins which are membrane proteins forming water channels (Tyerman et al., 1999; Maurel and Chrispeels 2001). Expression of aquaporin genes in certain cellular and environmental conditions such as physiological processes,

drought and salinity (Sakurai et al., 2005; and Suga et al., 2002), suggest their role in the control of water use and water loss observed under conditions of drought, salt and heat stresses. cDNA-arrays of *Populus euphratica* Oli., a salt-tolerant species that can cope with up to 450 mM NaCl, showed certain transcripts significantly up-regulated by salt stress and related to the control of water (Gu et al., 2004). Among these transcripts, the authors identified a seed germination-related protein, a plasma membrane intrinsic protein (aquaporin), the photosynthesis-activating enzyme Rubisco activase and photorespiration-related glycolate oxidase. Considering both results, the regulation of aquaporin expression appears to be important for adequate tissue and cellular water transport under salt stress.

1.2 Ionic constraint

When grown in NaCl solution, plants accumulate Na^+ and Cl^- according to their tolerance capacity. High Na^+ and Cl^- concentrations in soils not only cause water stress but also hyperionic stress effects. Plant growth inhibition can be seen due to the effect of both Na^+ and Cl^- to limit the absorption of other ions and nutrients required for growth. While Na^+ competes with K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}, Cl^- restricts the absorption of NO_3^-, PO_4^{2-} and SO_4^{2-} (Termaat and Munns, 1986; Romero et al., 1994). To grow on saline soils and limit the effect of Na^+ and Cl^-, plants must develop mechanisms controlling the salt specific effect of salinity. Mechanisms for salt tolerance can be attributed to many strategies: On the one hand those minimising NaCl entry into the plant and those reducing the concentration of salt in the cytoplasm on the other hand (Lutts, 1996b).

1.2.1 Effect of Cl^-

Morphological and physiological disturbances produced by salinity are often associated with Cl^- and Na^+ accumulation. In some species like *Citrus*, Cl^- is more important and more rapidly accumulated than Na^+ (Romero-Aranda et al., 1998). Maize seems to accumulate the same amount of Cl^- and Na^+ (Izzo et al., 1991). In rice Lessani and Marschner (1978) reported that there is a linear accumulation of the two ions. Levitt (1980) considered the accumulation of Cl^- in the aerial parts as the main reason of toxicity of the NaCl corroborating that in presence of salt, the resistant cultivars accumulate less Cl^- than the sensitive ones. Many studies have been conducted on the implication of Na^+ in the molecular mechanisms of salt tolerance. Among these, those linked to the regulation of the concentration of Na^+ in the cytoplasm appear particularly important. The absorption and the efflux of the Na^+ through the plasma membrane and the vacuole regroup a multiplicity of research that addresses the role

of K^+ channels as well as Na^+/H^+ antiporters associated to with H^+-ATPase pump (Munns, 2005). Thus, the capability of *Citrus* plants to tolerate salinity might intimately be related to the ability of the rootstock to exclude Cl^- (Cooper et al., 1952; Walker et al., 1983; Banfuls et al., 1997), although the nature of this mechanism remains totally unresolved (Storey and Walker, 1999). It is hypothesized that Cl^- uses channels that play a crucial role in controlling the ionic composition of cytoplasm and the volume of cells. According to Jentsch et al. (2002) this function is performed in a close interplay with various ion transporters, including pumps, co-transporters and other ion channels, for instance Na^+/H^+ and HCO_3^-/Cl^- exchangers needed as a parallel Cl^- shunt for recycling Cl^-. In addition, some cells using H^+-ATPases, may need parallel Cl^- channels for the maintenance of electro neutrality. Cl^- channels also play an important role in cell volume regulation, involving in some cases, the parallel opening of stretch-activated K^+ and Cl^- channels resulting in a net efflux of salt regulating at the same time the cytoplasmic pH of cells (Jentsch et al., 2002). Despite their key roles in various functions, little is known about the regulation of Cl^- transport systems in salt treated plants. According to Hechenberger et al. (1996) voltage-gated Cl^- channels of the CLC-type family are found in prokaryotic and eukaryotic organisms ranging from bacteria to animals and plants that mediate passive Cl^- transport which is driven by the electrochemical gradient. In animals, CLC-type channels function in the regulation of membrane potential and cellular pH homeostasis, therefore, mutational inactivation of CLC-channels causes diseases as e.g. nephropathies (Dutzler, 2004). Homologous plant CLC-channels have been identified in tobacco and *Arabidopsis*. The expression of the *Arabidopsis* channels *AtCLC*-c and *AtCLC*-d could functionally complement the CLC-type yeast mutant *gef1* (Hechenberger et al., 1996, Gaxiola et al., 1998). Heterologous expression of tobacco *CLC-NT1* in *Xenopus* oocytes induced hyper-polarization through activated Cl^- channels whereas no Cl^- currents were elicited by *AtCLC*-a,-b,-c and -d (Lurin et al. 1996, Hechenberger et al., 1996). Barbier-Brygoo (2000) suggested that plant CLC-type chloride channels are involved in regulation of stomata movement.

1.2.2 Competition between Na^+ and K^+

K^+ channels are of interest to physiologists because they are universal regulators of cellular properties. The function of many organs and many different cell types is modulated via activation or inhibition of K^+ channels. In plant cells, K^+ is a major macronutrient essential for many cell processes, including enzymatic activation, turgor formation, regulation of stomatal movement and maintenance of osmotic homeostasis (Shabala et al. 2003).

Counterbalancing the large excess of negative charge, K^+ is also equilibrated with Na^+ to provide a correct environment for protein synthesis in condition of hyperionic stress. In case of high salinity, control of homeostasis consists of Na^+ efflux from the cytoplasm while maintaining K^+ concentration. K^+ channels and K^+ transporters may regulate Na^+ transport either directly because they may be incompletely selective for K^+ and they transport Na^+. The vacuolar Na^+/H^+ antiporter SOS1 localized in the plasma membrane has been identified in *Arabidopsis* (Shi et al., 2000, Zhu 2003). The vacuolar Na^+/H^+ antiporter NHX is a transport system specific not only for Na^+ cations but it has a broad substrate specificity for at least four alkali metal cations (Na^+, Li^+, K^+ and Rb^+) (Kinclova-Zimmermannova et al., 2004).

At the tonoplast, the vacuolar H^+-ATPase generates a H^+ gradient that energizes the NHX-type Na^+/H^+ antiporter which induces Na^+ efflux in the vacuole and contributes to Na^+ detoxification. (Ratajczack, 2000; Wang et al., 2001, Golldack and Dietz 2001). Na^+ is also indirectly excluded from the cytoplasm by proteins regulating K^+ transport. Several types of transporters and channels have been identified that mediate K^+ uptake into the plant at micromolar and millimolar external K^+ concentrations. Inward-rectifying AKT1/KAT1-type (*Arabidopsis* K^+ transporter) that are structurally homologous to Shaker-type K^+ channel in *Drosophila melanogaster* and vertebrates have been characterized in various species (Anderson et al., 1992; Sentenac et al., 1992; Jan and Jan 1994; Cao et al., 1995, Ketchum and Slayman 1996, Pilot et al., 2003). Although first considered to be a component of low affinity K^+ uptake, expression and functional involvement in K^+ transport at micromolar K^+ concentrations have been reported (Lagarde et al., 1996 and Spalding et al., 1999). Function of the transporter *HKT1* isolated from wheat as a K^+/Na^+ symporter has been shown by heterologous expression in *Xenopus* oocytes and yeast (Schatman and Schroeder, 1994; Gassman et al., 1996). In *Arabidopsis* and rice, specificity of HKT-proteins as Na^+ transporters have been shown (Uozumi et al., 2000; Golldack et al., 2002).

Accordingly, *HKT1* may have only a minor role in plant K^+ transport but may function in Na^+ uptake (Rus et al., 2001; Horie et al., 2001). A third family that is involved both in low- and high affinity K^+ uptake are HAK-type proteins that share homology with bacterial KUP-transporters and *HAK1* from *Schwanniomyces occidentali*s (Schleyer and Bakker, 1993; Banuelos et al., 1995; Santa-Maria et al., 1997; Kim et al., 1998; Fu and Luan, 1998). In the completely sequenced genomes of *Arabidopsis* and rice HAK-type transporters are represented by multigene families with 13 members in *Arabidopsis* and 17 HAK homologues in rice. (Mäser et al., 2001; Banuelos et al., 2002). Expression of HAK has been detected for

all plant organs and tissues and localization at various cellular compartments as the tonoplast and the plasma membrane has been reported (Rubio et al., 2000; Senn et al 2001; Su et al., 2002, Banuelos et al., 2002). Although expression of HAK-transporters has been found at millimolar and micromolar external K^+ concentrations, transcription was stimulated by K^+ depletion for e.g. *AtHAK5* in *Arabidopsis* and *McHAK1* and *McHAK4* in *M. crystallinum* indicating a role in inducible high- affinity K^+ transport (Su et al., 2002 and Gierth et al., 2005). In addition, an involvement of HAK- type transporters in plant growth has been found in *Arabidopsis* mutants that showed defects in shoot cell expansion and root hair development (Rigas et al., 2001 and Elumalai et al., 2002).

1.3 Oxidative stress tolerance

In addition to water and ionic stress, salt stress as well as other environmental stresses by dysfunction of the photosynthetic machinery or other metabolic disorder could generate secondary stress called oxidative stress which is caused by the accumulation of reactive oxygen species. Most of them include for example hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS are usually generated by normal cellular activity such as photorespiration and ß-oxidation of fatty acids, but their level increase under biotic and abiotic stress conditions (Xiong and Zhu, 2002). ROS cause oxidative damage to the membrane lipids, proteins and nucleic acids. The capacity of plants to scavenge ROS and to reduce their damaging effects appears to represent an important stress-tolerant trait. Elimination of ROS is achieved either by antioxidant compounds such as glutathione, thioredoxin, ascorbate and carotenoids or by ROS scavenging enzymes as superoxide dismutase, catalase, glutathione peroxidases and peroxiredoxins. Plants tolerant to ROS have evolved the capacity either to avoid the production of ROS, or increase the detoxification or repair of ROS damage. For example superoxide dismutase (SOD) catalyses the conversion of superoxide anions to H_2O_2 and H_2O. Overexpression of this enzyme increased tolerance to abiotic stress such as salinity, low temperature (Bohnert and Shevela, 1998). Transgenic *Arabidopsis* plants with reduced catalase activity revealed increased sensitivity to salt stress (Willekens et al., 1997).

1.4 Salt induced gene expression

Plant adaptation to salinity requires alterations of various cellular physiological and metabolic mechanisms that are controlled by specific gene expression. These specific genes could encode for proteins implicated in Na^+ sequestration, (H^+-ATPases, NHX-type transporters), in synthesis of compatible osmolytes (proline, glycinebetaine, polyols), in the

detoxification of toxic compounds (ROS scavenging enzymes), in signal perception and regulating factors and other unknown functions. To improve the understanding of salt tolerance for further increasing the plant productivity, it is necessary to increase the knowledge on salt-induced genes. One of the attempts made to understand the mechanism of salt toxicity is the technique of array hybridization. The complete sequences of the *Arabidopsis* genome proved knowledge on all genes and allows to monitor changes in transcript abundance under salt stress. Thus, the possibility to compare salt sensitive and tolerant species is expected to give more and better ideas about genes responsible for salt tolerance and open the vision on their future integration in programs of amelioration of crops. The halophyte salt cress (*Thellungiella halophila*) shares with *Arabidopsis* 90-95 % sequence identities at cDNA level. Mechanisms of salt tolerance in this halophyte seem to be similar with those operated by glycophytes. Analysis of salt tolerance using a full length *Arabidopsis* cDNA micro-array revealed less genes induced in salt cress than in *Arabidopsis*. Among genes highly induced in salt cress, there were those previously denoted SOS1 (salt overly sensitive) and genes of antioxidant responses (Taji et al., 2004). Compared to *Arabidopsis*, salt tolerance of salt cress might be due to its ability to detoxify cells from ROS. Micro-arrays studies of the salt tolerant species *Populus euphratica* Oli. identified transcripts significantly upregulated by salt stress including ionic and osmotic homeostasis elements such as a magnesium transporter-like protein, a syntaxin-like protein, seed imbibition protein and plasma membrane intrinsic protein, metabolism regulators like cytochrome P450, zinc finger protein, cleavage factor and aminotransferase, and the photosynthesis-activating enzyme Rubisco activase and photorespiration-related glycolate oxidase. Several photosynthesis-related transcripts were down-regulated in response to 72 h of salt stress but were upregulated after long-term recovery (48 h). Sucrose synthase, an ABC-transporter, calmodulin, and aquaporin appeared to be actively involved in the process of plant recovery from salt stress. Several transcripts encoding proteins of unknown function were regulated by salt stress (Gu et al., 2004). In rice cDNA-arrays comparison between the salt tolerant line Nona and the salt sensitive line IR28, showed earlier and strong expression of genes related to transcription factors and signal transduction under salt treatment (Chao et al., 2005). Differences could be seen in the regulation of gene correlating with salt tolerance in different species. However, many authors seem to agree to the importance of earlier perception and transmission of signals in the tolerance to stress namely salt stress (Kawasaki et al, 2001; Chao et al., 2005).

1.5 Transcription factor and signal transcription.

Adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, transcription, and the expression of specific stress-related genes and metabolites. These activated stress response mechanisms re-establish ion homeostasis and protect and repair damaged proteins and membranes (Fig. 1.2) (Ashraf, 1994).

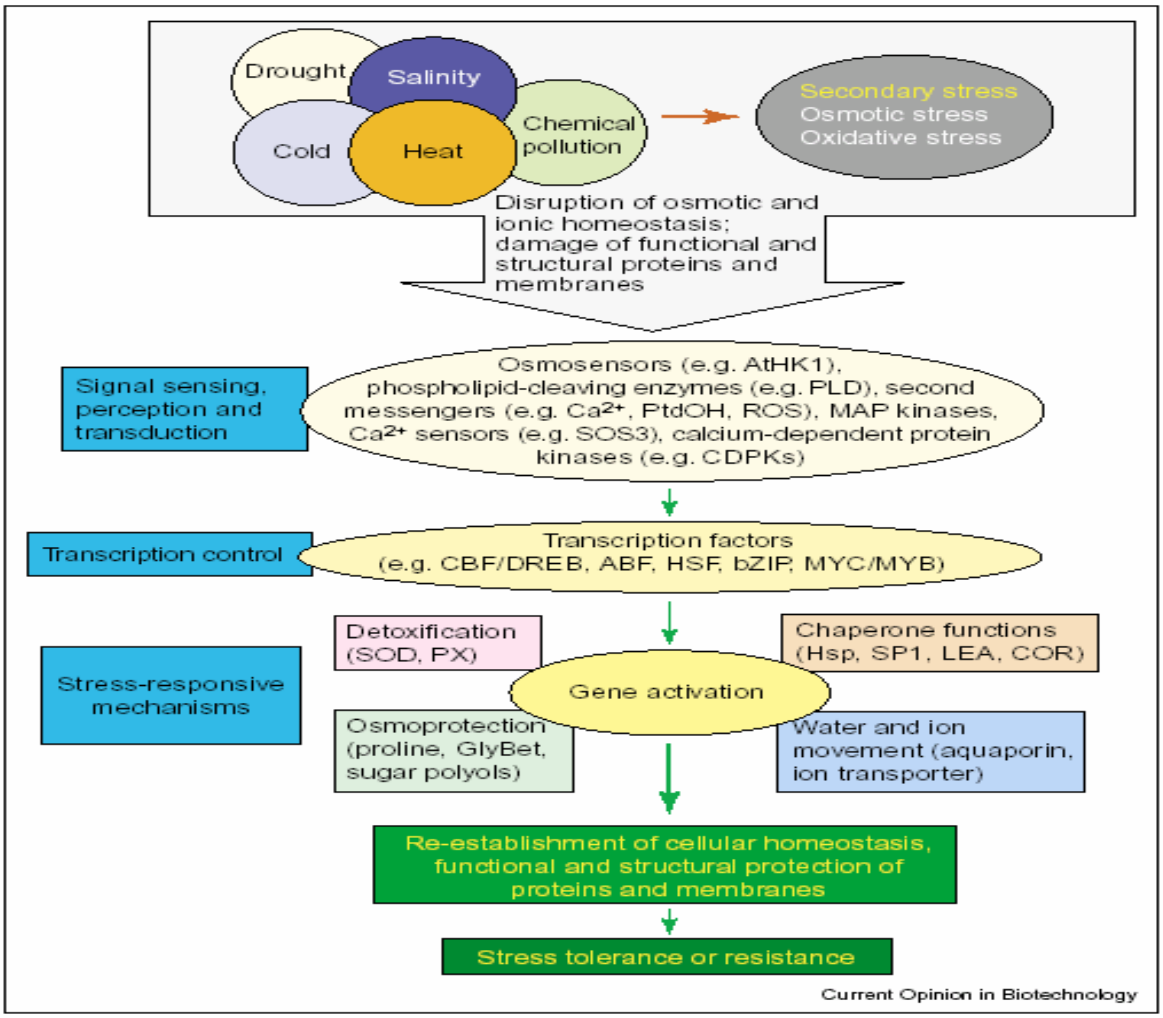

Fig. 1.2 The complexity of the plant response to abiotic stress (Wang et al., 2003). Primary stresses, such as drought, salinity, cold, heat and chemical pollution, are often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects or changes in temperature or membrane fluidity) trigger the downstream signaling process and transcription controls, which activate stress-responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes. Inadequate responses at one or more steps in the signaling and gene activation process might ultimately result in irreversible changes in cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death. Abbreviations: ABF, ABRE binding factor; *AtHK1*, *Arabidopsis* thaliana histidine kinase-1; bZIP, basic leucine zipper transcription factor; CBF/DREB, C-repeat-binding factor/ dehydration-responsive binding protein; CDPK, calcium-dependent protein kinase; COR, cold-responsive protein; Hsp, heat shock protein; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PtdOH, phosphatidic acid; PX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SP1, stable protein 1.

Several successful approaches to improve salt tolerance through the genetic engineering of specific genes belong to genes involved in signalling cascades and transcriptional control such as mitogen activated protein (MAP). Overexpression of the tobacco *NPK1 (Nicotiana* protein kinase 1) gene encoding an active form of protein kinase enhances the freezing, heat and salt tolerance in transgenic maize plants (Shou et al., 2004). The activated T/DSOS2 protein kinase (Salt overly sensitive) enhances salt tolerance in *Arabidopsis* transgenic plants (Guo et al. 2004). Overexpression of the rice transcription factor dehydration-responsive element binding protein (*OsDREB1*) in rice induced growth retardation but increased tolerance to drought, high salt and cold stress and enhanced the contents of proline and sugars in transgenic plants (Ito et al., 2005). Taken together these studies showed the determinant role of transcription factor and signals transduction in the mechanism of salt tolerance. Transgenic integration of these kinds of genes might improve crop plants.

The constraint salinity is a complex stress that implicates different adaptation responses of which several were above-stated. This study aimed to analyse the molecular mechanisms of salt tolerance in rice. To better understand these mechanisms, processes important for ion homeostasis under salt stress including ion accumulation as well as the gene regulation of the vacuolar ATPase, the Na^+/H^+ antiporter NHX, and a water channel, Cl^- channel and K^+ channel were investigated and compared in two rice lines: the salt sensitive line IR29 and the salt tolerant line Pokkali. Futhermore as ion accumulation, expression of the subunit B of the vacuolar ATPase, the Na^+/H^+ antiporter NHX1, water channel were investigated in the halophyte *Festuca* and the crop species wheat (*Triticum aestivum*). Considering that the tolerance to salt is complex and implicates the regulation of several other genes with determinant role in salt adaptation, rice- and *Festuca* cDNA-arrays using 192 and 480 genes were generated and hybridization patterns were compared under different salt concentration and different time of stress. Two genes (a translation initiation factor and a protein kinase) determinant in the tolerance to salt in *Festuca* were inserted and analysed in the rice sensitive line IR29.

2.1 Plant material

This research was concentrated on two rice (*Oryza sativa* L.) lines: IR29 and Pokkali. IR29 is a rice (indica) variety with a short stature; 55-80 cm long grain. However it has a good yield potential, IR29 is sensitive to salt. Pokkali is a rice (indica) variety highly salt tolerant. It commonly grows in costal areas of Kerala, India. Traditionally tall, it is photoperiod sensitive, presents long, broad, and droopy leaves. Its pericarp is red with a poor grain quality. Highly tolerant to salt stress, it produces vigorously growing seedlings with low yielding ability (Gregorio et al., 1997). For better understanding the molecular mechanism of salt tolerance, rice lines were compared with wheat (*Triticum aestivum)* and the halophyte grasses *Festuca rubra* ssp. *litoralis* and *Puccinellia distans* found in the European coast influenced therefore by the enrichment of sodium chloride. These species share with rice an actin-based relationship higher than 88% (Fig.2-1). Seeds of rice (IR29 and Pokkali) were obtained from International Rice Research Institute (IRRI; Laos Banos, Philippines).

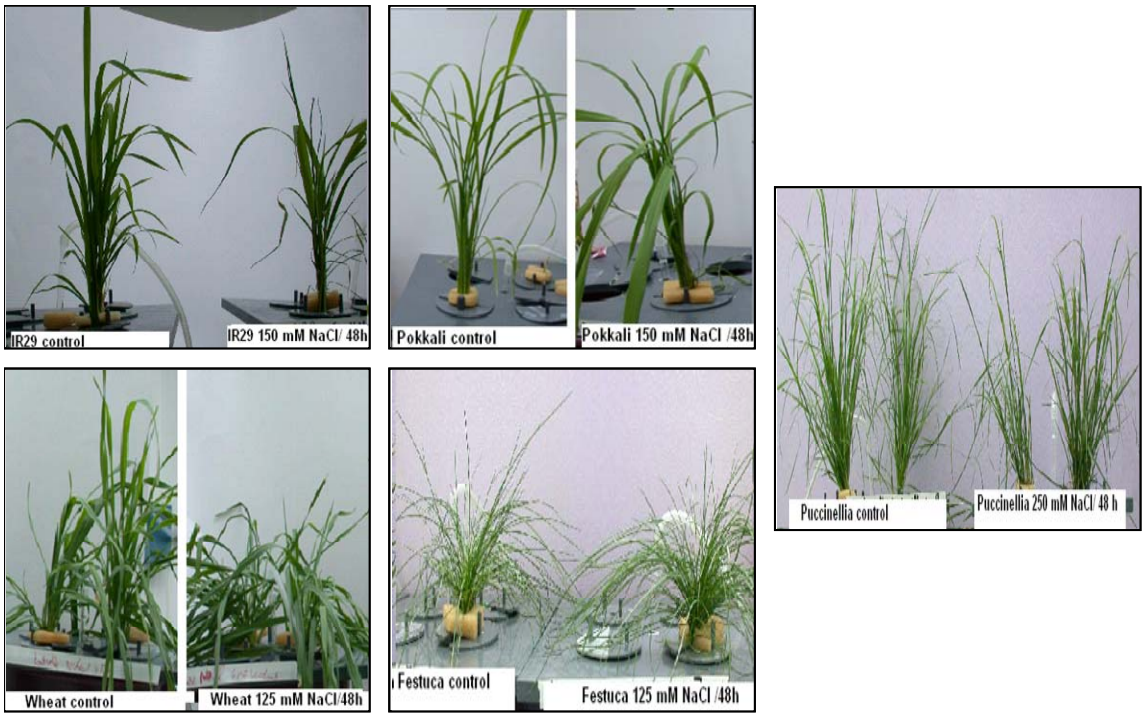

Fig. 2-1 Different species approached in this study exposed at control or salt stress conditions. IR29 and Pokkali: 150 mM NaCl, Festuca and wheat 125 mM NaCl and Puccinellia: 250 mM NaCl.

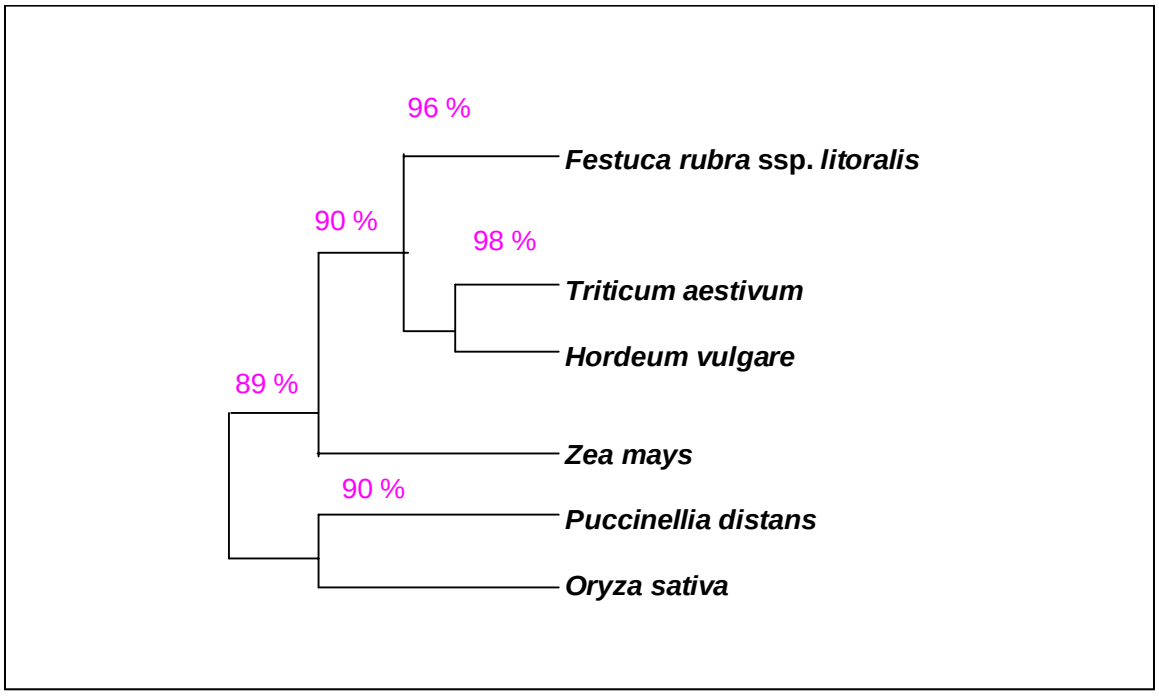

Fig. 2-2 Actin-based relationship of some crops and grasses.

2.2 Growth conditions and stress application

Non-dehusked seeds of all the species were germinated on two layers of filter paper (Whatman no 2) in sand. Moistened with Hoagland´s medium, the seeds were placed in a growth chamber at 25 /21°C under a 12-h daylight period. The plantlets were transferred to aerated hydroponic tanks containing 5 liters of nutritive solution (Hoagland medium) and grown for 3 to 8 weeks. The nutritive solution was renewed two times in a week. Stress conditions were 125 and 150 mM NaCl for rice, 125 mM NaCl for wheat and 125, 250, 500 mM NaCl for *Festuca* and *Puccinellia*. The salt constraint was applied after 3 weeks on rice and wheat and after 8 weeks on *Festuca*. Non-stressed plants were grown in parallel and harvested at the same time and served as a control. Plants were collected after 3, 6, 24 and 48 h of exposure and stocked at -80°C.

2.3 Nucleic acids extraction

2.3.1 RNA extraction

2.3.1.1 Caution in RNA extraction

Because of the risk of RNase contamination, working with RNA needs many cautions. Many sources of contaminating RNase exist: plasmid preps and nuclease protection assays, bacterial cultures, bacterial products such as some restriction enzymes, microorganisms in the air, on surfaces, solutions or water supply are environmental sources of RNase, other sources

include human skin and body fluids such as saliva, tears and mucus. Because RNases exhibit such a common presence in routine lab research, care should be taken. It is recommended to wear gloves when handling any reagents or reaction vessels, to bake glass-materials for 14 h at 180 °C, to incubate plastic-materials for 2 h in 0.1% NaOH and wash afterwards several times with DEPC water or to autoclave it and to treat all solutions with 0.1 % DEPC (diethylpyrocarbonate) water and afterwards to autoclave them (Chomczynski, 1992).
RNA was obtained using two methods: Acid guanidium thiocyanate-phenol-chloroform method and Trizol method

2.3.1.2 Acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacci, 1987)

Roots and shoots were separately frozen in liquid nitrogen and ground in a pre-chilled mortar. The tissue powder was transferred using a pre-cooled spatula into 50 ml-Falcon tubes with 10 ml of RNA extraction buffer (4 M guanidium thiocyanate-phenol-chloroform, 25 mM sodium citrate, 0,5% laurylsarcosyl and 0.1 M ß-mecaptoethanol) that facilitates cell lysis and the inhibition of RNases. After 30 seconds mixing, three reagents were added separately each one by 30 s vortexing:

1 ml of 2 M sodium acetate, pH 4.0,

10 ml of water-saturated phenol.

2 ml of chloroform: isoamyl alcohol (24:1).

The organic phase and RNA were separated by 15 min centrifugation at 2300 rpm. The upper phase containing RNA was mixed with isopropanol and kept overnight at -20°C. RNA was then precipitated by 30 min centrifugation at 6000 rpm at 4°C. The pellet was diluted in a half of RNA extraction buffer and mixed again with isopropanol. RNA was again precipitated by centrifugation during 30 m at 6000 rpm at 4°C after incubation at -20°C for 1 h. The obtained RNA pellet was washed with 10 ml of ice cold ethanol (75%) and by centrifuging 5 minutes at 4°C and resuspended with RNase free water (DEPC water), then kept at -80°C for further experiments.

2.3.1.3 Trizol method

Trizol- Reagent (Gibco-BRL, Karlsruhe Germany) is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol/guanidine-isothiocyanate/chloroform, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). The advantage of this

reagent is to maintain the integrity of the RNA during sample homogenization or lysis, while disrupting cells and dissolving cell components. Different steps constitute this method (Sambrock et al., 1989). Leaf or root tissue was ground in liquid nitrogen in a chilled motar and pestle. 50 to 100 mg of the powder was mixed with 1 ml of Trizol. After vortex, the sample was incubated for 5 min at room temperature and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and incubated for 5 min at room temperature (RT). 0.2 ml of chloroform was added and tubes were vigorously shaked by hand for 15 s and incubated at RT for 3 min. The obtained supernatant after centrifugation at 10000 rpm for 10 min was mixed in a fresh tube with 500 µl isopropanol and incubated for 10 min at RT and centrifuged before spin down at 10000xg for 10 min at 4°C. The RNA pellet obtained was washed once with 750 µl 75% ice cold ethanol (in DEPC treated water), centrifuged at 10.00xg for 5 min at 4°C, was briefly dried (5 min) and dissolved in an appropriate amount of DEPC treated water (heating to 65°C is likely to be necessary but should be as short as possible).

2.3.2 DNA extraction

2.3.2.1 Extraction of DNA from plant material

DNA was isolated according to the method of Edwards et al. (1991).

Plant materials were destroyed with plastic pestle for about 10 s or ground to powder in liquid nitrogen. Destroyed tissues were quickly mixed with 500 µl of Edwards's extraction buffer and after vortexing and 2 min spinning down, 300 µl of supernatant was mixed with 310 µl isopropanol. The mixture was vigorously shaken and allowed to precipitate for 2 min at root temperature and centrifuged for 7 min. The obtained pellet was dried for 10 to 15 min and re-suspended in 100 µl TE buffer.

Edwards extraction buffer: 200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS.

2.3.2.2 Extraction of plasmid DNA

The method used here is modified from Sambrock et al. (1989).

A single colony of *E.coli* or 10 µl of previously frozen cells containing the plasmid of interest were inoculated in 5 ml LB medium with 50 µg/ml Kanamycin. The mixture was incubated overnight at 37°C with shaking at 150 rpm. The overnight *E.coli* culture was spinned down for 1 min at 13.000 rpm in a microfuge tube, and the obtained pellet was resuspended with 100 µl of the lysis buffer solution I (1 M Tris-HCl pH 8, 0.5 M EDTA and 1 M glucose).

After 5 min incubation at RT, plasmid and chromosomal DNA were denatured with adding 200 µl of fresh solution II (10 N NaOH, 20% SDS), homogenized by inversion and incubation for 3 min at RT. Plasmid DNA was again renatured by adding 150 ml 3 M sodium acetate pH 5.2 and 150 µl ammonium acetate (10 M) successively mixed by inversion. 600 µl of the supernatant obtained after 5 min incubation in ice and centrifugation at 13000 rpm for 5 min at 4°C was mixed with 750 µl of isopropanol and spinned down at 13000 rpm at 4°C. The pellet was resuspended with 100 µl TE (1 M Tris-HCl, 0,5 M EDTA) and 250 µl ethanol was added and mixed by vortex for 15 s, incubated for 10 min at RT and centrifuged 10 min at 4°C. The new pellet was resuspended with TE (pH 8.0) and 100 µl 4 M LiCl. The resuspended cells were incubated for 2 h in ice and about 200 µl of solution obtained after centrifugation (10 min at 4°C) was mixed with 400 µl cold ethanol and a new pellet was collected by centrifugation (10 min at 4°C). The DNA was dissolved with 25 µl TE (pH 8.0) and kept at -20°C.

TE buffer: 10 mM Tris-HCl, 1mM EDTA

2.3.2.3 RNA and DNA quantification

RNA and DNA quantification is an important and necessary step prior to most RNA or DNA analysis methods. Total RNA or DNA were extracted from 3 or 6 -week-old seedlings (rice and *Festuca*) according the above-mentioned method and the concentration was measured using the Gene Quant (Amersham-Pharmacia-Biotech, Freiburg) UV spectroscope. The absorbance of a diluted RNA sample (2 µl of total RNA in 200 µl sterile water) was measured at 260 and 280 nm. An A_{260}/A_{280} ratio of 1.8 to 2.1 is indicative of highly purified RNA (Sambrock et al., 1989). The nucleic acid concentration was calculated according to the following equation:

Total RNA (µg/µl)= A_{260} reading number x 40 µg/ml x dilution factor,

Total DNA (µg/µl)= A_{260} reading number x 50 µg/ml x dilution factor.

An A_{260} reading of 1.0 is equivalent to ~40 µg/ml single-stranded RNA and ~50 µg/ml double-stranded DNA. The isolated RNA may be checked by 1% agarose formaldehyde denaturing gel electrophoresis. If two strong RNA bands were visible (25 S rRNA and 18 S rRNA), the RNA can be used for Northern blotting, PCR or cDNA hybridization (Sambrock et al., 1989).

2.4 Nucleic acids analysis

2.4.1 Northern hybridization.

2.4.1.1 Technique of Northern blot

To quantify the expression of researched genes, Northern blot analysis was used. This technique is characterized by the transfer or blotting of electrophoretically separated RNAs from a gel to a filter membrane for subsequent fixation and hybridization to a specific DNA probe. 20 µg total RNA was mixed with RNA denaturizing buffer and separated 30 min in agarose gel electrophoresis and then transferred to a solid support (nylon membrane Sigma) through capillarity transfer and covalently linked to the support: The membrane was dried and baked at 80°C for 2 h and then fixed by exposition to 0,13 J/cm² UV-light.

2.4.1.2 Hybridization

The fixed nylon membrane was first incubated in 10 ml DIG Easy Hyb solution (Roche) in roller tubes at 42°C for 2 h. This prehybridization allows to block the non-specific RNA. In 10 ml Dig Easy Hyb solution containing DIG labeled probe the membrane was incubated overnight at 42^{o}C. This step allows specific hybridization of the base pairs. Probes were removed by two washes in 2x SSC, 0,1% SDS for each 15 min at room temperature, however, non-specific probes were removed by two washes in 0.5x SSC, 0.1% SDS for each 15 min at 42^{o}C. To reduce the background, the membrane was incubated for 30 min in 10 ml of blocking solution (Roche). For future detection anti-digoxiginin-alkaline phosphatase conjugated Fab antibodies were added in fresh blocking solution in which the membrane was incubated for 30 minutes. After two washes with washing buffer by discarding the blocking solution, the membrane was equilibrated with the detection buffer and as a substrate, the chemiluminescence substrate CSPD (Roche) was added. The membrane was incubated for 15 min at 37°C and covered with X-ray film for different incubation times (30 min, 1h, 3h, 5h) Data analyses were performed using the Gelscan software (INTAS, Germany).

Stock solutions used in the hybridization:

Washing solutions: 2x SSC, 0.1% SDS and 0,5x SSC, 0.1% SDS,

Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl , pH 7.5,

Blocking buffer: 5 g blocking reagent in 45 ml maleic acid buffer,

Blocking reagent working solution: 1 ml blocking reagent stock solution diluted in 9 ml **maleic** acid buffer

Anti-DiG solution: 1 µl/5ml (anti-DIG alkaline phosphatase/blocking buffer)

Detection buffer: 1 M Tris HCl, 5M NaCl, pH 9.5 and add 1M $MgCl_2$

2.4.2 RT-PCR (reverse transcription-polymerase chain reaction)

The method of RT-PCR is a powerful technique used to quantify the expression of genes. It is simple and fast and was used as alternative to the other gene quantification methods like Northern blotting. In contrast to Northern blot, the RT-PCR uses cDNA and needs specific primers. Despite its simple use, RT-PCR presents two big disadvantages which recommend verification by other methods. The first inconvenience of RT-PCR is related to the fact that cDNA can be amplified only with partial lengths ranging from 200 to 1000 base pairs (bp). The second disadvantage is that annealing is sometimes not specific and amplifies non-specific sequences called “artefacts”.

2.4.2.1 cDNA synthesis

cDNA is a DNA copy synthesized from mRNA. The enzyme used is the reverse transcriptase RNA-dependent DNA polymerase isolated from a retrovirus (AMV or MMLV). As other polymerases, a short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the polymerase. This is provided by the poly (A) tail found at the 3' end of most eukaryotic mRNAs to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized poly (A).Together with all 4 deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesises a complementary DNA on the mRNA template.

In this work, 20 µg RNA for the cDNA arrays and 5 µg RNA for gene expression using RT-PCR were used. The cDNA synthesis procedure consists of adding 20 or 5 µg RNA to 2 µl oligo dT (500 µg/ml) primer in a total volume of 13 µl. After briefly mixing and centrifugation (2 s) RNA was denatured by heating 10 min at 70°C and quickly chilled on ice for 2 min. After brief centrifugation, 6 µl of 5x First Strand Buffer (Sigma) was added for maintaining a favourable pH, 3 µl of 0.1 M DTT for stabilising the strand and 5 µl DIG-dNTP for the synthesis. The mixture was briefly centrifuged and then incubated at 42°C for 2 min before adding 0.3 µl of SuperScript RT II. The enzyme (Invitrogen Netherlands) was mixed to the solution by gently pipeting five times up and down and incubated at 42°C for the synthesis. The reaction was stopped after 1 h and the enzyme was inactivated by incubating at 70°C for 15 min. The obtained cDNA was kept on ice for 2 min, centrifuged briefly and stored at -20°C for further study.

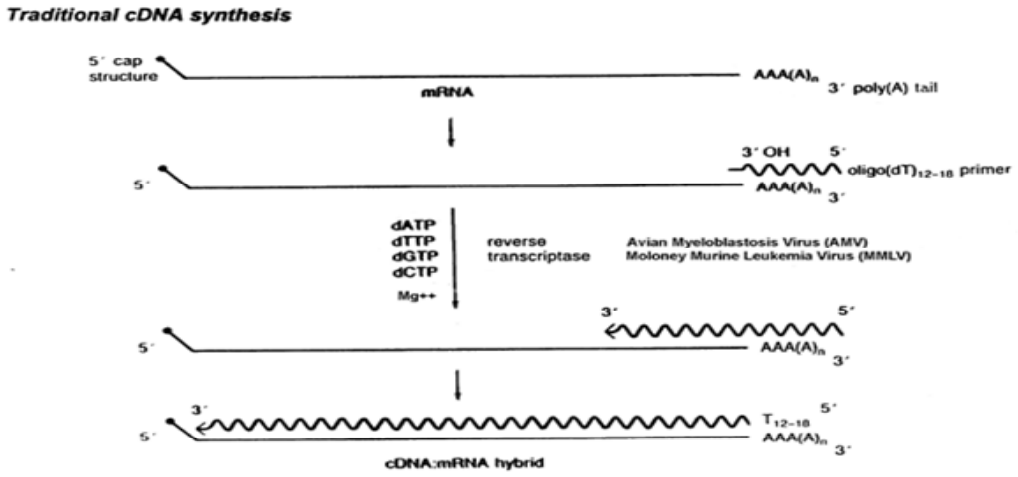

Fig. 2-3 synthesis of the first strand of cDNA using an oligo (dT) primer and reverse transcriptase (Clontechniques)

2.4.2.2 Control of cDNA-synthesis

Before using cDNA for other experiments, the transcript abundance of the obtained cDNA was controlled either in a test-actin PCR using 1 µl of cDNA synthesis reaction or by estimating the yield of DIG-labelled cDNA probe.

The Estimating method is made after purifying the cDNA

2.4.2.2.1 Purification of cDNA using the QIAquick PCR Purification Kit.

5 x Xµl cDNA of PB buffer (Quiagen) was added to the cDNA. The mixture was shaked and introduced to the QIAquick spin column and collected after 1 min centrifugation at 6000 rpm. The collected flow-through was discarded and cDNA was washed with 750 µl PE buffer. PE buffer was removed by 1 min centrifugation at 6000 rpm. Another centrifugation was performed to remove PE buffer completely from the column. To elute the cDNA, 50 µl of sterile water (preheated to 70°C) was added and after 1 min at RT the eluted cDNA was collected by centrifugation for 1 min at 13000 rpm.

2.4.2.2.2 Estimating the yield of DIG-labelled cDNA probe

On a piece of nitrocellulose membrane 1 µl of each control DNA (1/10 dilutions) was pipetted and in another row 1 µl of reference cDNA (1/10 dilutions) was pipetted. The membrane was then dried and fixed at 0.14 J/cm² UV-light. After a brief wash on maleic (section 2.4.1.2) acid buffer, the membrane was incubatd first for 30 min in 5ml blocking buffer (section 2.4.1.2) on a shaking platform and for 30 min in 5 ml anti-DIG solution by

shaking. While shaking, the membrane was washed twice for 15 min in maleic acid buffer (section 2.4.1.2). After 2 min incubation in 5 ml detection buffer (section 2.4.1.2**)** the membrane was placed in the color substrate solution and incubated in the dark until the spots appeared. The reaction was stopped by incubating the membrane in water. The yield of DIG-labelled cDNA was estimated by comparing the intensities of the sample spots to the control DNA

Color substrate solution: 100 µl NBT/BCIP, 5 ml freshly prepared detection buffer

2.4.2.2.3 PCR

The PCR is a powerful technique used to amplify a specific DNA sequence millions times in a few hours. To perform a PCR reaction, a small quantity of DNA is added to buffered solution (10x PCR buffer) containing DNA polymerase (Taq), two short oligonucleotide primers and four desoxynucleotides (dNTPs) and the cofactor $MgCl_2$.

10x PCR-buffer: 160 mM $(NH4)_2SO_4$, 15 mM $MgCl_2$, 670 mM Tris-HCl, pH 8 , 0,1% Tween 20

PCR assay	**Final concentration**
Sterilized distilled H_2O	40 µl
10x PCR buffer	5 µl
dNTPs	1 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq Polymerase	1 µl
Template DNA	1 µl
Total volume	50 µl

PCR program used in this work:

Step 1	94°C	1 min 30		1 cycle
	94°C	1 min	(denaturation)	
Step 2	55°C	1 min	(annealing)	40 cycles
	72°C	2 min	(polymerisation)	
Step3	72°C	10 min	(last polymerisation)	1 cycle

2.4.2.2.4 Specific primers used in this study

PCR reaction was performed with the gene specific oligonucleotide primers. The following sequence specific forward and reserve oligonucleotide primers were used for PCR amplifications and for the synthesis of DIG labelled probe:

According to the primers, different annealing temperature, and different cycles have been used.

VHA-B (48-50°C : 33-36 cycles), *OsSPK3* (52°C : 30-35cycles), *OsNHX1* (55-50°C : 50 cycles), Actin (55°C : 28-30 cycles).

The other genes were annealed at 55°C for 30-35 cycles.

OsCLC1, *OsHAK7*, catalase, ABC-transporter, dehydrine, oxidoreductase and *OsTIF*

5´-TGTACAAGCAGGACTGG-3´ and
5´-AGATAGGCCTTCACCTCA-3´ (***OsCLC1***),

5´-ATCTTCAATGGATTC-3´ and
5´-TGCATCCATCCCAACATA-3´ (***OsNHX1***),

5´-ATTGACAGGCA GCTGCAT-3´and
5´-GCAATGTCCATGCTAGT-3´ (***OsVHA-B***),

5´-GTGATCTCCTTGCTCATACG-3´ and
5´-GGNACTGGAATGGTNAAGG-3´ (**Actin**).

5´- GTCATGTACGTATGGCAC-3´and
5´-ACGATGCACCGGTACATA-3´ (***OsHAK7***)

5´- GGTGGCATGATCTTCRTYC-3´ and
5´- TGATGCCGGTKCCGGTGA-3´ (***OsPIP2;1***)

5'-ATGGAGAAGTACGAGGCGGTGAGGGA-3' and
5'-TGCGCAGTGAGCTCATACGGAG-3' (***OsSPK3***),

5'-ATGTCTGATCTCGACATTCAGATCCC-3'and
5'-GAAACCATGAATCTTGAT-3' (***OSTIF***),

5´-GGATGACACCAAGACATG-3´and

5´-TCACGTTGAGCCTATTCG-3´ (**Catalase**)

5´ TACTGTGCACCAGAGAAG -3´and

5´- CTCTCCATGGCATCTGT-3´ (**Oxidoreductase**)

5´-CGTGATGGGAATGGAGG-3´and
5´-CCATGAAGCCCTTCTTCTC-3´ (**Dehydrine**)

5´-TGTCTGCTCAGGATGATG-3´and
5´-CCTCGTTCCATCTTAGCA-3´ (**ABC-transporter**),

5´-TCAGCTGACATGGACATG-3´and
5´-CTACTTGTCCACTTCCTC-3´ (**OsDPCS**) (pyrroline-5-carboxylate synthetase)

2.4.2.2.5 Analysis of PCR products

The PCR products were separated on 1.7 % (w/v) agarose gels and stained with ethidium bromide. Photographic images were obtained with a gel documentation system (INTAS, Göttingen, Germany). Densitometric analyses were performed with the Gelscan software (INTAS, Germany).

2.4.3 *In situ* PCR

The *in situ* PCR allows identifying the tissue specificity of gene expression. Leaf sections of rice lines IR29 and Pokkali as well as *Festuca* treated respectively with 150 mM NaCl and 500 mM NaCl were fixed with FAA, dehydrated, and embedded with Paraplast Plus (Fisher Scientific) (Golldack et al., 2002b). 12 µm microtom sections of control and salt treated conditions were mounted on microscopic slides coated with aminoalkylsilane (silane-prep slides, Sigma, Germany). The tissue sections were de-paraffinized and re-hydrated, treated with Proteinase K and RNase free DNaseI (Popova et al., 2003). cDNA was synthesized with oligo-dT-primers and Superscript RT II (Invitrogen, Netherlands) as described in section

2.4.4 Isolation of transcripts

Conserved regions of *PIP2;1, NHX1* and *VHA-B* genes from *Triticum aestivum, Hordeum vulgare, Zea mays* and *Oryza sativa*, respectively, were identified by sequence alignments. The sense and the antisense oligonucleotide primers 5´-3´and 5´-3´ sequences described were generated and used for PCR amplifications from cDNA of *Festuca*. Single PCR products were obtained for ESTs of *FrPIP2;1 FrNHX1*, and *FrVHA-B*. The sequencing of the ESTs was performed by SeqLab and MWG (Germany). For the alignments, the following

Accession-No.: L11862), *OsVHA-B* (Oryza sativa , AF375052), *ZmVHA-B* (*Zea mays*, AY104180), *TaVHA-B* (*Triticum aestivum*, TC2649885), *McVHA-B* (*Mesembryanthemum crystallinum*, AJ438590), *GhVHA-B* (*Gossipium hirsutum*, U07052), *LeVHA-B* (*Licopersicum esculentum*, BT013016), *CuVHA-B* (*Citrus unshiu*, AB024277), *SmVHA-B* (*Suaeda maritima*, AY231438); PIP2;1 homologues: *HvPIP2;1* (*Hordeum vulgare*, AB009307), *TaPIP2;1* (*Triticum aestivum*, AF139815), *ZmPIP2;1* (*Zea mays*, AY243801), *OsPIP2a* (*Oryza sativa*, AF062393), *CpPIPC* (*Craterostigma plantagineum*, AJ001294), *AtPIP2A* (*Arabidopsis thaliana*, NM_115202), *BnPIP2;1* (*Brassica napus*, AF118383), *PtPIP2;1* (*Populus tremula*, AJ849324), *PcPIP2;1* (*Pyrus communis*, AB058678), *TbPIP2;1*(*Triticum, boeoticum*, AF388171), *McMIPC* (*Mesembryanthemum crystallinum*, MCU73466), *NtPIP2* (*Nicotiana tabacum*, CK720599), *RcPIP2;1*(*Ricinus communis***,** AJ605575), *JrPIP2;1* (*Juglans regia*, AY189973), *MtPIP2;1* (*Medicago truncata*, AY059380); NHX-type transporters: *HvNHX1* (*Hordeum vulgare*, AB0891979, *TaNHX1* (*Triticum aestivum*, AY040245), *TaNHX2* (*Triticum aestivum*, AY040246, *TheNHX1* (*Thinopyrum elongatum*, AF507044), *TheNHX2* (*Thinopyrum elongatum*, AY357107), *OsNHX1* (*Oryza sativa*, AB021878), *ZmNHX4* (*Zea mays*, AY270039), *ZmNHX5* (*Zea mays*, AY270040), *AtNHX1* (*Arabidopsis thaliana*, AY685183), *AtNHX2* (*Arabidopsis thaliana*, AF490586), *MsNHX1* (*Medicago sativa*, AY456096), *AgNHX1* (*Atriplex gmelini*, AB038492), *CgNHX* (*Chenopodium glaucum*, AY371319), *SeNHX1* (*Salicornia europea*, AY1312359, *McNHX* (*Mesembryanthemum crystallinum*, AF279670)

2.4.3 cDNA -array establishment

The technique of array Hybridization (Micro- and Macro-arrays) is a powerful technique for the simultaneous analysis of multiple transcripts on a single slide of membrane. The common use of this technique is in our study to determine which genes are activated or repressed under sodium chloride, lithium chloride and potassium starvation stress conditions. Different steps were performed:

2.4.3.1 Synthesis of DIG-labeled probes.

cDNA-array hybridizations become possible with the establishment of cDNA-labelled probe using Digoxiginin-11-dUTP. Compared to radioactive probe $[^{32}P]^{-}$, the probe with DIG-dUTP present two major advantages. It is simply used and can be reused many times after storage at -20 °C. The synthesis of a Digoxininin-labeled probes follow the same steps of a

standard PCR with the only difference of the use of Digoxiginin 11- dUTP in addition to non labelled dNTPs.

DIG-PCR assay (50 µl)	**Final concentration**
Sterilized distilled H_2O	34.5 x µl
10x PCR buffer	5 µl
DIG-11-dUTP dNTP mix	2.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq Polymerase	1 µl
Template cDNA	5 µl
Total volume	50 µl

2.4.3.2 Hybridization

The cDNA labelled probe (1 µl) was denatured at 100°C (10 min), fixed for 3 min in ice and mixed to 10 ml of the DIG easy hyb solution (Roche). cDNA hybridization used the same procedures of Northern blotting hybridization (section 2.4.1.2).

2.5 Transformation of rice mediated by *Agrobacterium*

Generation of cDNA-Arrays and of the constructs for *OsTIF* and *OsSPK3* for plant transformation was performed by Golldack and Popova (non–published).

2.5.1 RNA isolation and construct of subtraction cDNA-library

RNA used for cDNA-labrary was isolated as described in section 2.3 and mRNA was isolated using the polyATract kit (Promega, Mannheim, Germany). Synthesis of the subtraction cDNA-libraries of rice and *Festuca* was performed using the PCR-Select Kit (Clontech, Heidelberg, Germany) according to the manucfacturer´s protocol. Same amounts of mRNA from the following salt stress treatment of *Festuca* were pooled for the tester cDNA: 125 mM NaCl, 250, 500 for 6 h, 24 h, 48 h, and 7 days at the age of 6 and 12 weeks each leaf and root tissue. For generation of the rice cDNA-array, mRNA from the rice line IR29 and the rice line Pokkali were stressed with 150 mM NaCl for 6, 24 and 48 h. cDNA was synthesized for leaf and root tissue pooled for IR29 and Pokkali, respectively, and IR29 and Pokkali cDNA were used both as tester and driver in the subtraction procedure. For *Festuca*, same amounts of mRNA obtained from control plants of the same developmental

stage and harvested in parallel to the stressed plants were pooled for the driver cDNA. The subtracted cDNA was cloned into the vector PCR-Top II (Invitrogen, Karlsruhe, Germany) and the inserts were amplified by PCR using the primers1 and 2R (Clontech Heidelber Germany). PCR products were analysed on agarose gels and products that yielded single bands were selected for future procedures.

2.5.2 Preparation of cDNA-Arrays and labelling of probes

PCR product from the subtraction cDNA-library was purified using QIAquick spin columns (Qiagen, Hillden, Germany) and dissolved in 50 % (v/v) DMSO. cDNA-arrays were generated at the center of the Genome research (ZFG) at the University of Bielefeld. For *Festuca ,* 480 PCR products and for rice 192 products were transferred in duplicates to nylon membranes (Hybond-N, Amersham Pharmacia Biotech, UK) in a 3 x 3 pattern leaving an empty spot in the middle for local background subtraction. The membranes were fixed by baking and UV cross-linking. Labelled probes were prepared from each 25 µg RNA by incorporation of digoxigenin-11-dUTP (Roche, Mannheim, Germany) during the first strand cDNA synthesis using SuperScript II (Invitrogen) and oligo(dT)-priming. The probes were purified using QIAquick spin columns (Qiagen, Hillden, Germany) to remove unincorporated nucleotides. Prior to hybridization, the probes were denatured at 100^{o}C for 10 min.

2.5.3 Hybridization and data analysis.

The cDNA-Arrays were prehybridized in DIG Easy Hyb solution (Roche) for 2 h at 42^{o}C. Hybridization with digoxigenin-labeled probes was performed in the same buffer at 42^{o}C overnight followed by two washes in 2x SSC, 0,1% SDS for each 15 min at room temperature and two washes in 0,5x SSC, 0.1% SDS for each 15 min at 42^{o}C. Signal detection was performed with anti-digoxiginin alkaline phosphatase conjugated Fab fragments and CSPD (Roche) as substrate. Data analyses were performed using AIDA Array Vision and AIDA Array compare software (Raytest, Germany) Filters were normalised to actin and tubilin, respectively that were included as internal control gene. For identification of differential expression of clones, induction factors (IF) were calculated with thresholds set to IF>1.8 indicating upregulation of gene expression and IF< 0.5 indicating repression of expression. Reliability of results was verified by Northern analysis of selected differentially expressed clones for *Festuca*-array.

2.5.4 Generation of constructs and transformation of rice

Open reading frame cDNA of *OSTIF* (AF094774) and *OsSPK3* (AP003286) was amplified by RT-PCR (section 2.4.2) from rice RNA (variety IR29) with the following oligonucleotide primers:

5'-ATGGAGAAGTACGAGGCGGTGAGGGA-3',

5' GCGCAGTGAGCTCATACGGAG-3', respectively, (*OsSPK3*),

5'–ATGTCTGATCTCGACATTCAGATCCC-3'

5'-GAAACCATGAATCTTGAT-3', (*OSTIF*), respectively.

The sequences were cloned for overexpression and for overexpression with a C-terminal GFP-fusion derived from the vector under the control of the constitutive CaMV 35S promoter (Hajdukiewicz et al., 1994; Goossens et al., 2003). *Agrobacterium tumefaciens* strain LBA4404 was transformed with the constructs and used for transformation of rice IR29.

2.5.4.1-Detection of activity GFP reporter gene associated with researched gene

2.5.4.1.1 Preparation of competent *E. coli* cells

An overnight culture of *E.coli* DH5 α, was inoculated to 50 ml fresh LB medium and incubated by shaking at 37°C until 1,2.10^6 cells/ml (A600-0.25). Cells were harvested after 5 min incubation in ice and centrifugation at 2500 rpm at 4°C for 5 min. In 15 ml pre-chilled (T+B I) the sediment was resuspended and centrifuged at 2500 rpm at 4°C for 5 min. The new pellet was dissolved with 2 ml pre-chilled T+B II and incubated in ice for 5 min and then frozen in liquid nitrogen before storing at -80°C.

Transformation Buffer II (T+B I) : 30 mM KAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8 with acetic acid

Transformation Buffer II (T+B II): 10 mM MOPS, 10 mM RbCl, 75 mM CaCl2, 15% glycerol pH 7.0 with NaOH

2.5.4.1.2 Transformation of *E.coli* cells.

50µl DH5 α competent cells were thawed on ice and 2-4 µl DNA were mixed by inverting the tube several times. After 30 min incubation in ice, the mixture was warmed at 42°C for 30 s and incubated again in ice for 2 min before 250 µl SOC medium was added and gently mixed by pipetting. The transformation was achieved by shaking (120 rpm) for 1 h at 37°C. Selection to kanamycin was conducted by spreading 30-50 µl of the transformed competent cells onto LB plate containing kanamycin resistance. After incubation overnight at 37°C resistant colonies could be seen.

SOC medium: 2 % (w/v) trytopne 0. 5 % (w/v) Yeast extraction 10 mM NaCl , 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl, 20 mM Glucose.

2.5.4.1.3 Plasmid DNA isolation and purification

One colony of *E.coli* DH5 α resistant to kanamycin was picked and grown overnight in LB medium with kanamycin. The obtained pellet was resuspended by adding 0.4 ml of cell suspension buffer (E1) containing RNase and the plasmid DNA was separated from the genomic DNA by adding a modified alkaline /SDS Buffer (Cell Lysis solution (E2)) and by mixing gently by inverting 5 times. After incubation for 5 min at room temperature, 0.4 ml of Neutralisation Buffer was added and mixed immediately five times by inverting then centrifuged at the 12000 rpm for 10 min, at RT. The supernatant was allowed to drain by gravity flow over a loading column (Qiagen, Hillden, Germany) where the negative charges of phosphate DNA interact with the positive charges of the surface of the resin. RNA proteins and other impurities were removed by washing the column with 2.5 ml of wash buffer (E5). With 0.9 ml of the high salt concentration solution (E6) the plasmid DNA was eluted. The DNA was desalted and washed by centrifugation, respectively, with 0.63 ml isopropanol for 30 min and with 70% ethanol for 5 min at 4°C. The DNA was eluted with 30 µl TE buffer and stored at -20°C. The DNA concentration could now be measured (section 2.3.3)

Cells suspension buffer (E1): 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, RNase A,
Cells lysis solution (E2): 200 mM NaOH, 1% SDS (w/v),
Neutralisation buffer (E3): 3.1 M potassium acetate (pH 5.5),
Equilibration buffer (E4): 600 mM NaCl, 100 mM sodium acetate (pH 5 0), 0.15% Triton X-100 (v/v)
Wash buffer (E5): 800 mM NaCl, 100 mM sodium acetate (pH5.0),
Elution buffer (E6): 1.25 M NaCl, 100 mM Tris-HCl (pH 8.5),
TE buffer (TE): 10 mM Tris-HCl, 0,1 mM EDTA.

2.5.4.1.4 Transient expression in *Arabidopsis*: DNA preparations and PEG-mediated transformation

Histochemical assays to access the expression of the GFP gene associated with the researched gene in *Arabidopsis* tissues was carried out using the PEG method and observed using a fluorescence microscope.

2.5.4.1.4.1 Protoplast isolation

20 leaves of *Arabidopsis* (3-4 weeks old) were cut in small leaf strips (< 0.5 mm) and, submerged in a Petri dish with 5-10 ml enzyme solution and put for 3 min in a vacuum platform shaker. After checking under the microscope, the protoplasts were released by shaking at 80 rpm for 1 min and the enzyme solution containing the protoplasts was filtered with a 0.45 µm nylon mesh. Protoplasts were collected by centrifuging at 800 rpm for 1 min. The pellet was washed in 10 ml W5-solution resuspended in the same solution (10 ml), and incubated in ice for 20 min, then spinned down for 1 min at 800 rpm. The new pellet was resuspended in 5 ml MMG-solution. The aspect of protoplasts in microscope was controlled before PEG transfect ion.

Reagents:

Enzyme solution : 1.5% Cellulase R10, 0.4% Macerozyme, 0.4 M Mannitol, 20 mM MES pH 5.7, 10 mM CaCl

W5 solution 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl

(The enzyme solution was heated at 55°C for 10 min to inactivate the proteases and enhance enzyme solubility and cooled at RT before adding

MMG Solution: 0.1 % BSA Fraction V (Filtered trough 0.2 µm filter) 0.4 M Mannitol, 15 mM $MgCl_2$, 4 mM MES pH 5.7

PEG solution (10 ml): 1 M mannitol (2 ml), 1 M $Ca(NO3)_2$ or $CaCl_2$ (1 ml), 40% PEG 4000, 3.5 ml H_2O

2.5.4.1.4.2 PEG transfection.

Plasmid DNA containing the researched gene associated to GFP was mixed to *Arabidopsis* protoplasts according to the procedure below.

20 µl plasmid DNA (40 µg) were gently and thoroughly mixed into 100 µl of protoplast suspension by smooth movement of the pipette tip as DNA is expelled. 110 µl of PEG solution were immediately mixed by pipetting during 1 min and the all mixture was incubated for 15 min. After this incubation, the mixture was slowly diluted with W5/mannitol by adding 500 µl, 1, 2 and 4 ml at 15 min intervals. Then the washed protoplasts were collected in a sterile falcon tube and incubated for 24-36 hours in the dark at 25°C. GFP-derived fluorescence emission was detected by a Leica confocal laser scanning microscope.

2.6 Production of rice transgenic plants

2.6.1 *Agrobacterium* transformation

Agrobacterium strain (LBA4404) with the desired Ti-plasmid was grown in 50 ml of LB medium at 28°C until the culture reached 0.5-1 at OD_{680}. The culture was chilled on ice, centrifuged briefly at 1500 rpm to remove unwanted clumps of cells and then fresh cell suspensions were centrifuged in a fresh tube at 3000 rpm for 6 min at 4°C. The sediment of cells was re-suspended with 1 ml of ice-cold $CaCl_2$ concentration by gently pipetting up and down and 0,2 ml aliquot was distributed into two sterile microfuge tubes containing 5-10 µl DNA plasmid containing the inserted gene and 5 µl sterile H_2O that served as a control. The cells were frozen for 30 s in liquid nitrogen and incubated at 37°C for exactly 5 min. After adding 1 ml of LB medium, the solution was mixed by pipetting, inversed and set down on a shaker for 2-4 hours at 28°C and then centrifuged 30 s at 13000 rpm. The obtained pellet was resuspended in 0.1 ml LB medium and cells were plated in agar plates containing kanamycin. Resistant colonies were verified with colony PCR (section 2.4.2.2.3) and plasmid DNA could be isolated.

LB: medium 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl

2.6.2 Plant transformation procedure

Mature dehusked seeds were sterilized in 94 % ethanol for 30 seconds and formaldehyde (0.8 %) for 40 min and then in a solution of sodium hypochlorite (1.8 %) plus Tween 20 for 20 min. Then they are incubated at 42°C for 24 h in a Petri dish containing two Whatman paper and 7 ml sterile deionised water to allow the seeds to pre-germinate. The embryos were immersed for 10-15 min into 25 ml of liquid co-culture medium (CCL) containing Agrobacterium cells at a density of cells/ml (OD_{600}=1). Dry embryos were then transferred to a Petri dish containing solid co-culture medium (CCS) and then incubated for three days at 25°C in the dark. For eliminating Agrobacterium, pre-germinated embryos were kept in a new CCS medium containing the antibiotic cefotaxime (400) mg/l and incubated at 25°C under 12/12-h (day/night) photoperiod for three days. To promote vigorous tiller and root development, the plantlets were incubated at a photoperiod of 12/12h (day/night) in N6 medium without hormones. One medium contained kanamycin for the selection, another one without kanamycin served as comparison. Large plants were transferred for acclimatizing to hydroponic culture in Hoagland's medium. The presence of the inserted gene in the transgenic plants was verified by PCR using primers specific for the kanamycin used as gene

marker. Obtained seeds were sterilized and germinated under 50 mg/l kanamycin treatment. After 10 days the segregation for kanamycin resistance was calculated. The presence of the Kanamycin resistant gene in resistant plants was again verified by PCR using primers specific for the kanamycin (selection marker).

N6 or Chu (1978): Major salts, Minor salts and vitamins Nitsch-Nitscht

Major salts 2830 mg/l KNO_3; 166 mg/l $CaCl_2$, 2 H_2O; 185 mg/l $MgSO_4$, $7H_2O$; 400 mg/l KH_2PO_4; 463 mg/l $(NH4)_2SO_4$; Iron**:** 37.2 mg/l Na_2 EDTA; 27.8 mg/l $FeSO_4$ $7H_2O$

Minor salts: 0.8 mg/l KI; 4.4 mg/l $MnSO_4$, 4 H2O; 1.5 mg/l $ZnSO_4$, 7 H_2O; 1.6 mg/l H_3BO_3

Vitamins Nitsch-Nitscht: 100 mgl Myoinositol; 0.5 mg/l Thiamine-HCl 5 mg/l acid nicotinic 0.5 mg/l Pyridoxine HCl; 0.05 mg/l Biotin; 0.5 mg/l Folic acid; 1mg/l vitamin B12* ; 2 mg/l glycine

(vitamin B12 was added by filtration after autoclaving)

CCL=N6 modified + 2 mg/l naphthalene acetic acid, 1mg/l kinetin, 10g/l glucose, 100µM acetosyringone, pH 5,2

CCS= CCL + 7g/l agarose.

2.7 Physiological analyes of transgenic and non transformed plants

Germination and growth of transgenic plants were measured. Seeds were dehusked, sterilised and germinated in a sterile petridish. 50 seeds were germinated in H_2O and an other group of 50 seeds in H_2O plus 50 mM NaCl. The number of germinated seeds was estimated after 7 days. In the same time we measured the length of plantlets and estimated the percentage of growth. For each line, three repetitions have been conducted.

2.7.1 Ionic analyses.

Ions contents were analysed for wheat, *Festuca*, rice and rice transgenic plants in leaf and root tissue of salt-stressed plants and plants grown as control in normal growth conditions. Roots and leaves of 12 different plants were dried by incubation at 70°C and cation concentrations were measured with inductivity coupled plasma atomic emission spectrometer (ICPAES University of Würzburg). Cl^- concentration was measured by using fresh tissue. The measurement used a micro-chloro-counter (Marius-Utrecht, Netherlands). Extracts of stressed and non-stressed plants were titrated with silver ions generated from a set of silver electrodes immersed in the sample solution by electric current pulses. Each pulse generated a constant amount of charges which is transferred to the electrode and generates constant

amounts of silver ions. The number of pulses is directly correlated to the quantity of Cl^-. The amount of Cl^- in a sample was obtained by the operation:

$$\frac{\text{Count in unknown solution}}{\text{Count in calibration solution}} \text{ X 100 mg eq/l}$$

A sample size corresponded to 10µl

mEq/l or mg% (1 microequivalent Cl = 1000 counts , 1 µg Cl = 10 counts**)**

Titration solution:

Base solution 1: 100 mg Glacial acetic acid + 8 ml Nitric acid filled up to 1 l and boiled to eliminate dissolved oxygen,

Base solution 2: 600 mg white powder gelatine + 50 ml distilled H_2O +10 mg thymol blue + 50 ml distilled H_2O.

Calibration solution: 2922 mg NaCl in 500 ml distilled water**.**

2.7.2 Measurement of osmotic potential, chlorophyll fluorescence and K^+ content

The osmotic potential in leaf extracts of 3 week old rice lines IR29 and Pokkali grown under control conditions and treated with 150 mM NaCl was determined with an osmometer (Knauer, Germany) by freezing point depression. Chlorophyll a fluorescence was measured with a Mini PAM (Walz, Germany) in attached leaves using saturating light intensity (5000 µmol $m^{-23}s^{-1}$). Measurements were performed on the youngest fully expanded leaf, approximately on the middle of the leaf. The photosynthetic yield was calculated according to the manufacturer´s instructions.

The K^+ contents in leaves and in root were analysed by cation HPLC (Golldack et al., 2002). Root tissue and the second leaf of each plant were collected and ground in liquid nitrogen. After homogenization in ethanol: chloroform: water (12: 5: 3) and re-extraction with water, the aqueous phase was used for cation HPLC analysis (IonPac cation exchange; Dionex, Sunnyvale, CA, USA) (Adams et al., 1992). Alternatively, analyses of ion concentrations were performed with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer, Boston, MA, USA) according to Brune et al. (1995). The results shown are means from 5 plants.

3-1 Salt-dependent regulation of chloride channel transcripts in rice

3-1.1 Morphological aspects of IR29 and Pokkali under salt stress

When IR29 and Pokkali rice plants are grown in 150 mM NaCl, IR29 line showed a precocious wilting followed by complete necrosis of the leaves after 48 h, whereas Pokkali continued growing without visible symptoms of severe stress (Fig.3-1.1). Pokkali leaves remained green up to one week. Leaf wilting by Pokkali intervened only after one week stress.

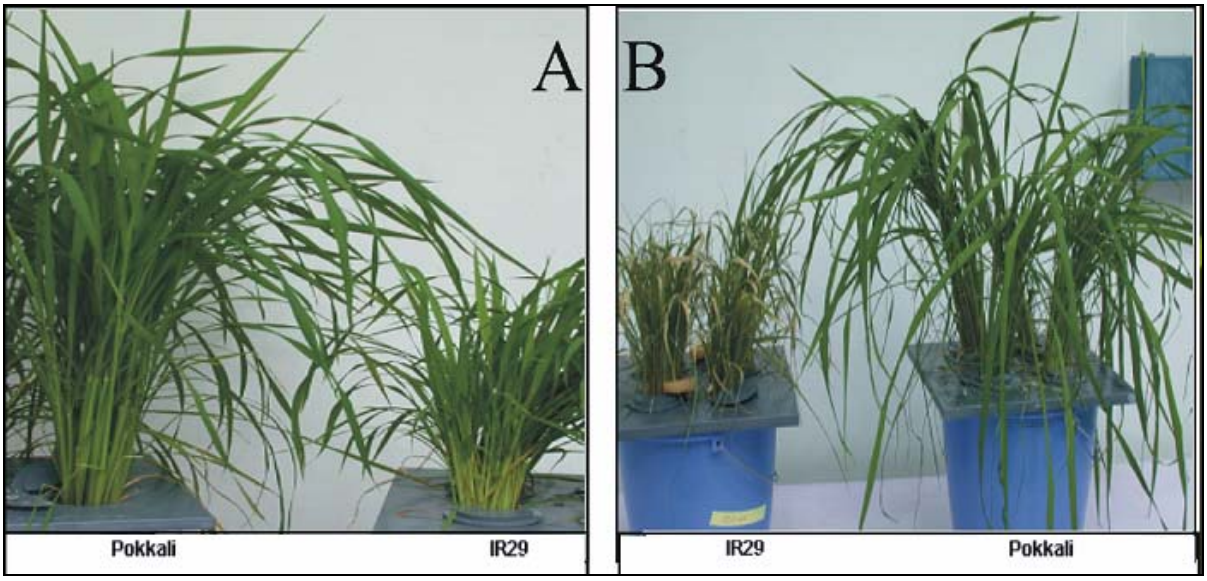

Fig. 3-1.1 Effect of a saline constraint on the habitus of two rice varieties: IR29 (salt sensitive), Pokkali (salt tolerant). Cultivated in Hoagland medium using hydroponics culture seedlings that were submitted to a stress of 150 mM NaCl at the age of 40 days. Non stressed plants served as control. A: control condition B: stress condition.

3-1.2 Different Cl^- accumulation in the rice varieties IR29 and Pokkali

When grown in a NaCl medium, plants accumulate Na^+ and Cl^- differently. In previous studies, Golldack et al. (2002a) reported higher accumulation of Na^+ in the salt sensitive line IR29 whereas the salt-tolerant line Pokkali excluded Na^+ when exposed to 150 mM NaCl. Measurement of net Cl^- uptake reported in this work and conducted at the same salt stress conditions indicated big accumulation differences between root and leaf and between the two lines (Fig. 3-1.2). Cl^- is generally progressively accumulated in both tissue and in both lines but the slope of this process is more pronounced in leaf than in root. In root tissue, Cl^- concentration remained almost, at the same level in the two lines, whereas the salt sensitive line IR29 accumulated more salt in leaves and reached at 48 h three times more the concentration of Pokkali. The salt sensitive line IR29 accumulated Cl^- faster in leaves, in contrast salt tolerant (Pokkali) efficiently limited the absorption of Cl^-.

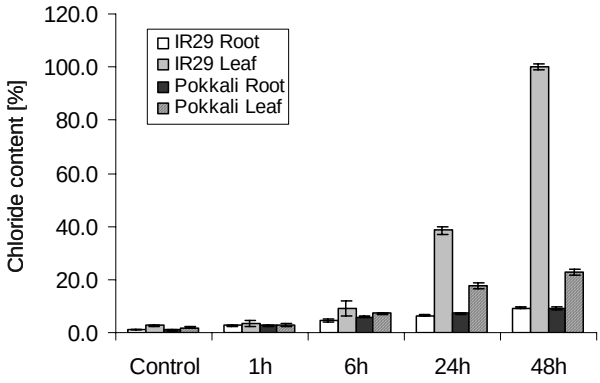

Fig. 3-1.2 Cl^- accumulation in the rice lines IR29 and Pokkali. The plants were grown in the control condition or treated with 150 mM NaCl for 48h. Data represent means ± SD. N=10

3-1.3 Tissue-specificity and salt stress dependence of *OsCLC1* transcript abundance.

Using the sequence information of rice genome data base, 7 sequences homologous to plant voltage-gated chloride channels were isolated. The predicted protein sequence of rice CLC-type channels shows homology ranging from 60 to 81% with *Arabidopsis* voltage gated chloride channel sequences (Fig. 3-1). Localization of putative rice Cl^- channels could be predicted in the plasma membrane, the thylakoid, and the ER membrane respectively (Table 3-1.1). Among these channels, our research has been concentrated on *OsCLC1* homologous to Arabidopsis *AtCLC*-c which shares 75% of identity with tobacco CLC-Nt1 and that could suppress salt sensitivity when heterologously expressed in a *gef1* yeast mutant (Gaxiola et al., 1998). Transcript abundance of *OsCLC1* gene was analysed by RT-PCR performed on total RNA isolated from root and leaf using gene specific oligonucleotide primers amplifying a partial cDNA-sequence of the coding region of the gene (Fig. 3.1.4). During 48h, 150 mM NaCl induced a significant reduction of O*sCLC1* transcript amounts in leaves of both lines, whereas in roots the expression looked very different. While the sensitive line showed a weak decrease, the tolerant one strongly increased.

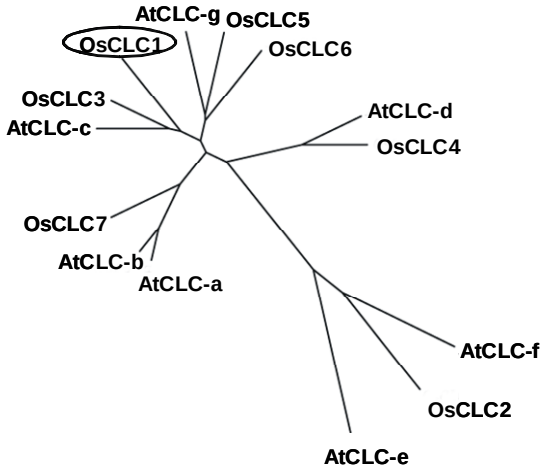

Fig. 3-1.3 Un-rooted tree of the deduced amino acid sequences of *OsCLC1* to *OsCLC7* as shown in the table 3-1.1 and of AtCLC-a (At5g40890), AtCLC-b (At3g27170), AtCLC-c (At5g49890), AtCLC-d (At5g26240), AtCLC-e (At1g55620), AtCLC-f (At4g35440), AtCLC-g (At5g33280).

Table 3-1.1 CLC-type Cl^- channel homologous genes were identified in rice genomic database (http:// www.tigr.org). TIGR (Institute for Genomic research).

Gene Name	Chromo-some	Predicted Molecular Mass (kDa)	Theoretical pI	Locus	Predicted Cellular Localization	Certainty of Prediction	Predicted Number of Transmembrane Helices
OsCLC1	1	84,2	8.00	OS01g65500	chloroplast thylakoid membrane	0.862	13
					plasma membrane	0.8	
OsCLC2	1	76,4	6,45	OS01g50860	chloroplast thylakoid membrane	0.783	10
					plasma membrane	0.6	
OsCLC3	2	87.0	7,85	3865.t00005	plasma membrane	0.8	14
					chloroplast thylakoid membrane	0.515	
OsCLC4	3	85,5	8,76	OS03g48940	plasma membrane	0.6	12
					chloroplast thylakoid membrane	0.508	
OsCLC5	4	87,1	8,18	*OS04g55210*	plasma membrane	0.8	13
					chloroplast thylakoid membrane	0.527	
OsCLC6	8	87.0	8,85	OS08g20570	chloroplast thylakoid membrane	0.642	12
					plasma membrane	0.642	
OsCLC7	12	67,8	8,86	OS12g25200	endoplasmic reticulum (membrane)	0.685	9
					plasma membrane	0.64	

3-1.4 Salt-dependent regulation of *OsCLC1* in correlation with *OsVHA-B, OsNHX1* and *OsPIP2;1* in the rice lines IR29 and Pokkali.

The first results obtained by RT-PCR (Fig. 3-1.4) indicated a decrease in the expression of *OsCLC1*, *OsVHA-B* and *OsNHX1* after 48 h salt stress in both lines. Since Na^+ and Cl^- accumulated to a higher concentration in leaves than in root, the study was conducted with a focus on leaves and less on root. The effects of salt stress on the expression of these genes were examined in leaves by Northern blot.

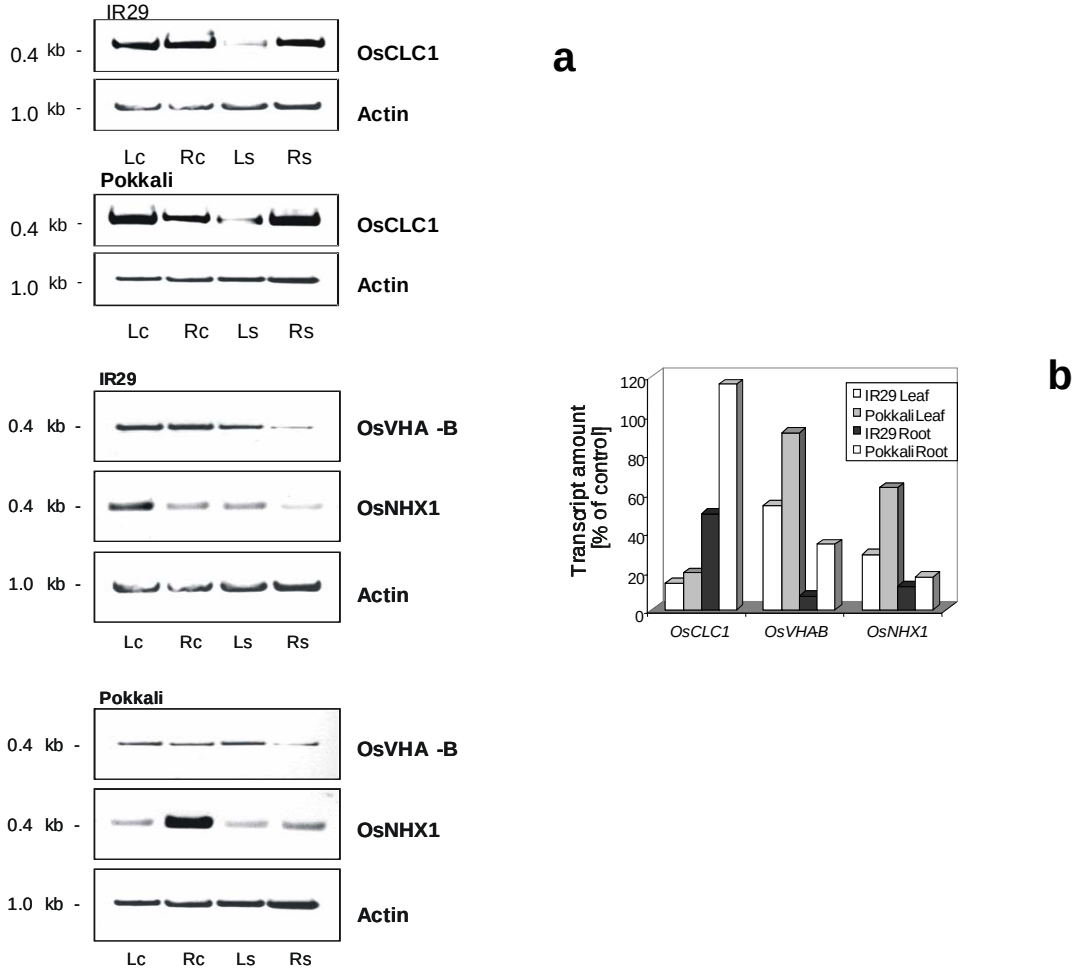

Fig. 3-1.4 Expression of *OsCLC1*, *OsVHA-B* and *OsNHX1* in control plants and after treatment with 150 mM NaCl for 48 h. **a:** RT-PCR amplification of fragments of the coding region of *OsCLC*, *OsVHA-B* and *OsNHX1* respectively. Lc-Leaf, control. Rc - Root control. Ls- Leaf salt stress. Rs- Root salt stress. **b:** Densitometric analysis of transcript levels shown in **a**. The transcript amounts of *OsCLC1*, *OsVHA-B* and *OsNHX1*, respectively, in leaf tissue of IR29 plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin (Diedhiou and Golldack, 2005).

3-1.5 Expression of the *OsCLC1* gene

Expression of *OsCLC1* in dependence of the duration of the salt stress (150 mM NaCl) was investigated in leaves of IR29 and Pokkali. In Pokkali transcript amounts of *OsCLC1* increased transiently after 3h of salt stress, then decreased slightly and were maintained until 24 h at the same transcript level of the control and finally significantly decreased after 48h salt stress. In contrast, expression in IR29 was slightly increased at 3 h, down regulated at 6h of stress and decreased drastically to about 10% of the control level at 24 h (Fig.3-1.5).

3-1.6 Expression of *OsVHA-B* and *OsNHX1*genes

One of the important ways to maintain low concentration of Cl^- and Na^+ in the cytoplasm is to sequester them in the vacuole. Expression of subunit B of V-ATPase (*OsVHA-B)* and *OsNHX1,* regulating the detoxification of the cell under 150 mM NaCl, are represented in the Fig. 3-1.5. Transcript levels obtained in leaves by Northern blot generally indicated a decrease in the two lines in the expression of both genes after 48 h salt stress. Considering the timescale of stress, Pokkali shows right from the beginning an increased expression of *OsVHA-B*. Moreover at 3h salt stress, this higher expression decreased progressively and reached the same levels of the control at 24h. In IR29, since the transcript amount of *OsVHA-B* remained unaltered at 3h, the down regulation started at 6h and continued with the time of stress. The expression of *OsNHX1,* regulating Na^+ storage in the vacuole, showed in Pokkali a strong up-regulation in the 24 h salt stress, then decreased to a level comparable with the control at 48 h. The up-regulation was limited to 6h in IR29 with a decrease less strict at 48h. The expression of the genes *OsVHA-B* and *OsNHX1* implicated in the Na^+ accumulation in the vacuole indicated a regulation parallel to the expression of the *OsCLC1* channel. In all obtained results, the tolerant line Pokkali showed a higher rate of transcript amount than the sensitive line IR29.

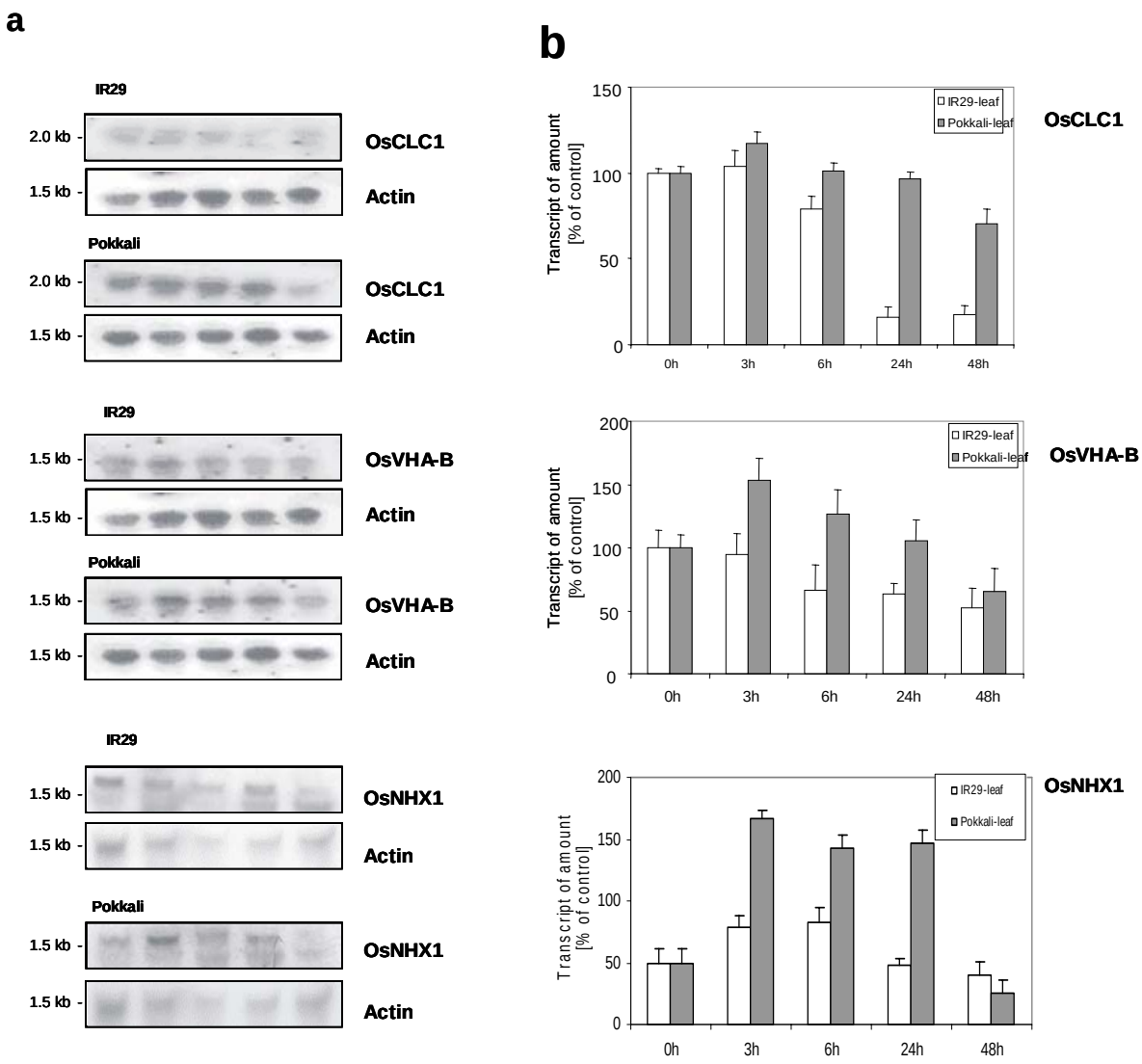

Fig. 3-1.5 a: Northern-type hybridization of the expression of *OsCLC1, OsVHA-B*and *OsNHX1* in leaves of IR29 and Pokkali during salt stress of 150 mM NaCl for 48h. 1 – control, 2 – 3h, 3 – 6h, 4 – 24h, 5 – 48 h of salt stress. **b:** Densitometric analysis of transcript levels shown in **a**. The transcript amounts of *OsCLC1*, *OsVHA-B*, and *OsNHX1* in leaf tissues of IR29 and Pokkali plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-1.7 Salt dependent regulation of *PIP2; 1* aquaporin

In their work, Moya et al. (2003) showed strong evidence that Cl^- and water uptake are directly linked. In agreement with this finding, this study concerning the expression *OsPIP2;1* water channel indicated the similar variation as *OsCLC1*(Fig. 3-1.6). Placed under 150 mM NaCl, IR29 and Pokkali showed an increase of the expression of *OsPIP2;1* in leaves during the first 6 h of stress followed with a decrease. The decrease became more intense at 24 h of salt stress, in both lines in leaf tissue. In the root tissue, the lowest level expression was noted at 6 h. In contrast to the leaf tissue, no increase was observed in root tissue where the expression remained below the control.

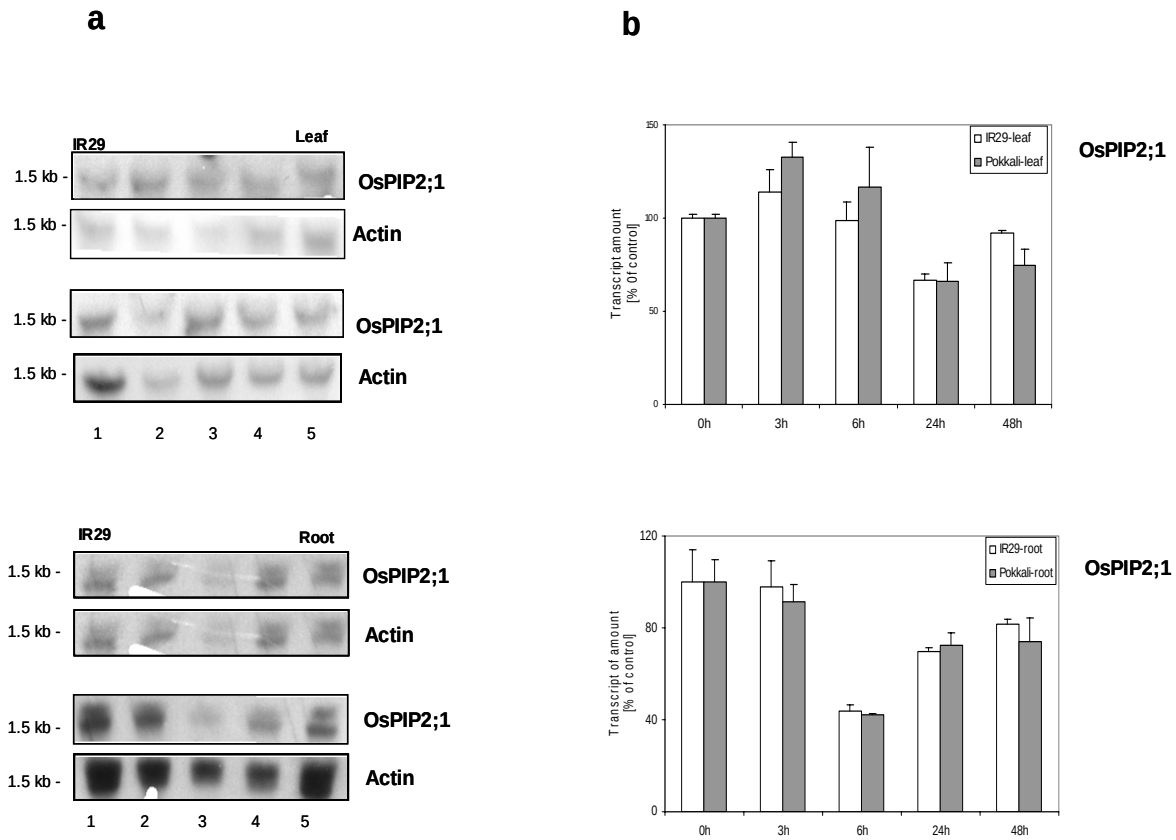

Fig. 3-1.6 b a: Northern-type hybridization of the expression of *OsPIP2;1* in leaves of IR29 and Pokkali during salt stress of 150 mM NaCl for 48 h. 1–control, 2– 3 h, 3– 6 h, 4– 24 h, 5– 48 h of salt stress. **b**: Densitometric analysis of transcript levels shown in **a**. The transcript amounts of *OsPIP2;1* in leaf and root tissues of IR29 and Pokkali plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-1.8 Cell-specific expression of *OsCLC1*

To provide information on the cell specific expression of *OsCLC1* gene, *in situ* PCR experiments were performed with leaf sections of IR29 and Pokkali. The CLC-type chloride channel was expressed in both rice lines with similar patterns. Signals were localized in the mesophyll cells and stomata whereas no transcripts could be detected in epidermal cells (Fig. 3-1.7). *OsCLC1* expression was observed in the vascular tissue in selected xylem parenchyma cells and in phloem in sieve elements as well as selected companion cells. In leaves of plants salt-stressed with 150 mM NaCl for 48 h the *OsCLC1* expression pattern changed. In both IR29 (Fig. 3-1.7b) and Pokkali (not shown) the signal strength decreased in mesophyll cells but was not affected in stomata. In IR29 the signals in the phloem and in the xylem parenchyma cells mostly disappeared (Fig. 3-1.7d). In contrast, Pokkali leaf section transcripts of *OsCLC1* could be detected in selected xylem parenchyma cells (Fig. 3-1.7e) whereas expression in the phloem was significantly repressed

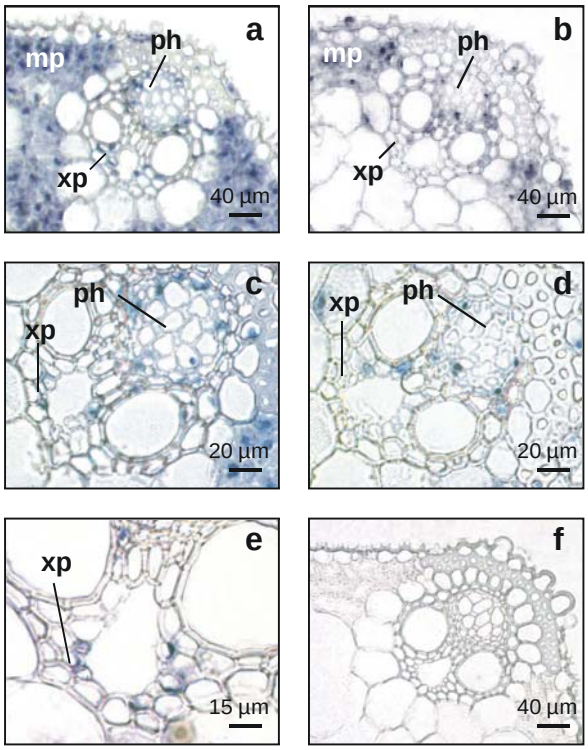

Fig. 3-1.7 ***In situ*** **PCR localization of** ***OsCLC1*** **in leaf cross sections of the rice lines IR29 and Pokkli**. **a** IR29, control. **b** IR29, 48 h 150 mM NaCl. **c** Vascular bundle of IR29 , control. **d** Vascular bundle of IR29 , 48 h 150 mM NaCl. **e** Section of a vascular bundle of Pokkali 48h 150 mM NaCl. **f** *In situ* PCR without gene specific oligonucleotide primers as background control. Xp – xylem parenchyma cells, ph – phloem, mp – mesophyll (Diedhiou and Golldack, 2005).

3-2 Salt stress regulates expression of the HAK-type K^+- transporter *OsHAK7* in rice

3-2.1 Different regulation of K^+/Na^+ homeostasis in the rice lines IR29 and Pokkali

3-2.1.1 Effect of salt stress on the fluorescence capacity of two rice lines IR29 and Pokkali

Chlorophyll fluorescence emission from the upper surface of the leaves was measured as a parameter for the photosynthetic yield. Salinity stress (150 mM NaCl) caused a reduction of 20 % of photosynthetic yield in both lines at 6 h after treatment. At 48 h the salt sensitive line IR29 showed 50% reduction and reached 70% at 72 h. The salt tolerant line Pokkali showed decreased photosynthetic activity at 24 h and kept it constant at 90% until 72 h salt stress (Fig. 3-2.1)

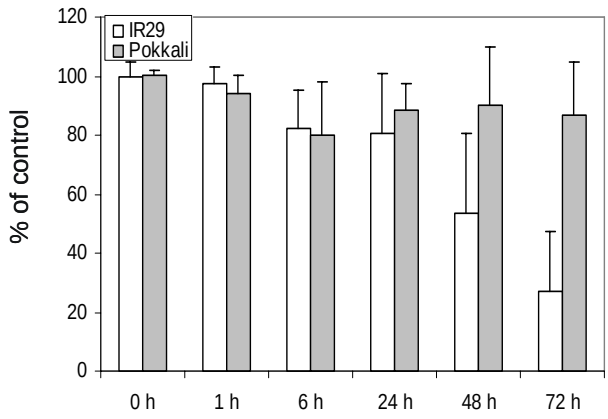

Fig. 3-2.1 Effects of salt stress on phototosynthetic activity calculated from chlorophyll a fluorescence in IR29 and Pokkali. The measurements were performed in attached leaves control plants and plants treated with 150 mM NaCl for up to 48 h. Data represent means ± SD, n=10.

3-2.2 Effect of salt stress on the osmotic potential of two rice lines IR29 and Pokkali.

The osmotic potential was very low (5 %) in root tissue and was not significantly changed in both lines in response to the duration of salt treatment (Fig. 3-2.2). In leaf tissue, the osmotic potential increased progressively and similarly in both lines, IR29 and Pokkali, and reached only a significant difference at 24 h after treatment in IR29. The increase of the osmotic potential at 24 h in the salt sensitive line was higher (50 %) than in Pokkali (30 %). At 48 h of 150 mM NaCl, the osmotic potential in IR29 reached 100 %, whereas the salt tolerant rice line Pokkali showed an osmotic potential of 35 %.

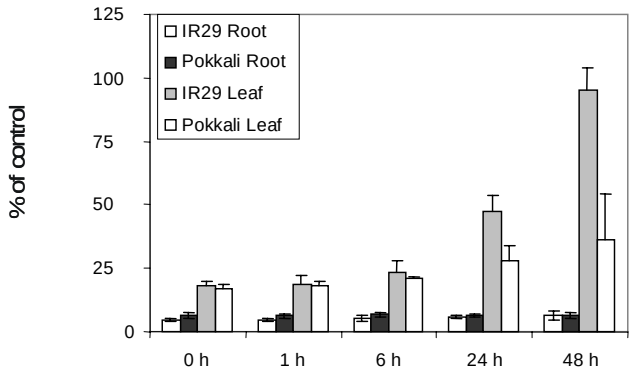

Fig. 3-2.2 Effects of salt stress on the osmotic potential in shoots and roots of IR29 and Pokkali plants that were either grown under control conditions or treated with 150 mM NaCl for up to 48h. Data represent means ± SD, n=10.

3-2.3 Effect of salt stress on K^+ uptake of the two rice lines IR29 and Pokkali

Both rice lines showed similar accumulation of K^+ under 150 mM NaCl. In roots, the K^+ contents decreased after salt treatment and reached 40 µmol/g fwt at 48h. This concentration is about 70% of the concentration of the control plants (Fig. 3-2.3a). In contrast, the increase was about 300 µmol/g fwt in leaves under the same condition of salt stress. As it was shown in previous studies, IR29 accumulated more Na^+ in leaves under salt stress, whereas Pokkali tended to limit the uptake of Na^+ in leaves. The Na^+/K^+ ratio increased in both rice lines but in roots, Pokkali showed the highest (Fig. 3-2.3b). In contrast, the leaves of IR29 showed more than 4-fold the concentration of Pokkali (Fig. 3-2.3c)

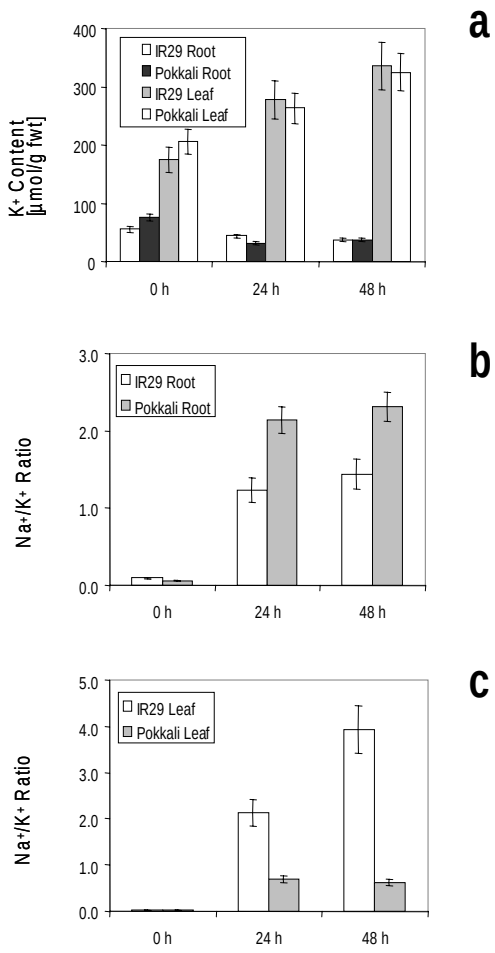

Fig. 3-2.3 Salt dependent K^+ accumulation in the rice lines IR29 and Pokkali. **a:** K^+ content in plants that were adapted either to control conditions or treated with 150 mM NaCl for 24 and 48h. **b:** Na^+/K^+ ratio of roots of IR29 and Pokkali grown in control medium and after exposure to 150 mM NaCl for 24h and 48 h. **c:** Na^+/K^+ ratio in leaves. Data represent means ± SD, n=7 (Golldack et al., 2002).

3-2.4 Tissue specificity and salt-stress dependence of *OsHAK7* transcript abundance

For analysis of transcriptional regulation of salt-dependent K^+ homeostasis in rice, expression of *OsHAK7*, a member of the HAK-type dual affinity K^+ transporter family in rice was studied. *OsHAK7* has a predicted localization in the plasma membrane and has 12 transmembrane domains. Transcript abundance of *OsHAK7* was studied by RT-PCR amplification with gene specific oligonucleotide primers that were designed for the coding region of the gene.

In non stressed conditions, *OsHAK7* was expressed more in roots than in leaves in both lines (Fig. 3-2.4). Expression of *OsHAK7* in dependence of salt stress was monitored by Northern-blot hybridizations and showed a similar expression in the roots in both lines (Fig. 3-2.5). Under 150 mM NaCl, Pokkali showed a slight increase of *OsHAK7* at 3 h. This level was maintained constant at 6 h, where IR29 showed its first increase. The only significant

difference of the increased expression was seen at 24 h with a signal similar in both lines. After one day of salt stress, the expression of *OsHAK7* decreased and became closer to the control at 48h. In leaves, *OsHAK7* was upregulated in both lines already at 3 h of 150 mM NaCl treatment (Fig. 3-2.5). While in IR29 the signal intensity remained uniform at 6 h, Pokkali indicated a down-regulation. After one and two days stress, the expression of *OsHAK7* decreased in both IR29 and Pokkali to a lower level than in the control.

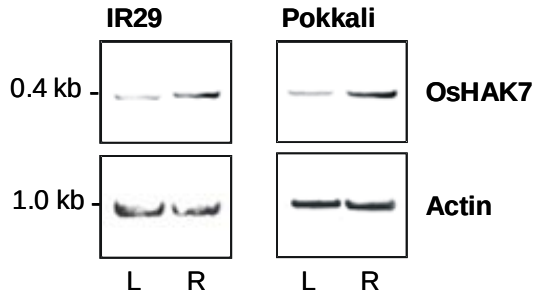

Fig. 3-2.4 Tissue specificity of *OsHAK7* expression in rice. Transcript amounts of *OsHAK7* were quantified in leaves (L) and roots (R) of the rice lines IR29 and Pokkali. A fragment of the coding region of *OsHAK7* was amplified by reverse transcription PCR. Actin was amplified as a loading region (Golldack, non-publisch).

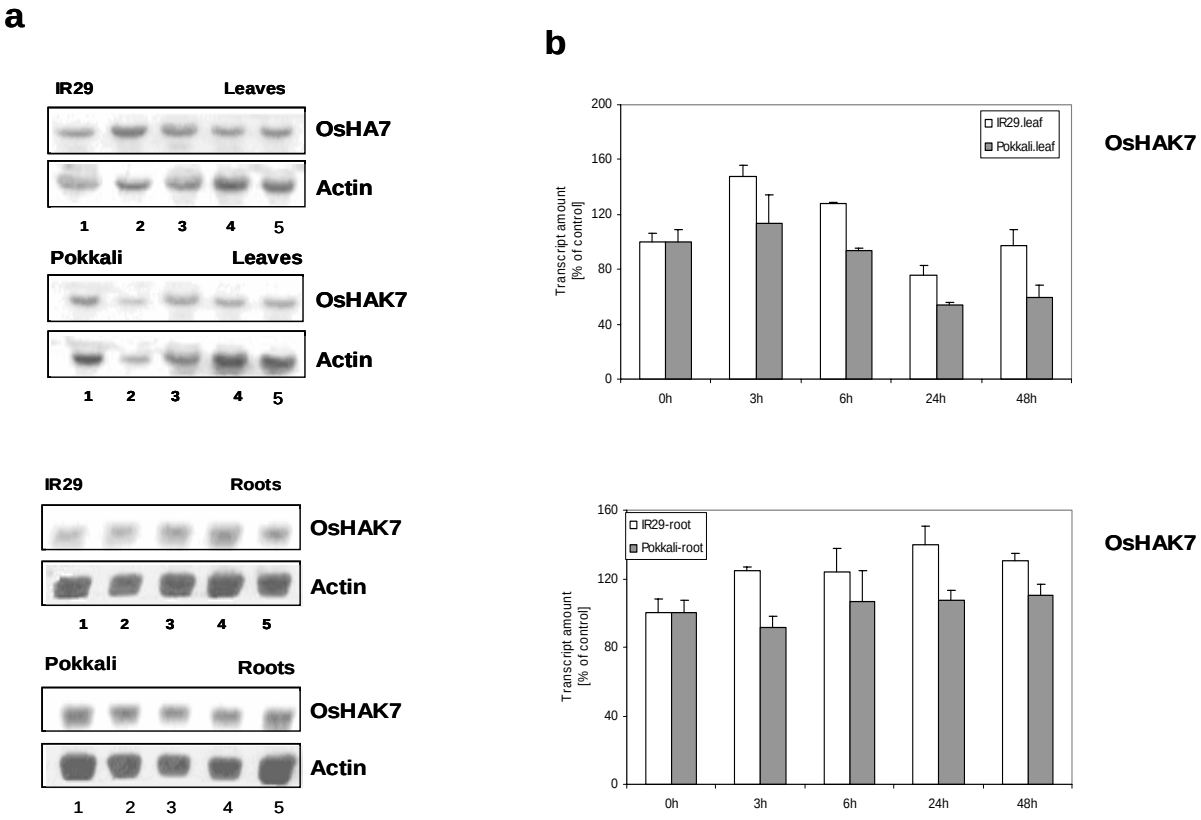

Fig. 3-2.5 a: Northern type hybridization of the expression of *OsHAK7* in roots and leaves of IR29 and Pokkali during salt stress of 150 mM NaCl. 1- control, 2- 3 h, 3- 6 h, 4- 24 h, 5-48h of salt stress. **b:** Densitometric analysis of the transcript levels shown in **a**. The transcript amounts of *OsHAK7* in root tissue of plants grown under control conditions were set to 100 %. Transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-2.5 Salt-stress and cell specific expression of *OsHAK7*

Cell specific expression of *OsHAK7* was evaluated by *in situ* PCR in leaf sections of IR29 and Pokkali. Under control conditions, *OsHAK7* is preferentially expressed in mesophyll cells in both lines (Fig. 3-2.6). A difference of signal expression in IR29 and Pokkali could be noted in vascular tissue. While no signals were detected in cells of the vascular bundles (Fig. 3-2.6a) in IR29 control, in Pokkali *OsHAK7* was expressed in the phloem, and transcripts were abundantly observed in xylem parenchyma cells adjacent to the metaxylem (Fig. 3-2.6c). In IR29 (Fig. 3-2.6b) and Pokkali (Fig. 3-2.6d) exposed to a treatment with 150 mM NaCl for 48 h, strongest signals of *OsHAK7* occurred in the mesophyll surrounding the vasculature, whereas the expression was down-regulated in mesophyll cells more distant to vascular bundles. In both lines, signals were detectable in the phloem and selected xylem parenchyma cells, besides, expression was also stimulated in epidermis cells.

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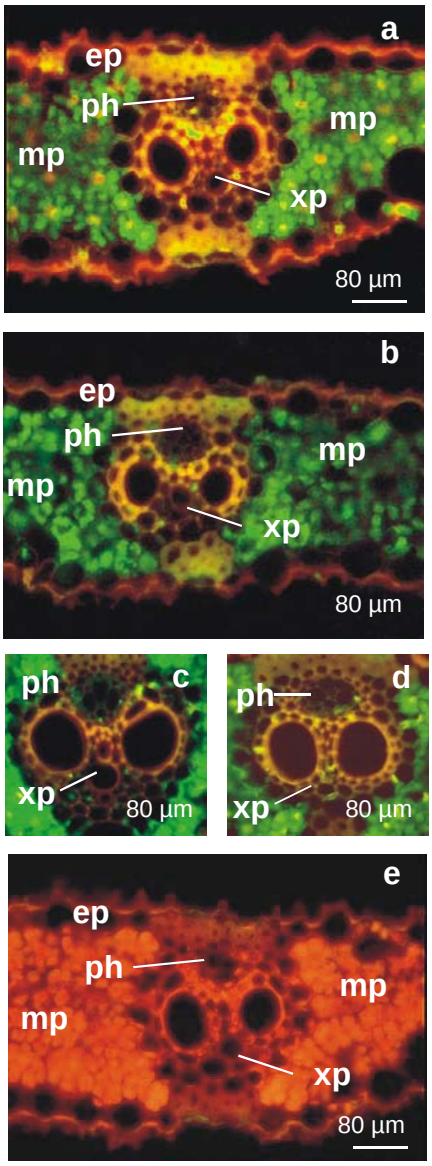


Fig. 3-2.6 *In situ* PCR localization of *OsHAK7* In leaf cross-section of the rice lines IR29 and Pokkali.a. IR29 control. **b**. IR29, 48h 150 mM NaCl. **c**. vascular bundle of Pokkali, control. **d**. vascular bundle of Pokkali 48h 150 mM NaCl. **e**. *In situ* PCR without with gene specific primers as a background control. xp- xylem parenchyma cells, ph-phloem, mp-mesophyll, ep-epidermis (Golldack, non-publisched).

3-3 Wheat and a salt-tolerant relative, *Festuca rubra* ssp. *litoralis*, regulate a plasma membrane aquaporin, the vacuolar H^+- ATPase and Na^+/H^+ antiporter differently

3-3.1 Ion accumulation in *Triticum aestivum* and *Festuca rubra*

A comparative analysis of salt stress responses in the salt sensitive *T. aestivum* and the halotolerant *Festuca* was performed to characterize salt induced ion uptake and transcriptional regulation of key adaptative mechanisms in both species. Accumulation of K, Na, Ca, Mg P, S and Fe in wheat and *Festuca*, were determined for leaf and root tissues and configured in Fig. 3-3.1 Grown under control conditions, ion contents in wheat was similar in leaf and in root except for Ca that showed higher amounts in leaves than in roots. In *Festuca* the content of Mg, Na, and Fe were higher in roots than in leaves, whereas compared to wheat, higher amounts of Ca, Mg and Fe were detected in leaves and in roots. When both species were exposed to 125 mM NaCl for 48h that is a severe salt stress for wheat but has minor effects on *Festuca*, wheat excluded Na from leaves and accumulated approximately 15 mg/g dwt of this element in roots. In contrast, *Festuca* accumulated Na to approximately 55 mg/g dwt and 30 mg/g dwt in leaves and roots, respectively. In addition, Mg and Fe content increased in leaves, whereas, the amounts of others ions were similar to non-stress control plants. When grown in hydroponic culture, *Festuca* continued growth and development at 250 mM NaCl for several weeks (not shown). In addition, *Festuca* can tolerate high concentrations of salt up to 500 mM NaCl. Exposure to 500 mM NaCl for 48 h causes in *Festuca* the accumulation of Na to approximately 65 and 80 mg/g dwt in leaves and roots, respectively, whereas, Mg and P contents increased in roots (Fig. 3-3.1). The Na^+/K^+ ratios were similar in leaves of wheat and *Festuca* grown under control condition but were higher in roots of *Festuca* (Fig. 3-3.2). In response of salt treatment, Na content increased above K in leaves of *Festuca* treated with 125 and 500 mM NaCl as well as in roots of plants exposed to 500 mM NaCl whereas salt-treated wheat accumulated less Na than K.

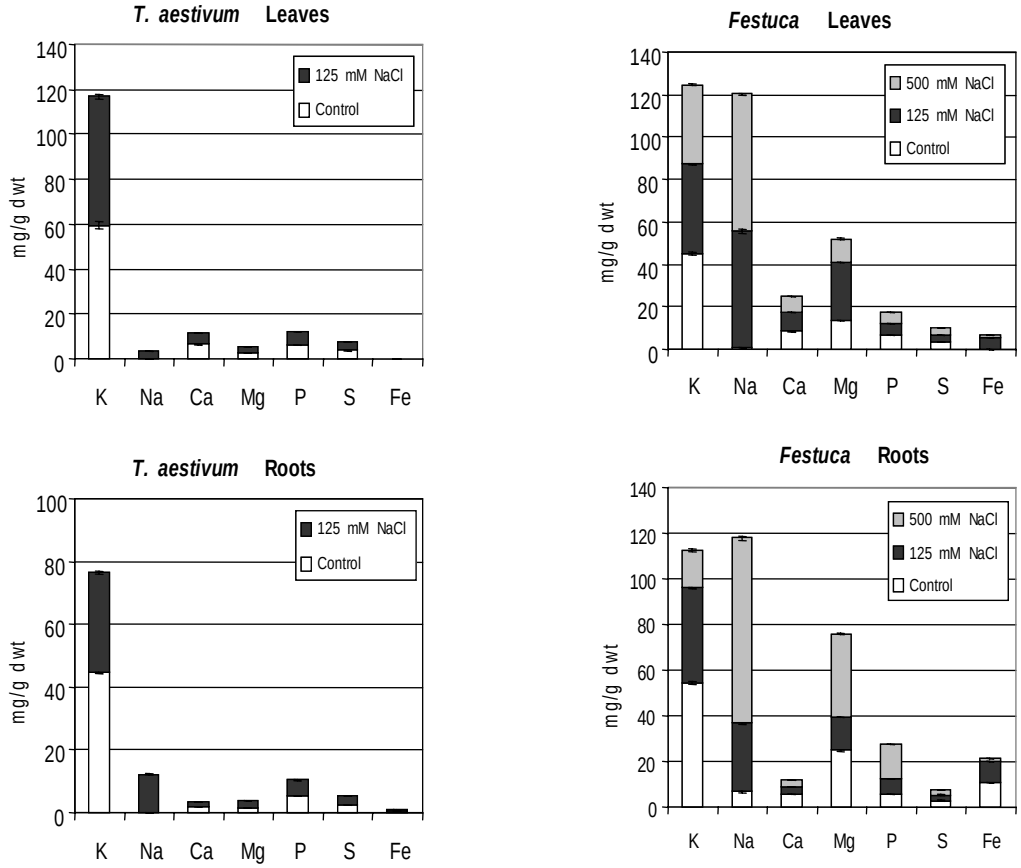


Fig. 3-3.1 Accumulation of ions in *Festuca* and wheat plants grown hydroponically. The plants were grown under control conditions or treated with 125 mM NaCl and 500 mM NaCl, respectively, for 48 h.

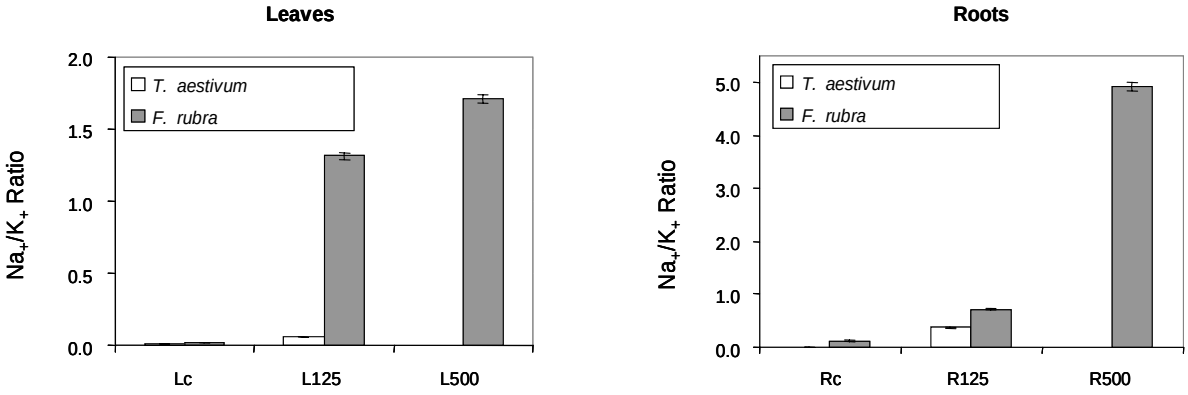


Fig. 3-3.2: **Na^+/K^+ ratio of *Festuca* and wheat grown in control condition medium and treated with 125 mM NaCl and 500 mM NaCl, respectively, for 48 h**.

3-3.2 Different salt-induced expression of a PIP2-homologue

Partial cDNAs of *FrPIP2;1*, *FrHNX1*, and *FrVHA-B* were obtained by RT-PCR from RNA of *Festuca* with degenerated primers. *Festuca* EST sequences share particulary high nucleotide identity with homologous sequences from *Hordeum vulgare*, *Triticum aestivum* and *Triticum boeoticum* ranging from 90 to 95 % whereas the degree of identity from *Oryza*

sativa and *Zea mays* ranges from 86 to 91 % (Fig. 3-3.3a). To obtain information on tissue specificity and salt-dependent expression of the PIP2-homologue in wheat and *Festuca*, Northern blots were performed with leaves and roots of plants grown under control and salt stress conditions for 48 h (Fig. 3-3.4). *TaPIP2;1* was expressed in leaves and roots of non stressed wheat plants with similar signal intensity. In response to treatment with 125 mM NaCl, the *TaPIP2;1* signal intensity decreased in both tissues (Fig. 3-3.4). In contrast in roots of *Festuca*, exposure to 125 mM NaCl induced an increase of *FrPIP2;1* transcript amounts, whereas expression was not modified in leaves. At higher salt concentration, namely 500 mM NaCl, the expression of *FrPIP2; 1* decreased in leaf and root tissues (Fig. 3-3.4).

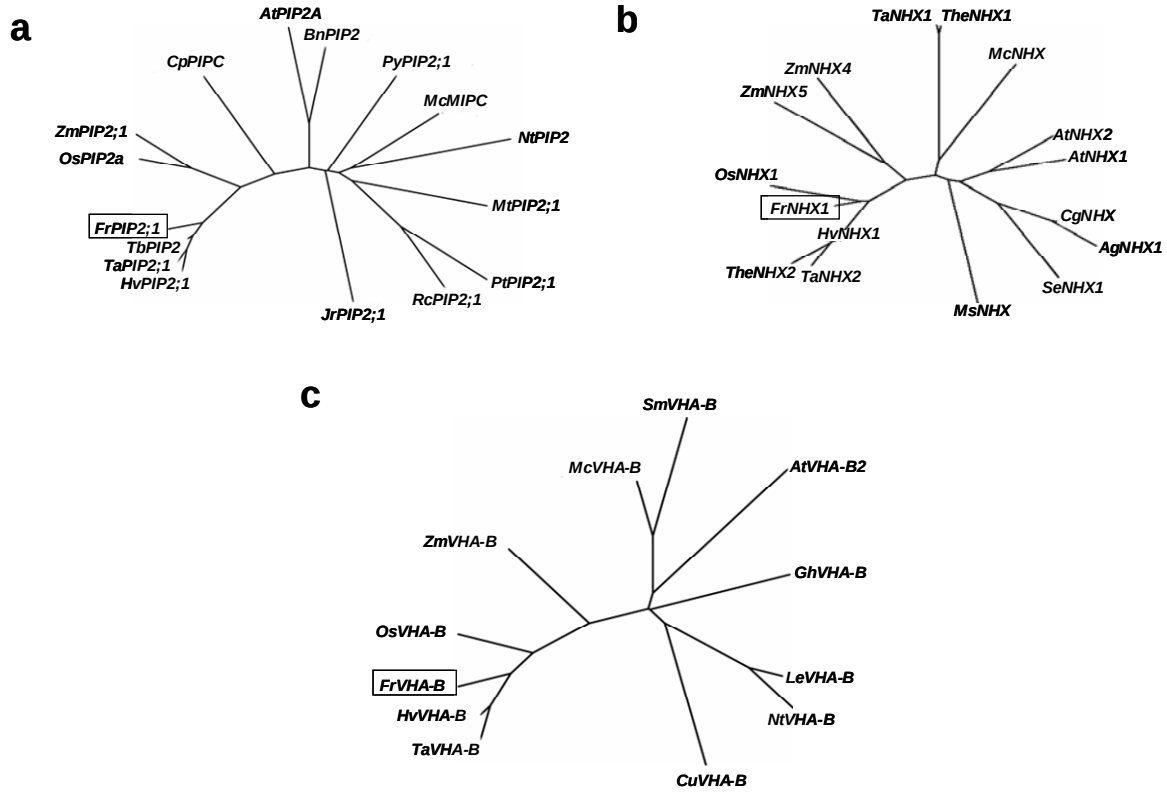


Fig. 3-3.3: Un-rooted tree of nucleotide sequences of *Fr PIP2;1* (a), *FrNHX1* (b), *FrVHA-B* (c), respectively, aligned to homologous sequences from other plant species. The species and accession numbers are described in Materials and Method section.

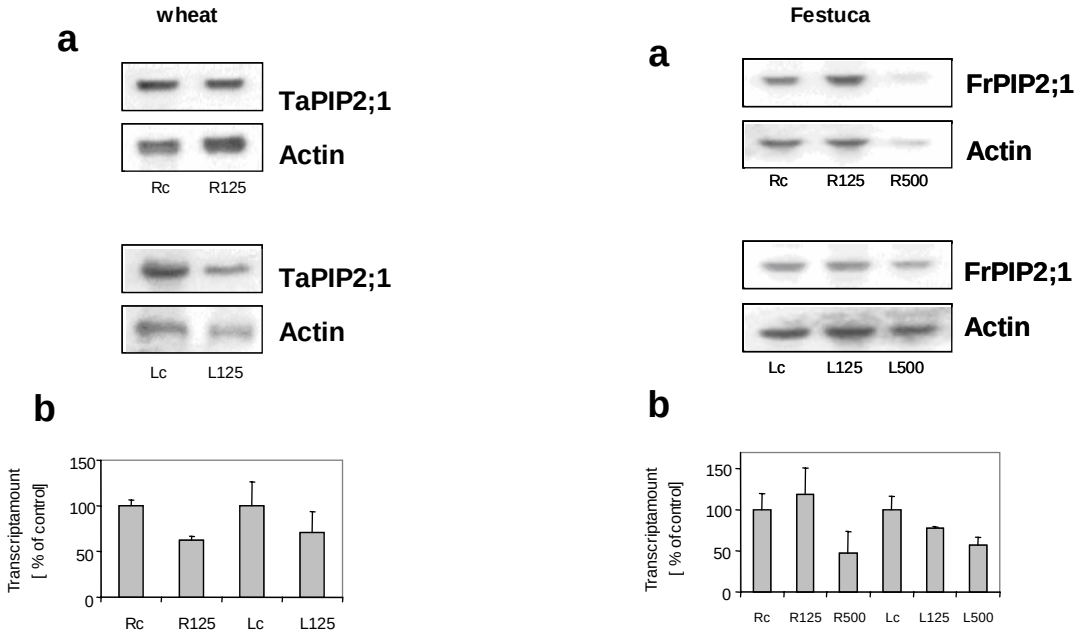


Fig. 3-3.4 a: Northern-type hybridization of the expression of *PIP2;1* in leaves and roots of wheat and *Festuca* under control, 125 and 500 mM NaCl. Hybridization of actin is shown as a loading control. Lc- leaf control, L125-leaves from plants treated with 125 mM NaCl L500- leaves from plants treated with 500 mM NaCl for 48 h, Rc-root control, R125-root from plants treated with 125 mM NaCl for 48 h, R500-root fom plants treated with 500 mM NaCl for 48h. **b:** Densitometric analysis of transcript levels. The transcript amounts of *TaPIP2;1* and Fr *PIP2;1* in leaf and root tissues of plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means of n = 3, ± SD.

3-3.3 Salt dependent regulation of *VHA-B* and *NHX*-homologous transcripts

To compare transcriptional regulation of genes involved in plant vacuolar Na^+ sequestration, Northern-type hybridizations for VHA-B and NHX-homologous transcripts in wheat and *Festuca* were performed. In wheat, *TaVHA-B* was highly expressed in leaves and in roots of control conditions and highly repressed when plants were treated with 125 mM NaCl. For the *TaNHX2* homologous to *FrNHX1*, expression was in control growth condition but under salt treatment a slight reduction of *TaNHX1* transcript level in roots, whereas in leaves the reduction became significant (Fig. 3-3.5). In *Festuca*, transcripts of *FrVHA-B* and *FrNHX1* were detected in both leaf and root tissues from non-stressed control plants (Fig. 3-3.6). Salt stress caused a decline of transcript levels of *FrVHA-B* and *FrNHX1* in leaves, whereas both genes showed stimulated expression in roots treated to 125 mM NaCl. In roots of plants stressed with 500 mM NaCl, transcript amounts of both *FrNHX1* and *FrVHA-B* decreased significantly.

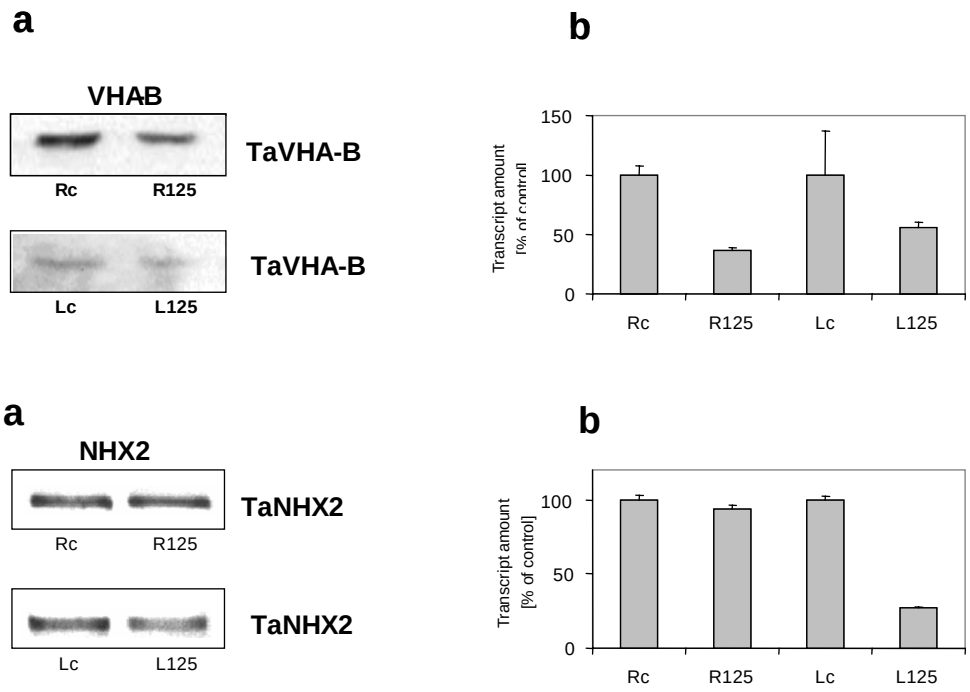


Fig. 3-3.5 a: Northern-type hybridization of the expression of *TaVHA-B* and *TaNHX1* in leaves and roots of wheat under control and 125 mM NaCl conditions. Lc- leaves control, L125-leaves from plants treated with 125 mM NaCl Rc-root control, R125-root from plants treated with 125 mM NaCl for 48 h. The same actin loading control was used as shown in Fig 4A. **b:** Densitometric analysis of the transcript levels. The transcript amounts of Ta*VHA-B and TaNHX1* in leaf and root tissues of plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means of n = 3, ± SD.

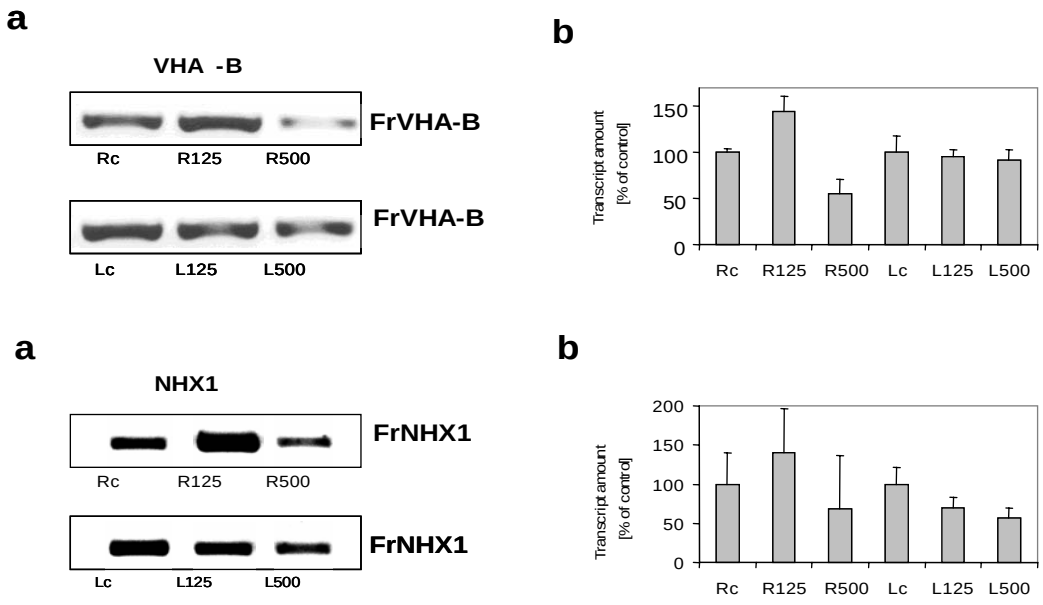


Fig. 3-3.6 a: Northern-type hybridization of the expression of *FrVHA-B* and *FrNHX1* in leaves and roots of wheat under control, 125 and 500 mM NaCl. Lc- leaves control, L125-leaves from plants treated with 125 mM NaCl, L500-leaves from plants treated with 500 mM NaCl Rc-root control, R125-root from plants treated with 125 mM NaCl for 48 h, R500-root from plants treated with 500 mM NaCl. The same actin loading control was used as shown in b**:** Densitometric analysis of the transcript levels. The transcript amounts of *TaVHA-B* and *TaNHX1* in leaf and root tissues of plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means of n = 3, ± SD.

3-3.4 Cell-specific expression of *FrPIP2;1*, *FrVHA-B* and *FrNHX1*

In situ PCR experiments were performed with root sections of *Festuca* control plants and plants exposed to 500 mM NaCl to analyse cell specificity of expression of *FrPIP2;1*, *FrVHA-B* and *FrNHX1* in response to salinity. In control plants faint signals of *FrNHX1* were detected in epidermal and neighbouring cortex cells (Fig. 3-3.7). *FrVHA-B* showed weak expression in all cell types in the root cross sections, whereas *FrPIP2;1* signals were more pronounced and could be detected in all cell types. In response to 500 mM NaCl the strongest signals of *FrPIP2;1*, *FrVHA-B* and *FrNHX1* appeared in endodermal and pericycle cells (Fig.3-3.7b,d,f). In addition, while *FrPIP2;1* and *FrVHA-B* were expressed in vascular cells and cells of the inner cortex, *FrNHX1* showed weaker signals in the vasculature.

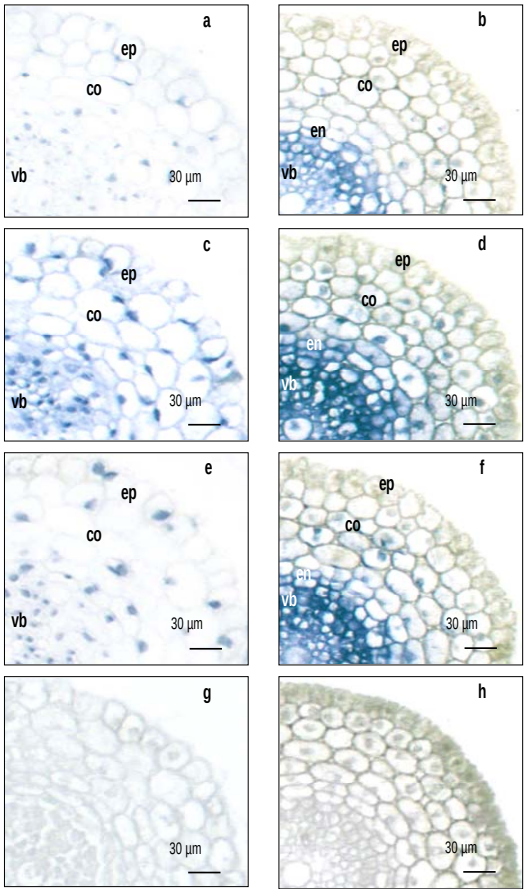


Fig. 3-3.7 ***In situ*** **PCR localization of** ***FrPIP2;1*****,** ***FrVHA-B*** **and** ***FrNHX1***. In root cross sections approximately 5 mm from the tip of *Festuca* **a, c, e, g** control plants.**b**, **d, f, h** plants treated with 500 mM NaCl for 48h . **a,b** *FrNHX1*. **c,d** *FrPIP2;1*. **e,f** *FrVHA-B*. **g, h** in situ PCR whih gene specific oligonucleotide primers as a background control . ep-epidermis, co-cortex, en-endodermis, vb-vacuolar bundle (Diedhiou and Golldack 2006).

3-4 Salt-responsive genes in rice and *Festuca rubra* ssp *litoralis* and induction of salt tolerance in the line IR29, rice sensitive

In this study, salt stress-induced transcriptional responses in the salt-sensitive rice variety *Oryza sativa* ssp. *indica* line IR29 were compared with that of the salt tolerant grass *Festuca rubra* ssp litoralis to identify genes that are differentially responsive to salinity in both species. *Festuca* is characterized by substantial salt resistance and tolerates up to 500 mM NaCl and continues growth and development in the presence of 250 mM NaCl when grown in hydroponic culture (Diedhiou and Golldack, 2005). In contrast, the rice line IR29 is severely damaged by exposure to salt concentrations of 150 mM NaCl (Golldack et al., 2002; 2003).

3-4.1 Molecular mechanisms of salt stress adaptation in the rice lines IR29 and Pokkali

3-4.1.1. Expression comparison between IR29 and Pokkali

To understand the molecular aspects of salt tolerance in the salt sensitive line IR29 and in the salt tolerant line Pokkali, a genomic scale analysis of gene expression was initiated by cDNA-array hybridizations. Genes from 192 expressed sequence tags (ESTs) from a salt stress cDNA rice library were used. The transcript changes were compared to unstressed plants from 6 h to 48 h after 150 mM NaCl salt treatment. The effect of potassium starvation under salt stress as well as the effect of LiCl stress was also tested. Expression analysis using Aida software indicated after different times of exposition to salt stress, in comparison to control conditions, a progression in the regulation of transcripts in both lines and at different time points (Fig.3-4.1a). Apparently, there was no significant difference between Pokkali and IR29 in the regulation of genes after 6h salt stress, however, Pokkali showed a slightly increased number of upregulated genes in leaf tissue. At 24 h of salt stress, IR29 seemed to recover from the delay showing more up- and downregulated genes mainly in roots. After 48 h of salt stress, the transcriptional changes decreased in both lines, but the expression remained still well detectable in IR29.

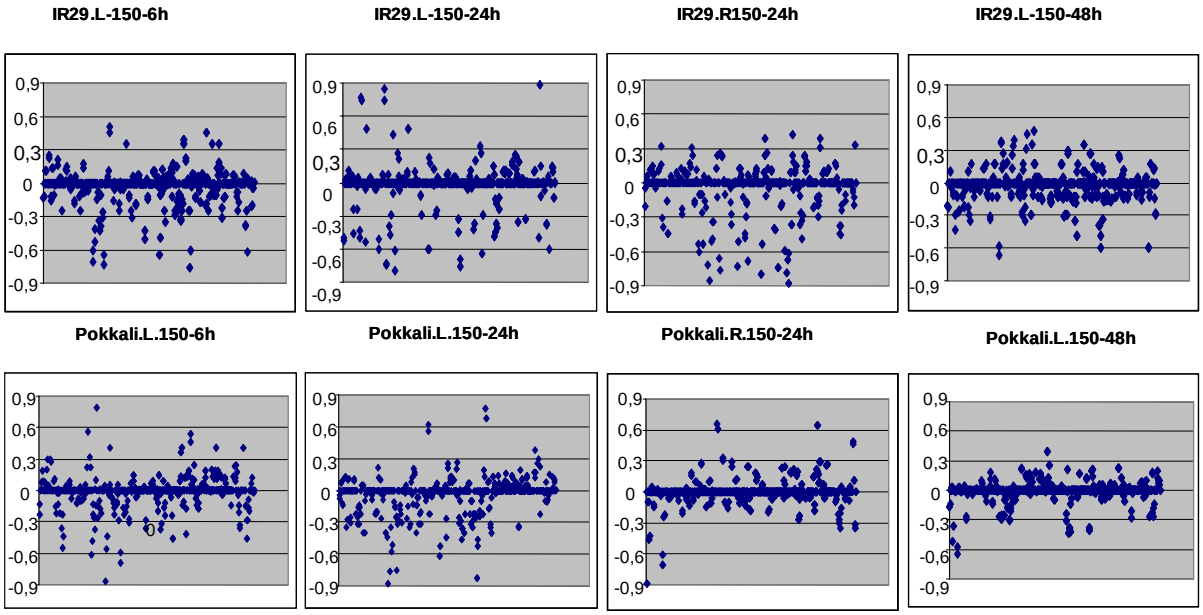


Fig. 3-4.1 Time course of transcript expression in the rice lines IR29 and Pokkali:

Transcript levels after 150 mM NaCl stress. The expression ratios of transcripts [log10 stress/control] from a series of time course experiments are plotted against the order of ESTs printed on the macro array (X axis). RNA from control conditions (IR29 and Pokkali) labelled with digoxigenin dUTP cDNA derived from RNA extracted from 150 mM NaCl stress from different time points were hybridized to ESTs deposited on the macro array membrane. L=leaf, R= root.

Under potassium starvation, the transcripts were more down-regulated in leaves of IR29 (Fig. 3-4.2). In roots, the number of upregulated genes seems to be the same as the number of down-regulated genes, whereas Pokkali showed a different pattern expressing more downregulated genes in roots and more upregulated genes in leaves. Two strategies could be seen in R29 and Pokkali in leaf. While Pokkali showed more genes upregulated, IR29 showed more repressed genes.

Lithium is considered as a sodium analogue with higher toxicity and can be used as a growth inhibitor, at concentrations lower than that of sodium, to reduce the osmotic effect (Serrano, 1996). Under lithium stress, most genes were downregulated in both lines notably in root (Fig. 3-4.3)

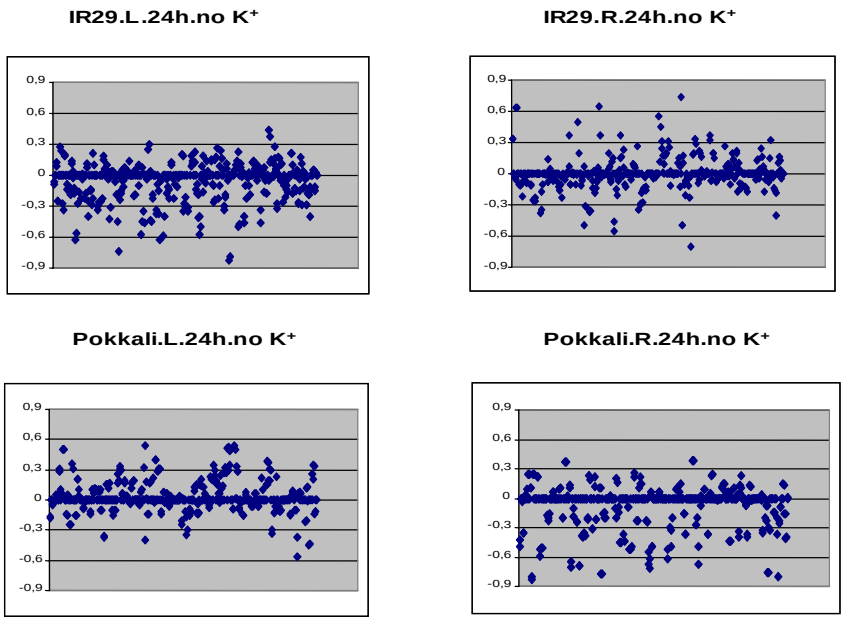


Fig. 3-4.2 Transcript expressions in the rice lines IR29 and Pokkali under salt stress and potassium starvation: Transcript levels after 150 mM NaCl stress. The expression ratios of transcripts [log10 stress/control] from a series of time course experiments are plotted against the order of ESTs printed on the macro array (X axis). RNA from control conditions (IR29 and Pokkali) labelled with digoxigenin-dUTP cDNA derived from RNA extracted from 150 mM NaCl stress at 24 h were hybridized to ESTs deposited on the macro array membrane.L=leaf, R= root.

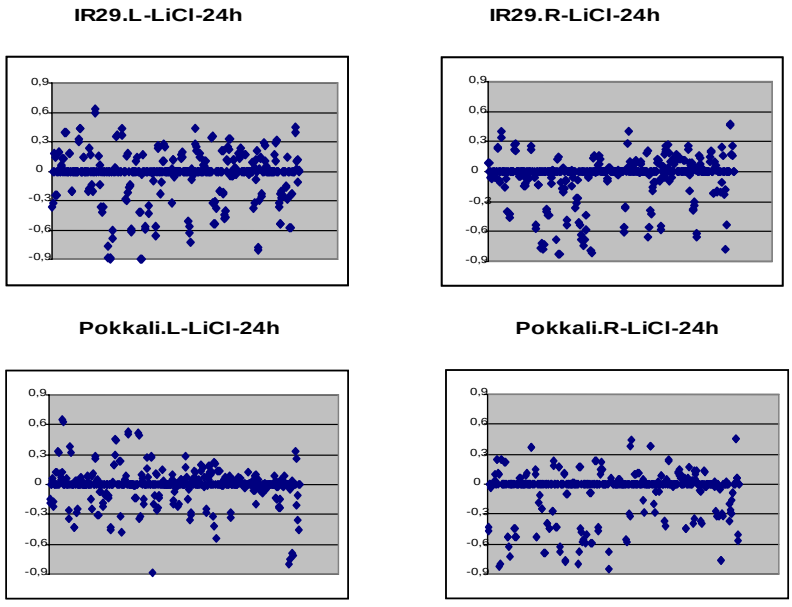


Fig. 3-4.3 Transcript expression in the rice lines IR29 and Pokkali under LiCl treatment: Transcript levels after 150 mM NaCl stress. The expression ratios of transcripts [log10 stress/control] from a series of time course experiments are plotted against the order of ESTs printed on the macro array (X axis). RNA from control conditions (IR29 and Pokkali) labelled with digoxigenin-dUTP cDNA derived from RNA extracted from 150 mM NaCl stress at 24 h were hybridized to ESTs deposited on the macro array membrane. L=leaf, R= root.

The array analysis indicated differences in relation to the timescale of salt constraint in both lines. The most apparent could be summarised by the number of expressed genes (Table 3-4.1). Generally under 150 mM NaCl, we noted more genes down-regulated (240) than genes upregulated (75) in both lines. The number of expressed genes in IR29 (193) is almost two times higher than in Pokkali (122). The growth in the presence of 150 mM NaCl and under K^+ starvation, showed 100 genes regulated in Pokkali and 82 genes in IR29. Treatment with LiCl stress induced more genes regulated in IR29 than in Pokkali.

Table 3-4.1 Summary of the number of up-and down-regulated genes in IR29 and in Pokkali under different times of stress and different conditions of stress

Up-regulated	down-regulated	IR29
IF>+0,3	IF<-0,3	
8	24	Ls150-6h
7	0	Rs150-6h
10	36	Ls150-24h
6	52	Rs150-24h
12	18	Ls150-48h
43	**150**	total up or down regulated genes
193		total up and down regulated genes
2	50	Ls150-24noK
16	14	Rs150-24noK
18	**64**	total up and down regulated genes
82		total up and down regulated genes
18	50	Ls150-LiCl
4	50	Rs150-LiCl
22	**100**	total up and down regulated genes
122		total up and down regulated genes

Up-regulated	down-regulated	Pokkali
IF>+0,3	IF<-0,3	
12	22	Ls150-6h
6	10	Rs150-6h
6	38	Ls150-24h
6	8	Rs150-24h
2	12	Ls150-48h
32	**90**	total up or down regulated genes
122		total up and down regulated genes
26	8	Ls150-24noK
4	62	Rs150-24noK
30	**70**	total up or down regulated genes
100		total up and down regulated genes
14	22	Ls150-LiCl
6	72	Rs150-LiCl
20	**94**	total up or down regulated genes
114		total up and down regulated genes

3-4.1.2 cDNA-array verification: Expression analysis of some transcripts

To see the expression of genes in detail, selected rice cDNA-array ESTs were sequenced. The analysis identified some salt induced genes, among them, the Os*HAL2* - Ca^{2+} sensitive 3'(2'), 5-diphosphonucleoside 3'(2'), phosphohydrolase homologue to the yeast *HAL2*. This gene encodes the enzyme 3'(2'),5'-bisphosphate nucleotidase which catalyses a reaction that converts 3'-phosphoadenosine-5'-phosphate (PAP) to adenosine-5'-phosphate (AMP) and inorganic phosphate (Pi). The activity of this enzyme is Mg^{2+} dependent, sensitive to Ca^{2+}, Li^+, and Na^+ and activated by K^+. Overexpression of the yeast *HAL2* under salt stress

improves salt tolerance in yeast (Peng and Verma, 1995). The second gene was a putative TATA-binding protein-associated that belongs to the transcription factor TFIID a major core promoter recognition factor implicated in the initiation of transcription mediated by RNA polymerase II (Lago et al., 2004).

3-4.1.3 Gene expression at different times of NaCl stress

Expression of these genes in IR29 treated to 150 mM NaCl showed that *OsHAL2* is more expressed in roots than in leaves (Fig. 3-4.4). The expression decreased from 6 h to 48 h in root tissue, whereas in leaves it increased significantly at 48 h. The putative TATA-binding protein showed at 6 h high and very low expression, in root and leaf tissues respectively. At 48 h there was almost no expression in roots, whereas in leaves the expression reached the induction factor 0.8.

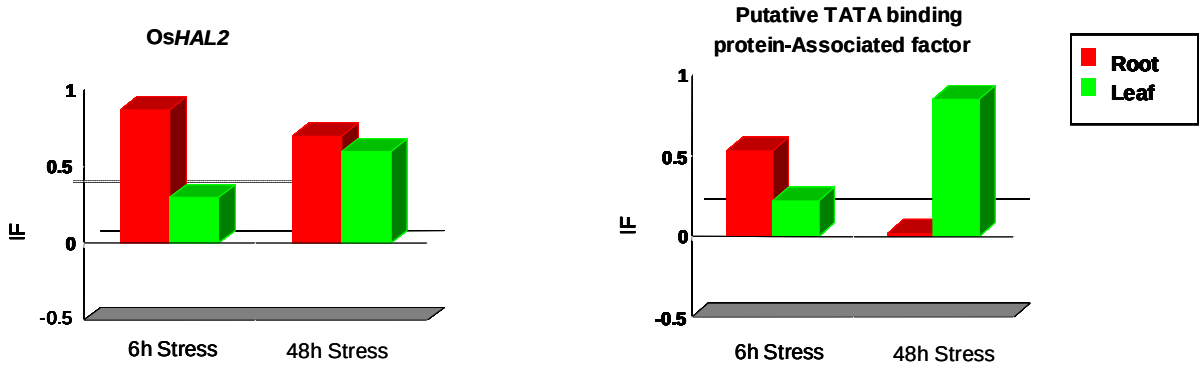


Fig. 3-4.4 Effects of 150 mM NaCl on selected transcripts in IR29.

3-4.1.4 IR29 gene expression compared to *Puccinellia*

The expression of these genes in IR29 at 48 h was also compared with the halophyte *Puccinellia*. *OsHAL2* was highly induced in the salt sensitive rice line at 150 mM NaCl in contrast to the salt tolerant *Puccinellia* which showed no changes. The putative TATA-binding protein was significantly induced in leaf of IR29, whereas in *Pucinellia* this gene was trongly repressed namely in root (Fig. 3-4.5)

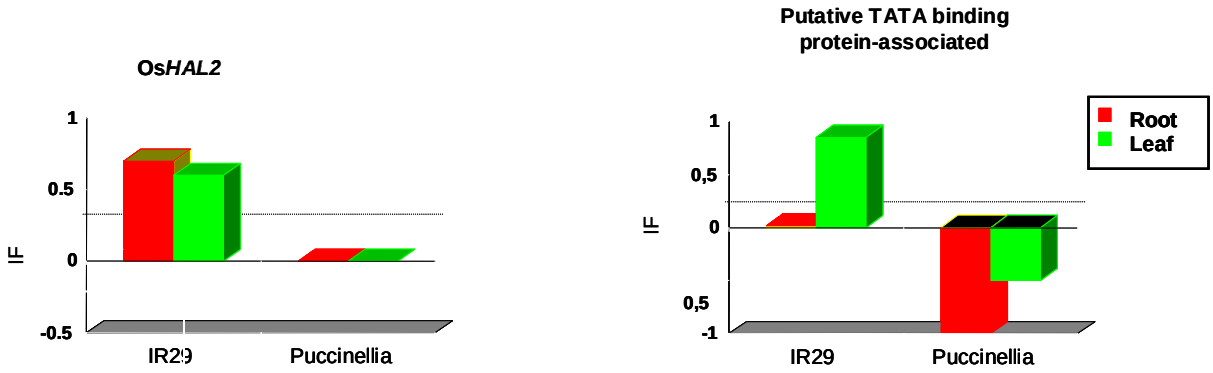


Fig. 3-4.6 Effects of 48h 150 mM NaCl on selected transcripts.

3-4.1.5 Effect of different stresses on the expression of analysed genes

The effect of other stresses was also examined in respect to the regulation of these genes. NaCl and LiCl repressed the expression of *OsHAL2* and the putative TATA-binding protein differently. While NaCl induced a moderate repression of both genes, LiCl strongly repressed the expression of *OsHAL2*. In contrast to NaCl and LiCl, treatment with rubidium increased the expression of *OsHAL2* slightly and the expression of the putative TATA-binding protein significantly (Fig. 3-4.7)

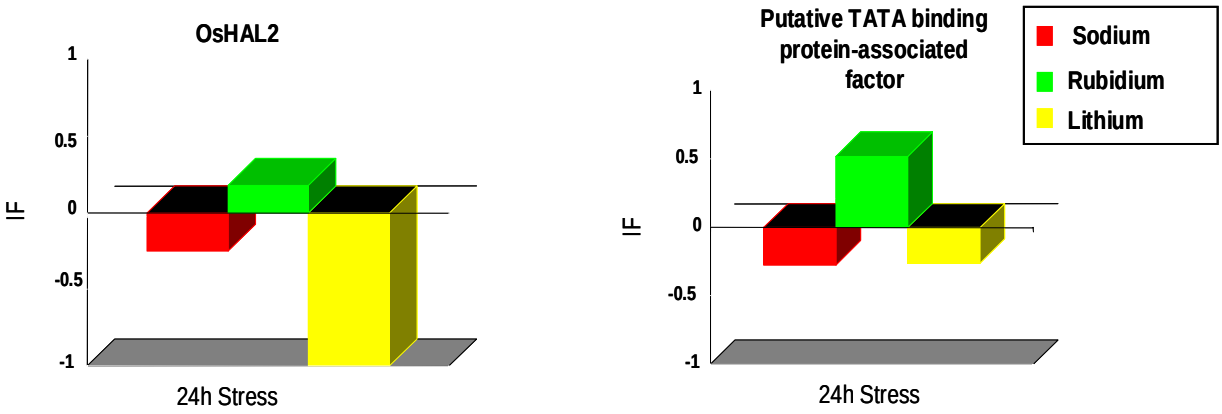


Fig. 3-4.7 Effects of NaCl, LiCl and RbCl on selected transcripts in roots.

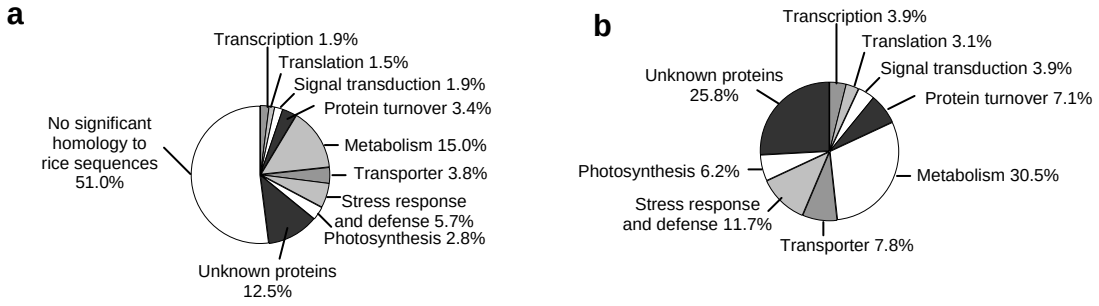
Differences could be seen between the salt sensitive rice IR29 and the halotolerant *Puccinellia* in the regulation of *OsHAL2* and TATA-binding protein genes. Thus, while both genes were significantly induced under salt stress in IR29, in *Puccinellia* the expression of these genes and as well as the expression other genes (not shown) seem to be non-affected or repressed. Although some differences in the regulation of genes in IR29 and in Pokkali (Table 3-4.1), the rice cDNA-array did not show significant differences in the expression of

genes under salt stress conditions. A comparative expression was then initiated between the salt sensitive line IR29 and the halophyte *Festuca* which share 89 % actin identities.

3-4.2 Comparison of the rice line IR29 with the halophyte *Festuca*

3-4.2.1 Annotation of genes on the *Festuca* cDNA-array

A subtracted cDNA library enriched for salt- responsive transcripts was established from the halotolerant grass *Festuca*. Sequence annotations of the *Festuca* cDNAs was performed by searching the rice genomic data base (www.Tigr.org). The *Festuca* cDNAs showed that 51 % had no significant homology to rice sequences, 15 % were related to the metabolism, 12.4 % were homologues to genes of unknown function, and 5.7 % were correlated to cell mechanisms of stress response and defence (Fig. 3-4.8 a). *Festuca* cDNA was also enriched for genes with high homology to rice sequences. Of genes with known function, we noted 30.5 % of the genes that were linked to metabolism, 25.8% belong to the genes with unknown function and 11.7 % for stress response and defence (Fig. 3-4.8 b).



a: Functional distribution of transcripts on the *Festuca*-cDNA-array.
b: Functional categories of transcripts on the *Festuca*-cDNA-array with significant homology to rice sequences with kown function identified in the rice genomic database.

Fig. 3-4.8 Comparison of functional representation of transcripts in *Festuca*-cDNA-arrays and their homologues in rice.

3-4.2.2-Expression analysis of *Festuca* cDNA-arrays

cDNA-arrays were hybridized with digoxigenin-dUTP labelled cDNA. Following normalization of the results representing leaf and root tissue under conditions of salt stress of rice and *Festuca* identified a number of differently regulated genes with high and low expression (Fig. 3-4.9). Considering the induction factors, the proportion of upregulated genes increased with the concentration of salt in *Festuca*, whereas in rice the proportion seemed to be reduced according to the duration of the constraint. 50 upregulated genes were

identified in *Festuca* at 125 mM NaCl after 6 h, whereas at 500 mM NaCl stress for the same time, this number was doubled. The same results were observed at 48 h salt stress. The number of repressed genes increased at low salt concentration. For example 150 genes were down-regulated when *Festuca* was treated with 125 mM NaCl for a 6 h period and less than 50 at 500 mM NaCl for the same time of salt exposition. In contrast, at 48 h 50 genes were downregulated at 125 mM NaCl, whereas the highest level of downregulated genes was noted at 500 mM NaCl. In rice, at 125 mM NaCl, the number of salt-induced genes was lower than those in *Festuca* for similar conditions of stress. In *Festuca*, more salt-induced genes were identified at 1 h than at 6 h in leaves, whereas in roots (results not shown), the number of salt-induced genes was below 50 and remained unchanged from 1 to 6 h salt treatment. In *Festuca* and rice respectively, 60 and 50 genes were upregulated at 1 h, and 50 and 40 after 6 h salt stress in leaf tissue. The number of repressed genes in rice was also lower in leaves, while it reached 150 at 6 h salt stress in *Festuca*.

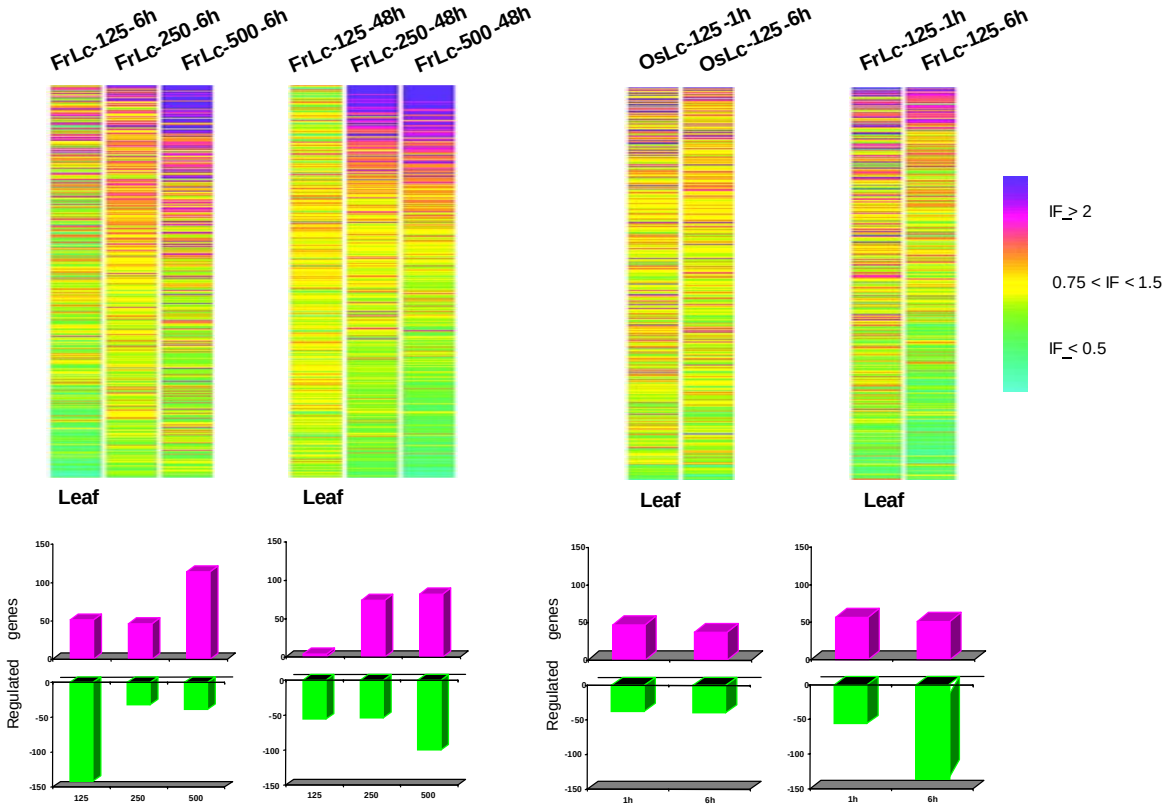


Fig. 3-4.8 Comparison of salt responsive gene expression in salt-stressed *Festuca* and IR29 plants using hierarchical cluster analysis. The log-2 ratio values of salt response ESTs were used for hierarchical cluster the violet areas represent up-regulated genes, the green areas represent downregulated genes.

3-4.2.3 Gene expression and functional distribution of *Festuca* cDNA-arrays

According to its function, genes of the *Festuca* cDNA-arrays were grouped and the expression was compared in accordance to salt concentration and the duration of the constraint. Thus, expression specifically showed similar gene regulation of IR29 and *Festuca* with almost a few genes upregulated at 125 and 250 mM NaCl 6 h in all groups (Fig. 3-4.10). The halotolerant *Festuca* showed a high upregulation only at 500 mM NaCl. Genes linked to metabolism, defence and stress response, and those with unknown functions mostly increased at 500 mM NaCl 6 h. The other significant difference could be seen in the repressed genes. More genes related to the metabolism and the group of unknown proteins were repressed in *Festuca* at 6 h. The number of down regulated genes in those groups decreased at 250 and 500 mM NaCl and reached the level of IR29 treated with 125 mM NaCl at 6 h.

The functional categories of channel mediated transport as well as transcription, translation and signal transduction were influenced by salt stress with a small number of genes in both species only. However, at the same salt treatment (125 mM NaCl), IR29 induced more genes related to channel dependent transport, whereas *Festuca* induced more genes related to transcription and signal transduction (Fig. 3-4.9).

In contrast to short exposition, salt stress treatment of 48 h tended to increase the number of repressed genes according to the concentration of salt (Fig.3-4.10). Except for the genes related to channels and other transporters where the highest number of gene were regulated at 250 mM NaCl, the proportion of salt induced genes in all groups increased and reached the maximum at 500 mM NaCl 48 h.

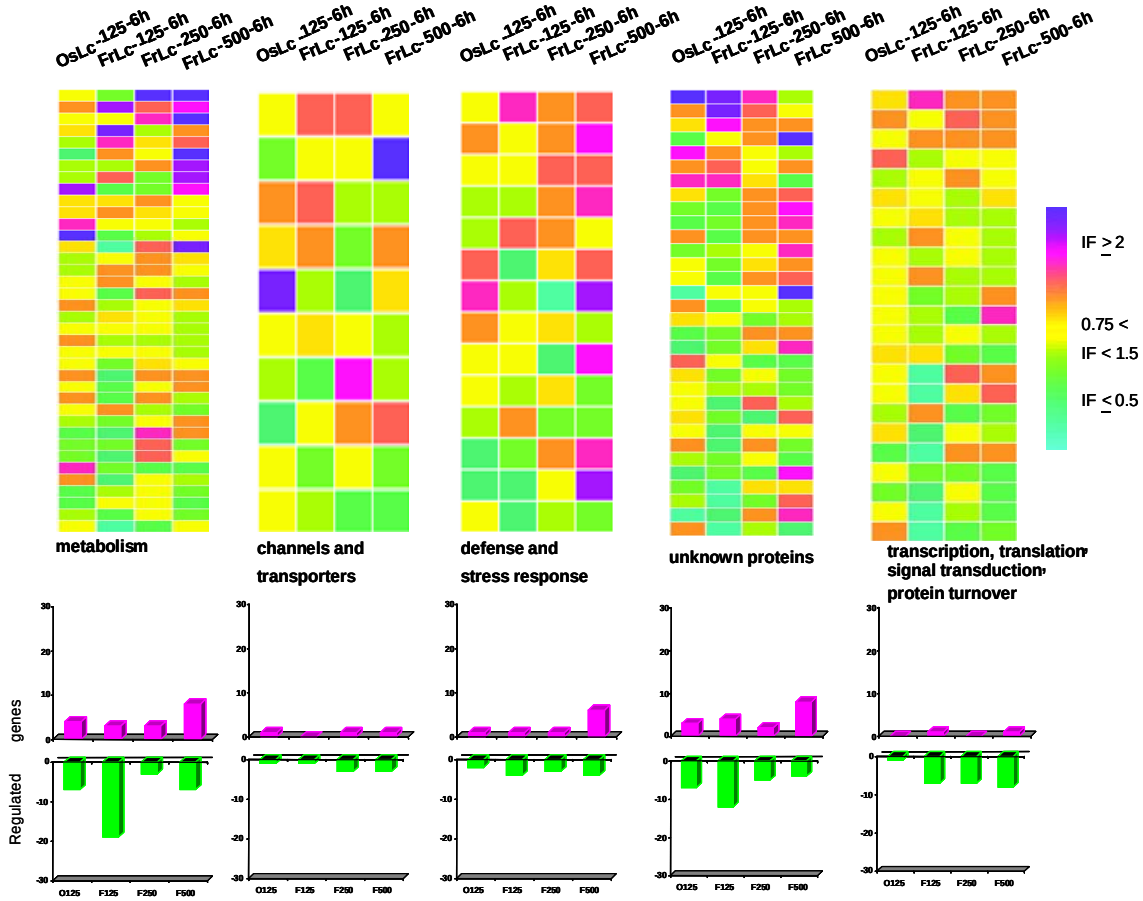


Fig. 3-4.9 Comparison of the salt response of functional groups. Salt stress was administrated to *Festuca* and rice plants for 6 h ; the data from array analysis were processed using hierarchical cluster algorithms. The log-2 ratio values of salt response ESTs were used for hierarchical clustering. The violet areas represent upregulated genes the green ones downregulated genes.

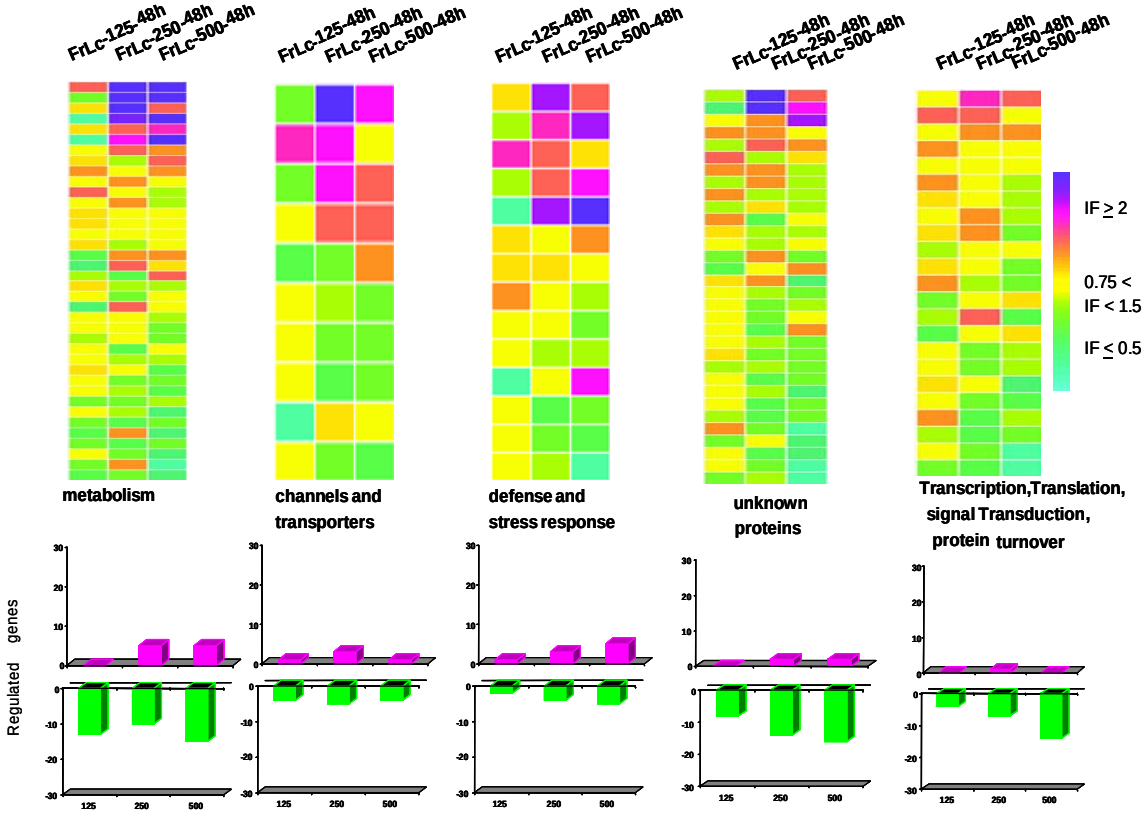


Fig. 3-4.10 Comparison of the salt response of functional groups. Salt stress was administrated to *Festuca* plants for 48 h at different concentrations; the data from array analysis were processed using hierarchical cluster algorithms. The log-2 ratio values of salt response ESTs were used for hierarchical clustering. The violet areas represent upregulated genes the green ones downregulated genes.

3-4.2.4 Verification of cDNA-array expression by Northern-blot

To confirm the expression data obtained by cDNA-array hybridizations, Northern blots using 4 probes were performed. The chosen probes were identified as salt-induced genes and had an induction factor higher than 1 in *Festuca* (Fig. 3-4.11 a) and in rice (not shown). These salt induced genes were: the metabolic gene delta 1-pyrroline-5-carboxylate synthetase (*OsDPCS*), a transporter gene with a putative ABC-transporter, and two genes responsible for stress response and defence: Dehydrine rab 16b (*OsRAB16*) and a putative catalase. The Northern blot analyses confirmed the expression status obtained by cDNA-arrays and showed a parallel increase of transcript amount with the salt concentration for *OsRAB16*, *OsDPCS* and the ABC-transporter, whereas the transcript abundance of catalase gene decreased with the increase of salt concentration (Fig. 3-4.11 a).

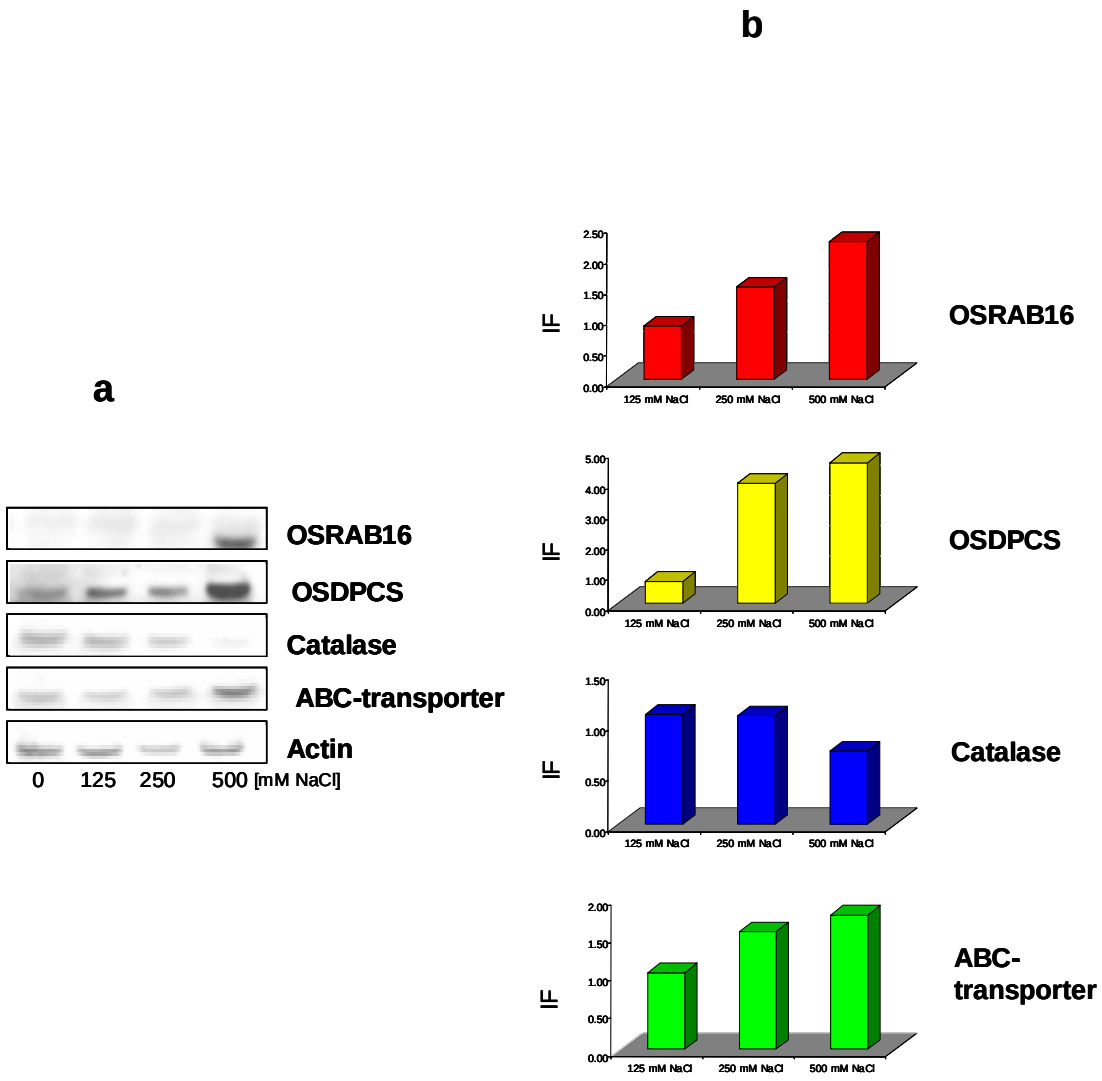


Fig. 3-4.11 Northern blot confirmation of *Festuca* cDNA-array results. **a:** Northern blot was used to examine the expression of four genes in *Festuca* plants under 0, 125, 250 and 500 mM NaCl. Each lane was loaded with 20 µg total RNA. **b:** Gene expression in *Festuca* cDNA-array exposed at 125, 250 and 500 mM NaCl during 48 h. IF represents the induction factor. *OsRAB16*-Dehydrine rab 16b, *OsDPCS*-1-pyrroline-5-carboxylate synthetase

3-4.2.5 Functional classification of salt induced genes

Expressed genes were classified in relation to their function. 7 groups have been identified (Table 3-4.2 Appendix 1) among them metabolism, unknown function protein, transcription, translation factors, signal transduction, protein turnover, channel and transport, and stress response. Genes linked to the metabolism constitute the most abundant group and on the 48 genes that constituted this group, an average of 38 genes were up-regulated in conditions of salt stress in *Festuca* and in rice. Among these induced genes, genes linked to antioxidant activity such as oxidoreductase and dehydrogenase. Signal transduction, transcription and

translation constituted a small group with some minor differences in expression between rice and *Festuca*. In this group, the putative protein kinase SPK3, the putative kinase *OSK4* and a translation initiation factor (*OsTIF*), were identified. Published studies have already revealed the advantage of some transcription and signal transduction genes in tolerance to salt stress. For example, expression of the protein kinase SOS2 in *Arabidopsis* and in *Brasica napus* revealed slight increases of leaf SOS2 mRNA under salt stress treatment (Liu et al., 2000 and Wang et al., 2004). Subsequently, Guo et al. (2004) showed that the salt tolerance increased in the plants over-expressing the SOS2 kinase suggested that the level of activated kinase may be limited in *Arabidopsis in vivo*. By overexpression of SOS2, the authors demonstrated that increasing active SOS2 levels *in planta* improved the tolerance to salt significantly when compared to the wild type. In agreement with these data, the reinforcement of *OsSPK3* and *OSK4* genes in the salt sensitive rice line IR29 might improve the tolerance to salt. In order to investigate whether the overexpression of protein kinases may improve the salt tolerance of the salt sensitive rice line IR29, transformations were made and transgenic rice plants carrying these genes have been studied.

In contrast to the SPK3 protein kinase, transcript abundance of the translation initiation factor SU1 (*OsTIF*) under the same stress conditions was higher in *Festuca* than in rice. In *Beta vulgaris*, under control and salt stress conditions, Rausell et al. (2003) noted a strong increase of a translation initiation factor (*BveIF).* Expression of *BveIF* in *Arabidopsis* and in eIF1A deficient yeast strain, improved the adaptation of *Arabidopsi*s plants and yeast to growth in salt stress medium as well as the tolerance to LiCl stress in yeast. In addition, under salt stress, *BveIF* improved translation and therefore the enhancement of protein production. These results suggested that the translation initiation factor eIF1A is an important determinant of sodium tolerance in yeast and plants. Therefore it was interesting to know if overexpression of eIF1-type translation initiation factor (*OsTIF*) in the salt sensitive rice line would improve its tolerance to salt.

3-5 Characterization of *OSTIF* transgenic plants

3-5.1 Expression of *OsTIF* in the rice line IR29 and in *Festuca*

To determine how the endogenous gene *OsTIF* encoding a translation initiation factor was expressed in the salt sensitive rice line IR29 and in the halophyte *Festuca*, Northern-blot analyses were made for control and salt stress conditions. Results showed that the *OsTIF* was expressed in rice control conditions at a level similar to the expression of actin, whereas in *Festuca* the expression of *OsTIF* seemed to be repressed in the same conditions. Submitted to salt stress, the salt sensitive line IR29 showed a slight decrease of its transcript amounts 48h after 125 mM NaCl, whereas in the halophyte *Festuca*, expression of this translation factor increased progressively from 125 mM to 500 mM NaCl and reached the maximum at the high salt concentration (500 mM NaCl) (Fig.3-5.1). These results suggest that salt stress induces the expression of *OsTIF* translation factor. It might be possible to suggest that this gene is correlated to the salt tolerance in *Festuca*, thus, overexpression of this gene in the salt sensitive line IR29 might improve the tolerance of IR29.

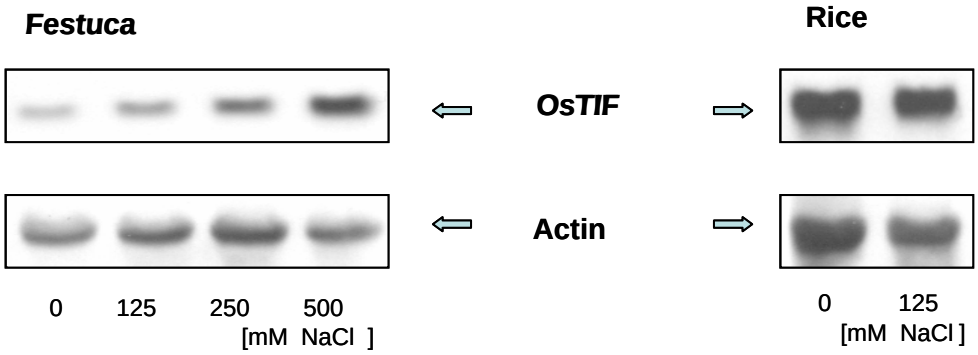


Fig. 3-5.1 Northern blotting analysis of the *OsTIF* expression in *Festuca* and in rice under different concentrations of salt. 0, control; 125 mM NaCl; 250 mM NaCl; 500 mM NaCl.

3-5.2 Molecular characterization of *OsTIF* in transgenic plants.

3-5.2.1 Overexpression of *OsTIF* in IR29

To better understand the role of *OsTIF* in salt tolerance, IR29 transgenic plants were generated by introducing a construct for overexpression of *OsTIF*. Transgenic rice plants carrying the *OsTIF* cDNA insert were examined by Northern blot in the control growth conditions. The plants showed no significant difference in the expression of *OsTIF* gene

between transgenic and wild-type plants although transgenic lines showed a slight increase (Fig. 3-5.2).

In Fig.3-5.1, salt stress strongly induced the expression of *OsTIF* in the halophyte *Festuca*. To determine the expression conditions of the transgene *OsTIF* in the transgenic plants, plants were treated with 200 mM NaCl during 3 h. The results obtained by Northern blot (not shown) and RT-PCR showed an overexpression of all IR29 transgenic lines, while the wild type decreased (Fig. 3-5.3 a).

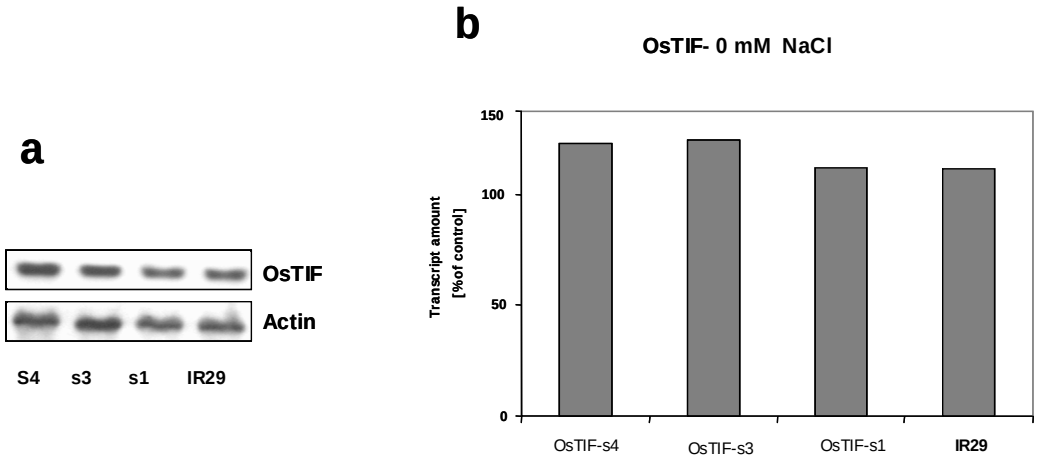


Fig. 3-5.2 a: Northern blot analysis of the *OsTIF* expression in transgenic rice lines and wild-type plants under normal growth conditions. S4, S3, S1 represent transgenic *OsTIF* plants. IR29 represents the wild-type **b:** Densitometric analysis of transcript levels shown in **a** .The transcript amounts of *OsTIF* in leaf tissues of transgenic rice lines and wild-type plants grown under control conditions were set to 100%. The transcript amounts were normalized to actin.

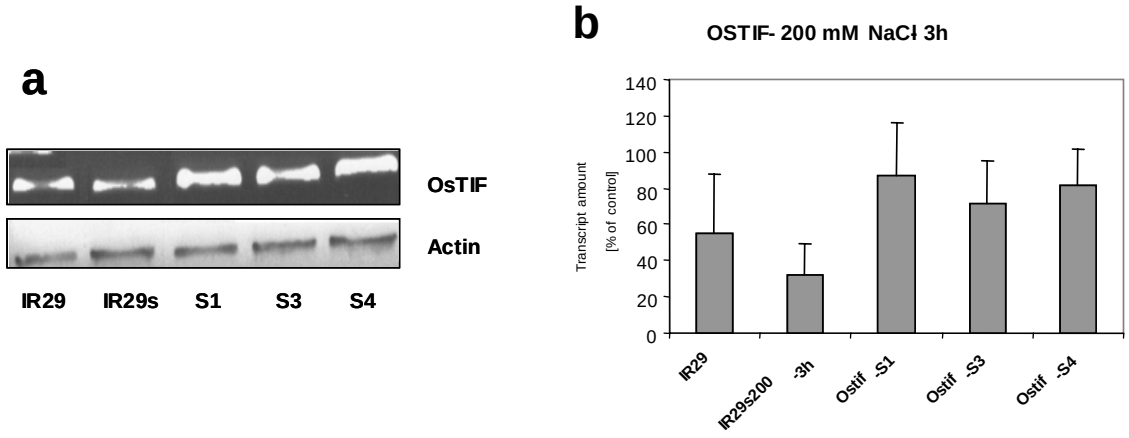


Fig 3-5.3. **a:** ***OsTIF*** **expression in transgenic rice lines and in wild-type plants under 200 mM NaCl 3 h**. S4, S3, S1 represent transgenic *OsTIF* plants.,IR29s represents the wild-type salt stressed, IR29 represents the wild type in control condition. **b:** Densitometric analysis of RT-PCR transcript levels shown in **a**. The transcript amounts of *OsTIF* in leaf tissues of transgenic rice lines and wild-type plants were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-5.2.2 Overexpression of *OsTIF* in IR29 and effect on the expression of *OsVHA-B* and *OsNHX1* genes

Expression of subunit B of V-ATPase (*OsVHA-B)* and *OsNHX1*, regulating the Na^+ detoxification of the cells, were also examined in the transgenic rice plants. Results represented in the Fig. 3-5.4 showed a different mode of expression in transgenic plant compared to the wild-type. The transgenic plant lines showed a higher expression of *OsVHA-B* while the wild-type decreased it whereas the expression of *OsNHX1* in transgenic and wild-type was significantly increased. However, more transcript amounts were seen in the wild-type than in the transgenic plants.

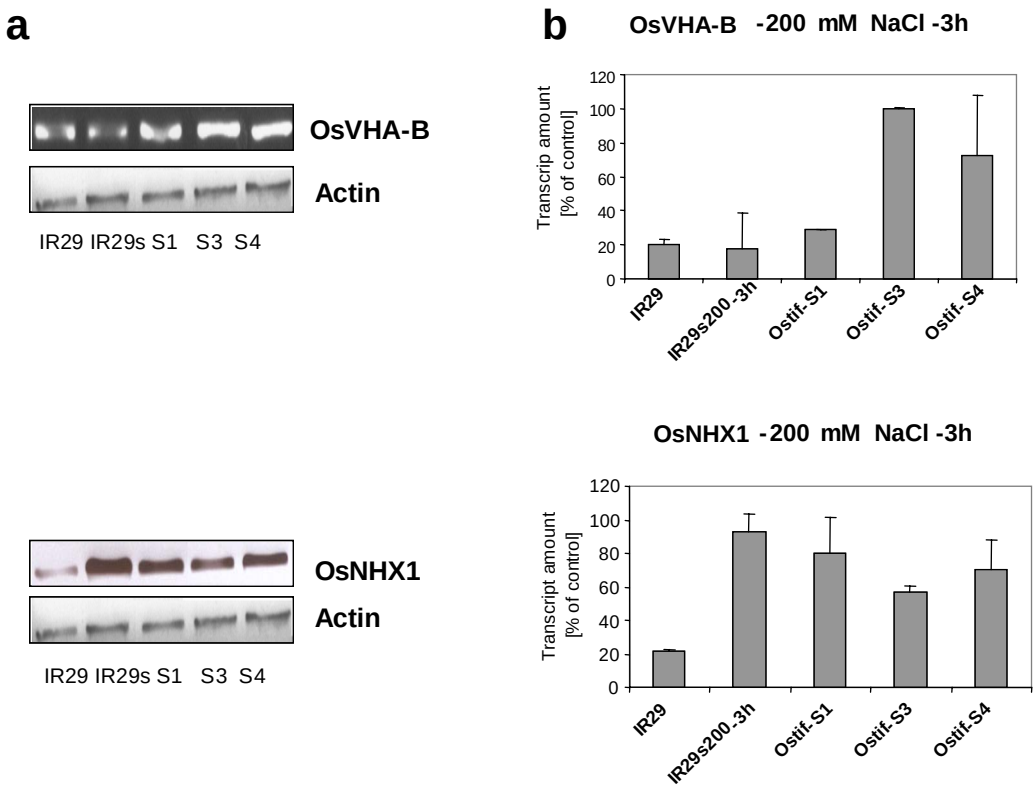


Fig. 3-5.4 *OsTIF* transgenic plants and expression of the specific genes *OsVHA-B* and *OsNHX1* under 200 mM NaCl 3 h. S4, S3, S1 represent transgenic *OsTIF* plants, IR29s represents the wild-type salt stressed, IR29 represents the wild type in control condition. **b:** Densitometric analysis of RT-PCR transcript levels shown in **a** The transcript amounts of *OsVHA-B* and *OsNHX1* in leaf tissues of transgenic rice lines and wild-type plants were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-5.2.3 Overexpression of *OsTIF* in IR29 and effect on the expression of other genes

3-5.2.3.1 cDNA-arrays

In addition to Northern-blotting and RT-PCR analysis, expression profiles of *OsTIF* transgenic plants under normal growth conditions have been compared with the wild-type plants. Probes from leaves of wild-type and transgenic plants grown under control conditions were hybridized to the *Festuca* cDNA-array. The analysis of the hybridization identified some genes induced under normal conditions (Table 3-5.1). Functional classification of these genes indicated many genes with a role in the metabolism, for example, those linked to the antioxidant activity**.** Among these genes, the oxidoreductase, short chain dehydrogenase/reductase showed an induction factor of 2.5 in *OsTIF* overexpression under control conditions. Oxidative stress is increased in conditions of salt stress and might enhance the expression of genes with antioxidant activity as for example oxidoreductase (Sunkar et al., 2003). To verify this hypothesis, the expression of the oxidoreductase was analysed by RT-PCR under 200 mM NaCl. As in control condition (Table 3-5.1), oxidoreductase was

strongly expressed in transgenic plants under salt stress, while it was repressed in the wild-type (Fig. 3-5.5).

Table 3-5.1: Genes induced in *OsTIF*-sense transgenic plants under normal growth conditions. Genes were identified by hybridization of digoxigenin-dUTP-labelled cDNA-array transgenic rice plants to *Festuca* cDNA-arrays.

Gene Name	Functional Category	Induction Factor
Protein kinase, putative	Signal transduction	3.5
ATP-dependent Clp protease	Protein turnover	2.8
Glycine dehydrogenase	Metabolism	2.6
Oxidoreductase	Metabolism	2.5
RuBisCO activase	Photosynthesis	2.2
Pyruvate kinase, putative	Metabolism	2.2
Hypothetical protein	Unknown	2.2
PAP fibrillin, putative	Transcription	1.9
Glutaredoxin	Stress response	1.9
Chaperonin, putative	Protein turnover	1.9
Expressed protein	Unknown	1.9
Phosphatidylglycerolphosphate synthase	Metabolism	1.7
Hypothetical protein	Unknown	1.6
Cysteine proteinase 1 precursor	Protein turnover	1.5
RNA recognition motif, putative	Transcription	1.5
Peptide chain release factor eRF/aRF	Translation	1.5
Carboxymethylene-butenolidase, putative	Metabolism	0.4
Catalase (EC 1.11.1.6)	Stress response	0.4
Hypothetical protein	Unknown	0.3

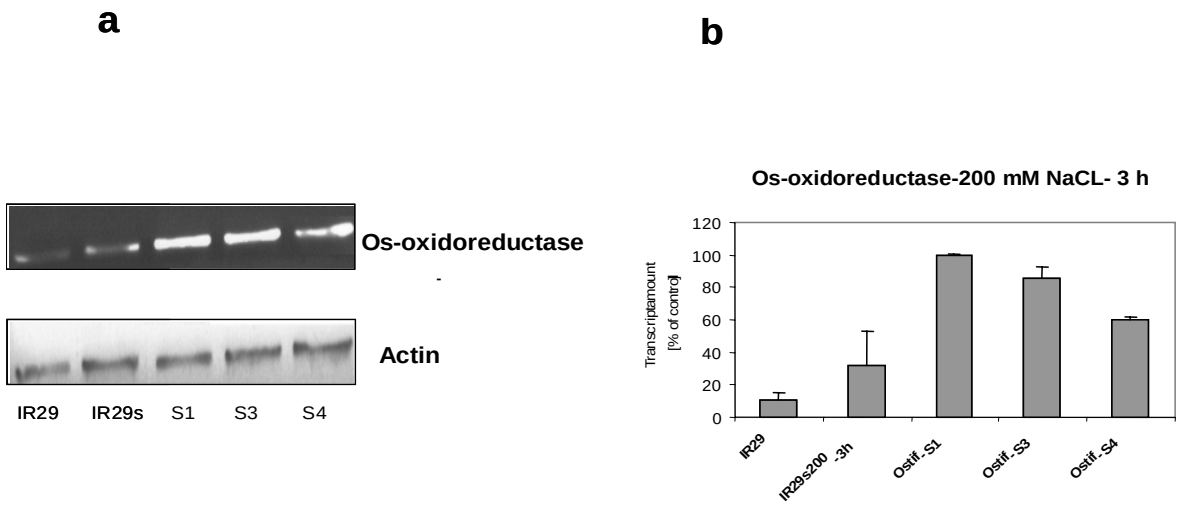


Fig. 3-5.5 *OsTIF* transgenic plants and expression of the Os-oxidireductase specific gene under 200 mM NaCl 3 h. S4, S3, S1 represent transgenic *OsTIF* plants, IR29s represents the wild-type salt stressed, IR29 represents the wild type in control conditions **b:** Densitometric analysis of RT-PCR transcript levels shown in **a** The transcript amounts of Os-oxidoreductase in leaf tissues of transgenic rice lines and wild-type plants were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3.

3-5.3 Physiological and morphological characterization of transgenic rice plants

3-5.3.1 Effects of overexpression of *OsTIF* gene on vegetative phases of growth

3-5.3.1 Germination

The germination of transgenic plants in control conditions showed a higher rate with 80 to 100%, while the wild-type showed a low rate (60 %) (Fig 3-5.6). Treatment with 50 mM NaCl, decreased strongly the germination rate of the wild-type, whereas transgenic plants were less affected and showed a germination improvement for example in the line S4 (Fig. 3-5.7).

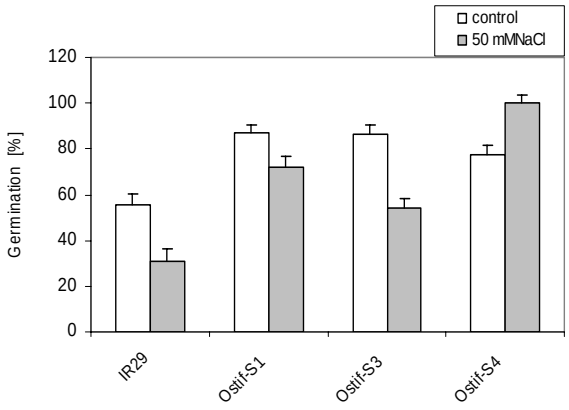


Fig 3-5.6 Germination of wild type and transgenic seeds in H_2O (control) and 50 mM NaCl. Germination percentage were determined after 7 days. Data represent means ± SD, n=3.

Fig. 3-5.7 Habitus seed lines after 7 d germination in H_2O and 50 mM NaCl.

3-5.3.2 Young seedlings

Under normal growth conditions, transgenic and wild type plants showed a uniform growth (Fig. 3-5.8a). When submitted to 50 mM NaCl salt stress, transgenic plants kept constant growth or improved their shoot-growth rates (S1 and S4), while the wild type decreased it significantly (Fig. 3-5.8b).

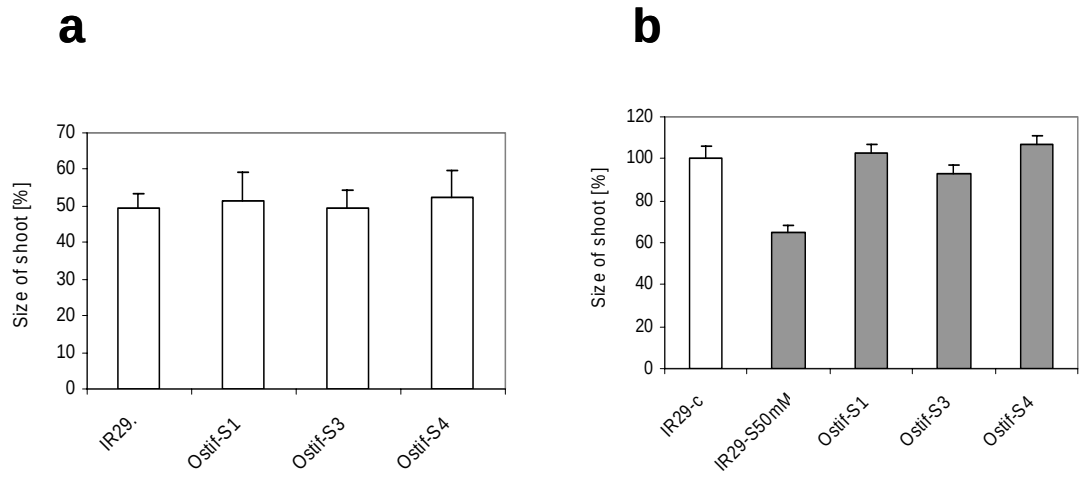


Fig. 3-5.8 Growth of wild-type and transgenic young seedlings under control growth conditions (a) and in 50 mM NaCl (b).

3-5.3.13 Photosynthetic activity

The photosynthetic activity realised after 100 and 150 mM NaCl treatment showed no difference in transgenic versus control plants in the two different NaCl concentrations (Fig. 3-5.9 a, b). After 48h salt stress, transgenic plants kept constant or slightly reduced photosynthetic yield however in the wild type the reduction reached 30 %. The S4 line maintained a good photosynthetic activity under 150 mM NaCl treatment for 48 h. At 96 h, 1/2 of wild-type leaf became necrotic and reduced therefore the photosynthetic activity. However, S4 and Pokkali conserved a photosynthetic activity in the large part of the leaves (Fig. 3-5.9 c). After 2 weeks in 150 mM NaCl, wild-type plants were completely dead, whereas the salt tolerant line Pokkali and the transgenic line S4 maintained yet green leaves (Fig. 3-5.10).

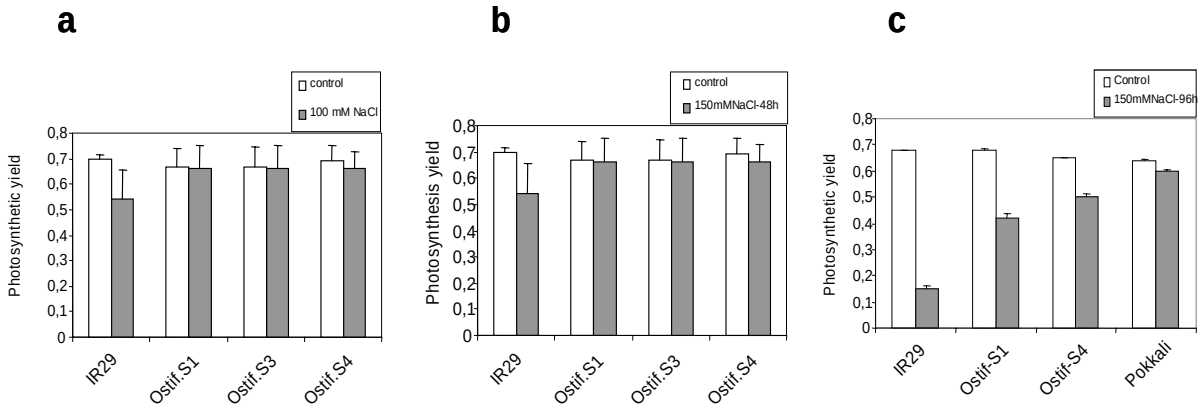


Fig. 3-5.9 Photosynthetic yield obtained at 48 h under control and 100 mM NaCl conditions (a) and under control and 150 mM NaCl conditions (**b**). **c-** photosynthetic yield obtained after 96 h after treatment with 150 mM NaCl.

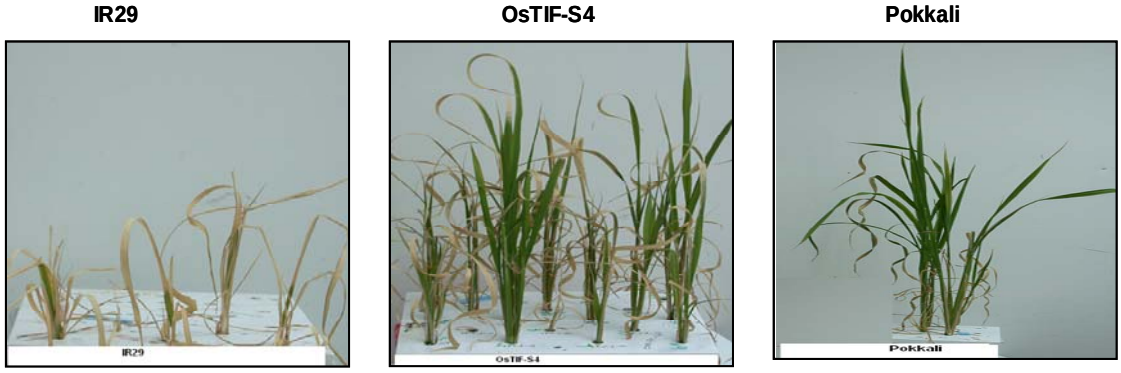


Fig. 3-5.10 Phenotype of wild-type and transgenic rice and Pokkali plants after 17 days in 150 mM NaCl.

3-5.3.14 Ion uptake in transgenic plants

In rice the capacity of salt tolerance might be related to Na^+ and Cl^- uptake (Golldack et al., 2002; Diedhiou and Golldack, 2005). To verify how transgenic plant proceed, ion content was measured under salt stress and indicated that Cl^- and Na^+ were abundantly accumulated in the wild-type plants (100%), whereas transgenic plants showed only 30 % accumulation in leaves (Fig. 3-5.11). Ca^{2+} content was very low (25%) in both transgenic and wild type plants, whereas K^+ was highly accumulated in both transgenic and wild-type leaf tissues, however, wild-type plants showed a slight increase.

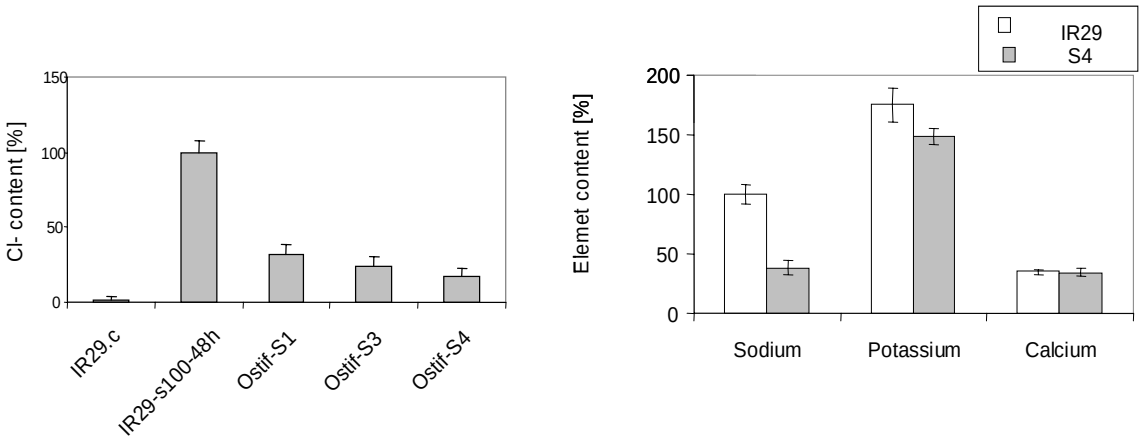


Fig. 3-5.11 Ion accumulation in leaves of wild-type and transgenic plant lines grown for 48 h under 100 mM NaCl (Cl^-) or 150 mM NaCl (Na^+, $K^+$and Ca^{2+}). Data represent means ± SD, n=7.

3-6 Characterization of *OsSPK3* transgenic plants

3-6.1 Differences of salt-dependent expression of *OsSPK3* in rice and in *Festuca*.

The serine-threonine protein kinase *OsSPK3* was identified in a subtracted cDNA library from *Festuca* enriched for salt-responsive genes, and in the present study a detailed analysis was performed on the role of the kinase in plant salt adaptation. By Northern-type RNA hybridizations, expression of *OsSPK3* was detected in non-stressed control plants of *Festuca* and rice (Fig. 3-6.1). In *Festuca*, salt stress of 125 mM NaCl for 2 days had no major effect on the transcript level of *OsSPK3*. At 250 mM NaCl the transcript level decreased drastically whereas at 500 mM NaCl the transcript abundance increased to a higher level than in the control. In rice, treatment with 125 mM NaCl for 48h caused a slight decrease of *OsSPK3* transcript abundance.

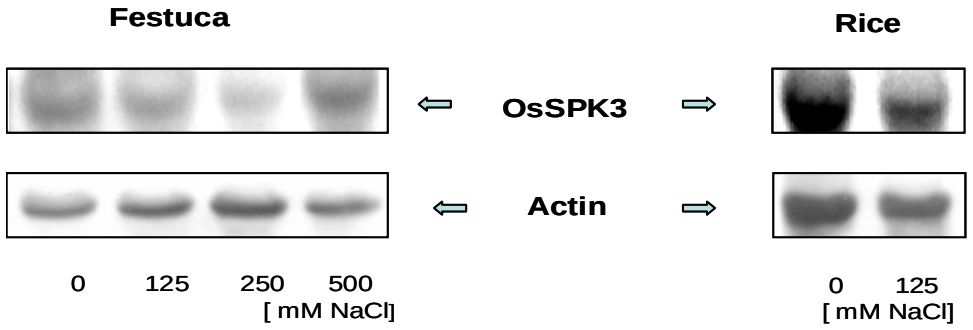


Fig. 3-6.1 Northern blotting analysis of the *OsSPK3* expression in *Festuca* and in rice under different concentrations of salt. 1, control; 2, 125 mM NaCl; 3, 250 mM NaCl; 4, 500 mM NaCl 48 h. Data represent means ± SD, n= 3.

3-6.2 Tissue specific expression of *OsSPK3*

OsSPK3 was inserted in rice plants and investigated for its function in salt tolerance by overexpression under control of the 35S CaMV promoter. For an analysis of the subcellular localization of the *OsSPK3* protein, constructs for the expression of the *OsSPK3* open reading frame cDNA were fused to the green fluorescent protein (GFP) reporter gene driven by the 35S-CaMV promoter. Onion epidermis cells were transformed with the translational fusion and fluorescence emission of GFP was monitored under a confocal laser scanning microscope. In cells incubated for 24 hours in 0.5 x MS nutrient medium, strong GFP signals

were detected in the nucleus (Fig. 3-6.1 a, b), whereas cells bombarded with the empty vector as a control showed no fluorescence (Fig. 3-6.1 c). To extend the results obtained from the onion epidermal cell system, *Arabidopsis* mesophyll protoplasts were isolated and similarly transformed with the *OsSPK3*-GFP transcriptional constructs. After incubation of transformed protoplasts for 24 hours, GFP-derived fluorescence emission was also detected in the cytoplasmic compartment (Fig. 3-6.1 d).

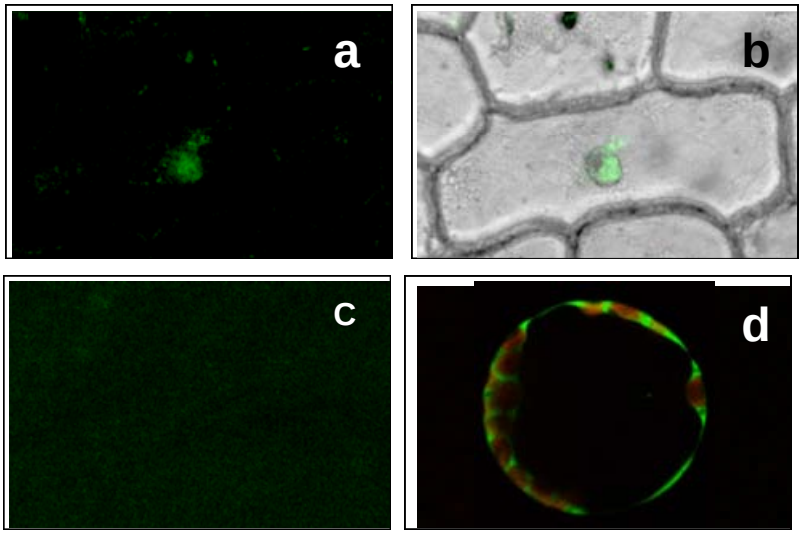


Fig. 3-6.2 Localisation of *OsSPK3* associated to GFP in onion cells [**a**, **b** and **c** (non transformed) Popova and Golldack unpublisch] **and in *Arabidopsis*** cells (**d**).

3-6.3 Functional Characterization of *OsSPK3* transgenic plants

To investigate how the serine-threonine protein kinase *OsSPK3* activity is regulated, transcript abundance was analyzed in wild-type and in transgenic plants submitted to normal and salt stress conditions. Northern blot and RT-PCR analyses were carried out to establish first the transcript level of *OsSPK3* in control growth conditions in leaves of transgenic plants compared to the wild type. Northern blotting analysis indicated no significant difference in the expression of *OsSPK3* in both wild-type and transgenic plants in control growth conditions. Despite this expression, transgenic lines showed a slight increase compared to wild-type plants (Fig. 3-6.3).

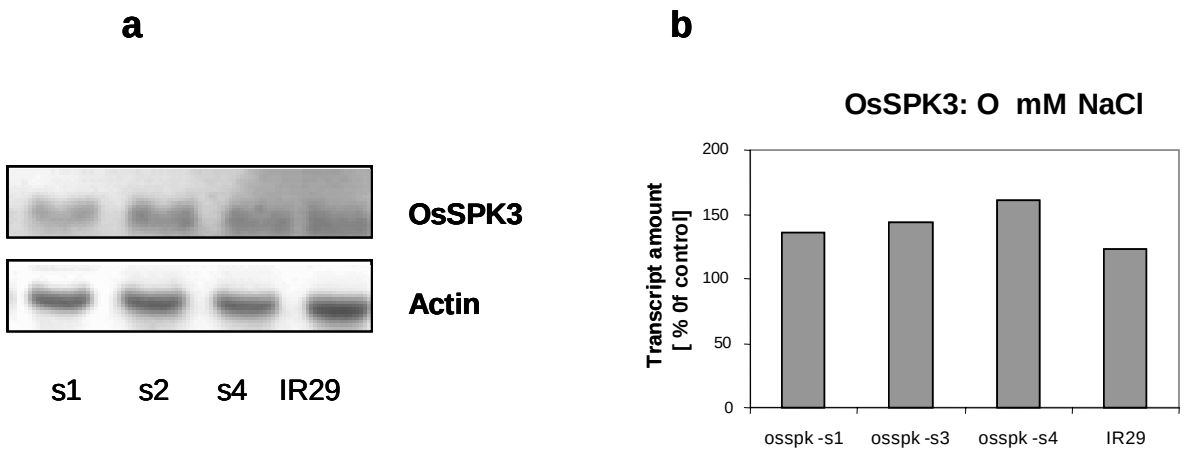


Fig. 3-6.3 a: Northern blotting analysis of the *OsSPK3* expression in transgenic plants grown at control growth conditions. S1, S2, S4, represent transgenic *OsSPK3* plants, IR29 represents the wild type. **b:** Densitometric analysis of transcript levels shown in **(a)**. The transcript amounts of transgenic lines in leaf tissues grown under control conditions were set to 100%. The transcript amounts were normalized to actin Data represent means ± SD, n=3.

3-6.2.1 *OsSPK3* transcriptome in transgenic plants under salt stress

To investigate the expression of *OsSPK3* in the transgenic plants under conditions of stress, Northern-blot and RT-PCR were performed. Analysis of the amplified fragments showed that *OsSPK3* was differently expressed in wild type and transgenic plants under salt stress conditions (Fig.3-6.4). While expression of *OsSPK3* in the wild-type was reduced to 150 mM NaCl, transgenic lines S1 and S4 were up-regulated significantly.

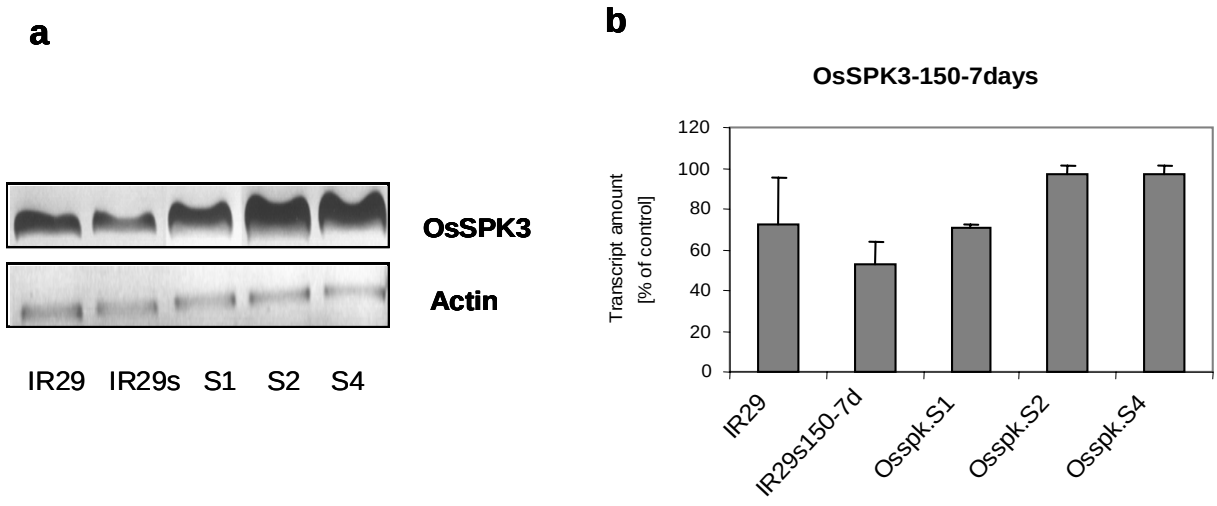


Fig.3-6.4 a: Expression of transgenic plants overexpressing *OsSPK3* compared to wild-type plants. S1, S2, S4, represent transgenic *OsSPK3* plants, IR29s represents the wild-type salt stressed and IR29 represents the wild type in control conditions. **b:** Densitometric analysis of RT-PCR transcript levels (**a**). The transcript amounts of *OsSPK3* in leaf tissues of transgenic rice lines and wild-type plants grown 7 days under control and 150 mM NaCl conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n= 3.

3-6.3.2 Effect of *OsSPK3* on the expression of other genes.

To investigate the role of *OsSPK3* insert, wild type and transgenic plants were stressed and RT-PCR were constructed using specific oligonucletide primers. Among the examined genes, V-ATPase subunit B (*OsVHA-B)* and the Na^+/H^+ antiporter *OsNHX1* which are implicated in the detoxification of cells and Na^+ sequestration into the vacuole were investigated. According to Golldack and Dietz (2001) and Zhang and Blumwald (2001) respectively, *OsVHA-B* and *OsNHX1* are involved in salt stress adaptation and improve the tolerance to NaCl. In comparison with those findings, expression of both genes in transgenic rice plants carrying an *OsSPK3* insert showed an opposite regulation (Fig. 3-6.4). Expression of *OsVHA-B* energizing the vacuolar sequestration of Na^+, showed a similar expression in wild-type and *OsSPK3*. *OsVHA-B* was abundantly induced in transgenic plant exposed for 7 days to 150 mM NaCl, while wild-type plants showed no changes. In contrast, *OsNHX1* was strongly increased in the salt-treated wild-type whereas the transgenic lines showed only 20 to 30 % increases (Fig 3-6.4)

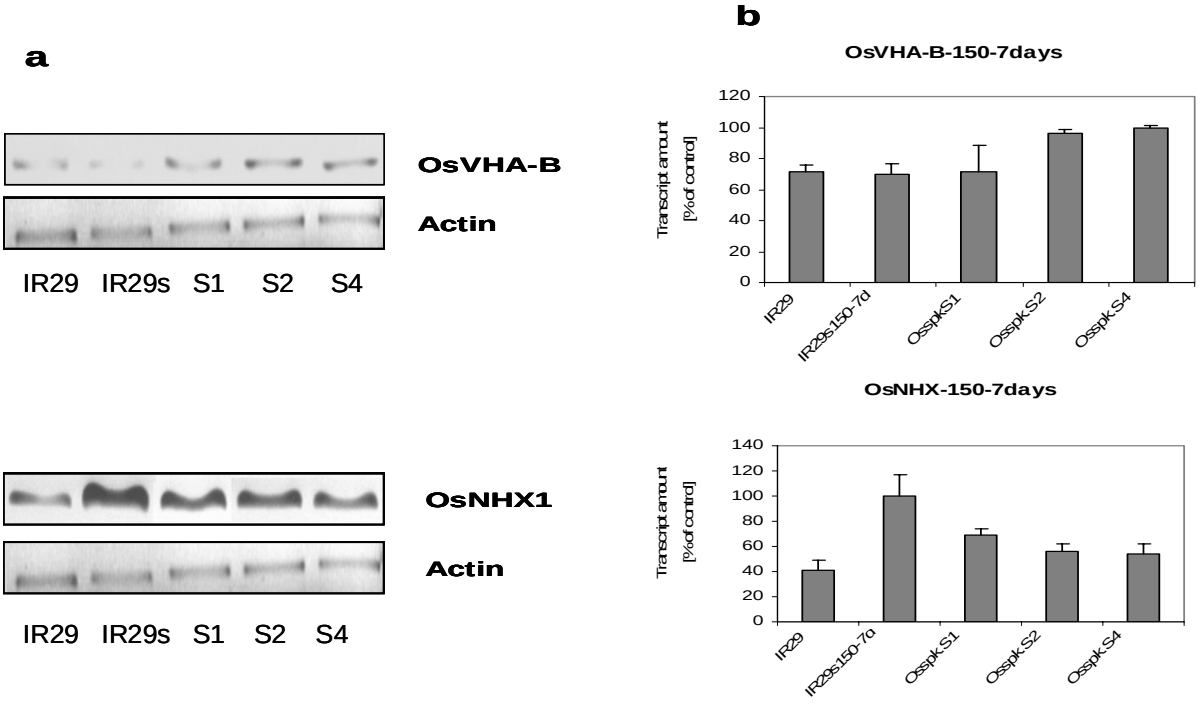


Fig.3-6.5 The effect of overexpression of *OsSPK3* on transcripts amounts of *OsVHA-B* and *OsNHX1*. S1, S2, S4, represent transgenic *OsSPK3* plants, IR29s represents the wild-type salt stressed and IR29 represents the wild type in control conditions **b:** Densitometric analysis of RT-PCR transcript levels shown in **a**. The transcript amounts of *OsNHX1* and *OsVHA-B* respectively in leaf tissues of transgenic rice lines and wild-type plants grown under control and 150 mM NaCl during 7 days were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n= 3.

The effect of *OsSPK3* in regulation of other genes was also investigated. Hybridization of transgenic plant cDNA samples to the subtracted cDNA library array from *Festuca* enriched for salt-responsive genes showed a high expression of genes with antioxidant activity when *OsSPK3* transgenic lines had been submitted to control growth conditions (Table 3-6.1). Therefore, we attempted to study the transcript abundance of an oxidoreductase and a catalase. According to the previous results, expression of both genes under the control of the wild type revealed an induction factor higher than 2 (Table 3-6.1).

Table 3-6.1 Genes induced in *OsSPK3* transgenic plants under normal growth conditions. Genes were identified by hybridization of digoxigenin dUTP-labelled cDNA from transgenic rice plants to *Festuca* cDNA-arrays.

Gene Name	Functional Category	Induction Factor
Chaperonin, putative	Protein turnover	3.2
Oxidoreductase	Metabolism	2.9
PAP fibrillin, putative	Transcription	2.8
Catalase (EC 1.11.1.6)	Stress response	2.7
ATP-dependent Clp protease	Protein turnover	2.5
Pyruvate kinase, putative	Metabolism	2.2
Hypothetical protein	Unknown	2.1
Expressed protein	Unknown	1.8
Cysteine proteinase 1 precursor	Protein turnover	1.5
Oxidoreductase (aldo/keto reductase)	Metabolism	1.5
ABC transporter, putative	Channels and transporters	0.5
Hypothetical protein	Unknown	0.1

RT-PCR analysis using mRNA from stressed probes indicated an opposite regulation in transgenic and wild-type plants (Fig. 3-6.3). The catalase expression was significantly induced in wild–type under control conditions, while it dropped very strongly to its least expression under 150 mM NaCl treatment for 7 days. In transgenic plants, catalase expression under 150 mM NaCl for 7 days was reduced by 50 % to 70 %. In contrast, oxidoredutase regulation was very low in control conditions and reached the highest level under salt treatment in the wild-type and was decreased in transgenic lines.

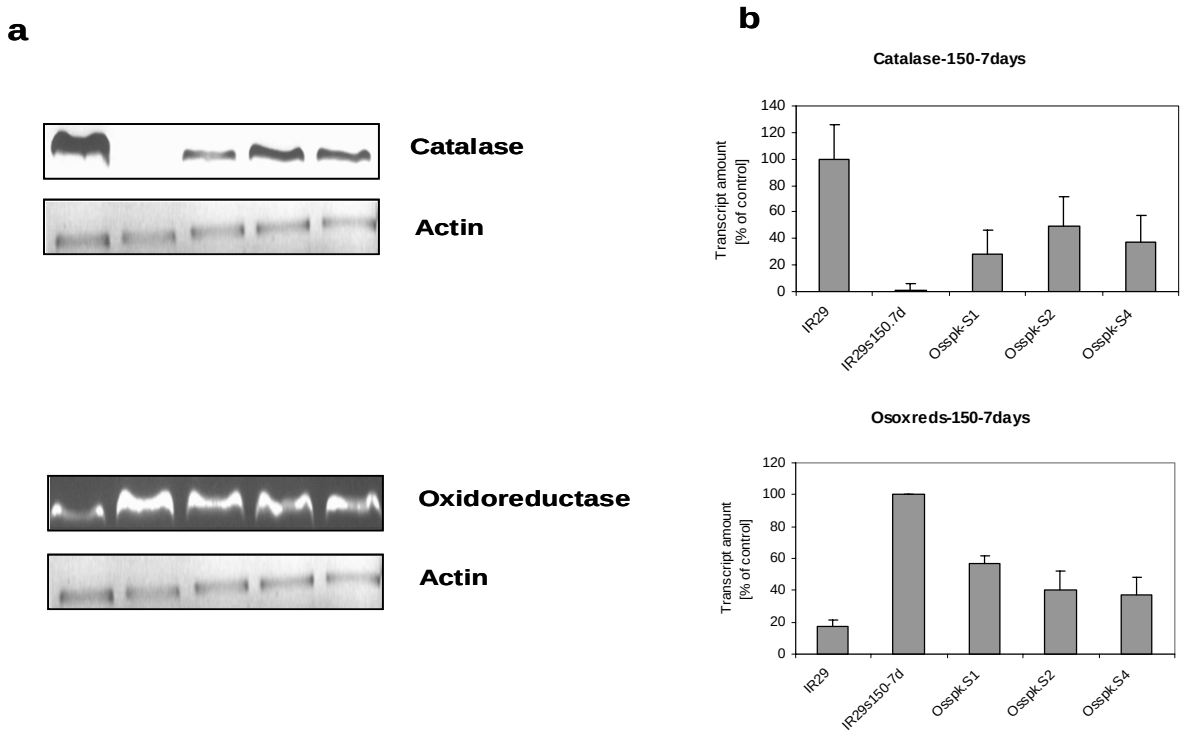


Fig.3-6.6 a: Influence of *OsSPK3* overexpression on the expression of catalase and oxidoreductase genes under 150 mM NaCl during 7 days. S1, S2, S4, represent transgenic *OsSPK3* plants, IR29s represents the wild-type salt stressed and IR29 represents the wild type in control conditions **b:** Densitometric analysis of RT-PCR transcript levels shown in **a**. The transcript amounts of *OsSPK3* in leaf tissues of transgenic rice lines and wild-type plants grown under control and 150 mM NaCl conditions were set to 100%. The transcript amounts were normalized to actin. Data represent means ± SD, n=3

3-6.4 Morphological and physiological characterization of transgenic plants.

3-6.4.1 Effect of the inserted *OsSPK3* gene during vegetative growth phases.

3-6.4.1.1 Germination

The germination rate showed almost the same level under control growth conditions in both transgenic and wild-type plants. When seeds are grown in 50 mM NaCl, transgenic lines increased their germination rate whereas, the wild-type showed a significant decrease (Fig. 3-6.6).

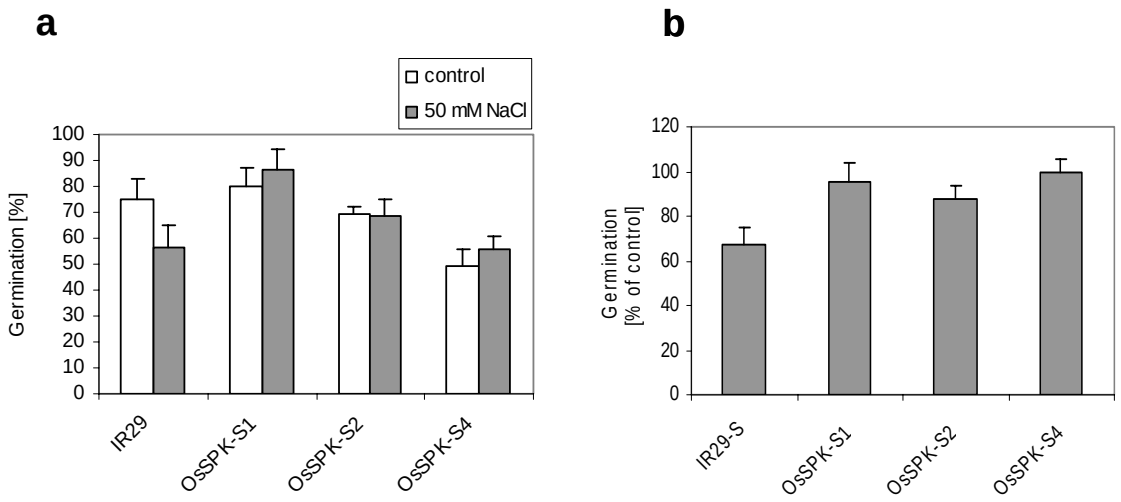


Fig. 3-6.6 a: Germination of wild type and transgenic seeds in H_2O (control) and 50 mM NaCl. **b:** Germination percentage was calculated in relation to the control and were determined after 7 days. Data represent means ± SD, n=3.

3-6.3.1.12 Young seedling

Shoot size of seedlings was measured after 7 days of salt stress. As the germination, the growth rate was significantly affected under salt stress in wild-type, whereas transgenic plants showed a constant or an improved growth under 50 mM NaCl (Fig. 3-2.7).

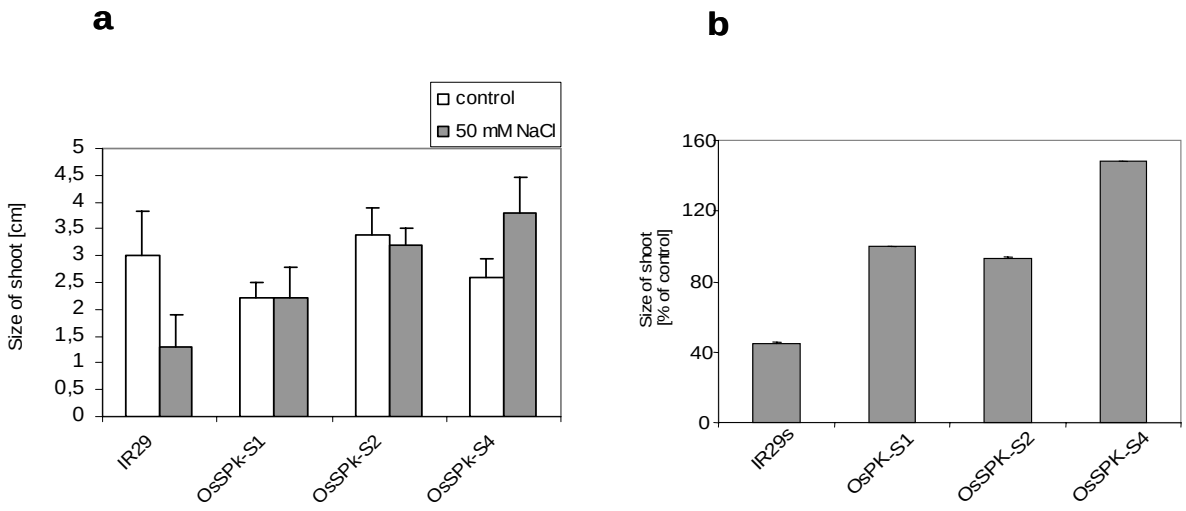


Fig. 3-6.7 Growth evaluation of wild-type and transgenic plants in control and 50 mM NaCl conditions (a) and percentage of growth calculated under the percentage of control. Data represent means ± SD, n=3.

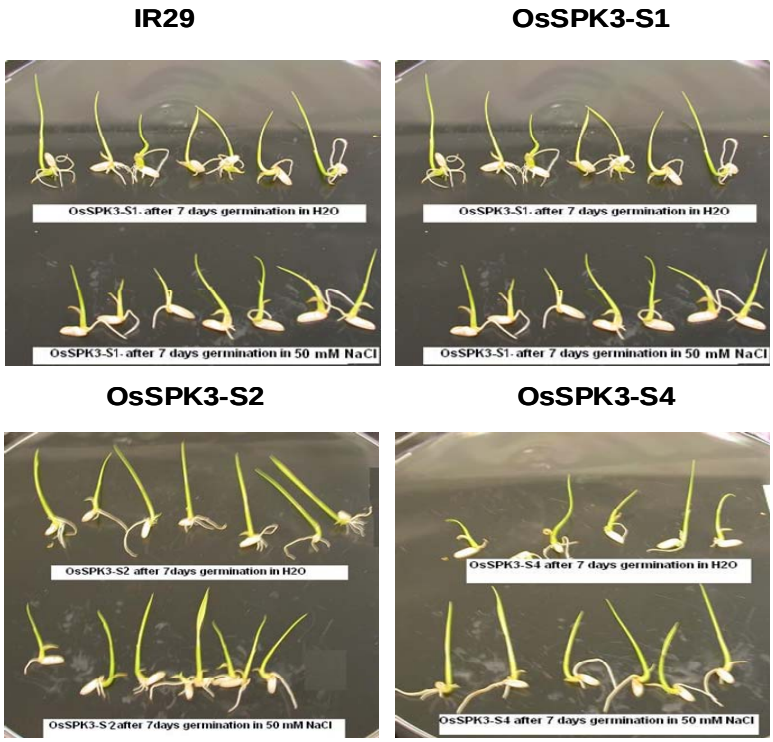


Fig. 3-6.8 Phenotype seedlings of wild type and *OsSPK3* transgenic plants after 7 days in 50 mM NaCl.

3-6.3.1.3 Photosynthetic activity

The photosynthetic activity was measured in wild-type and tansgenic plants at 0, 100 and 150 mM NaCl. Under normal growth conditions, wild type and transgenic plants exhibited the same constant photosynthetic yield, whereas 100 and 150 mM NaCl treatment during 2 days induced a decrease of the photosynthetic yield in wild-type and transgenic plants. The decrease was more pronounced in the wild-type plants (Fig. 3-6.9 a, b). When the stress was prolonged to 96 h in 150 mM NaCl, transgenic plants maintained a high photosynthetic activity comparable to the salt tolerant line Pokkali.(Fig. 3-6.9 c). Photosynthetic efficiency is also shown in percentage of the control (Fig. 3-6.9 d). It was maintained at higher levels in transgenic lines and seemed to have the same efficiency in the salt tolerant rice line Pokkali. The leaf morphology correlated with the flurescence activity, thus at 10 days of salt stress, Pokkali and transgenic plants kept their leaves green. Significant differences were only seen after 2 weeks when the wild-type had died while the transgenic plants only became affected. The morphological aspect observed at 17 days salt stress showed that Pokkali maintained a good habitus of their leaves while the leaves of transgenic plants began to roll up (Fig. 3-6.10).

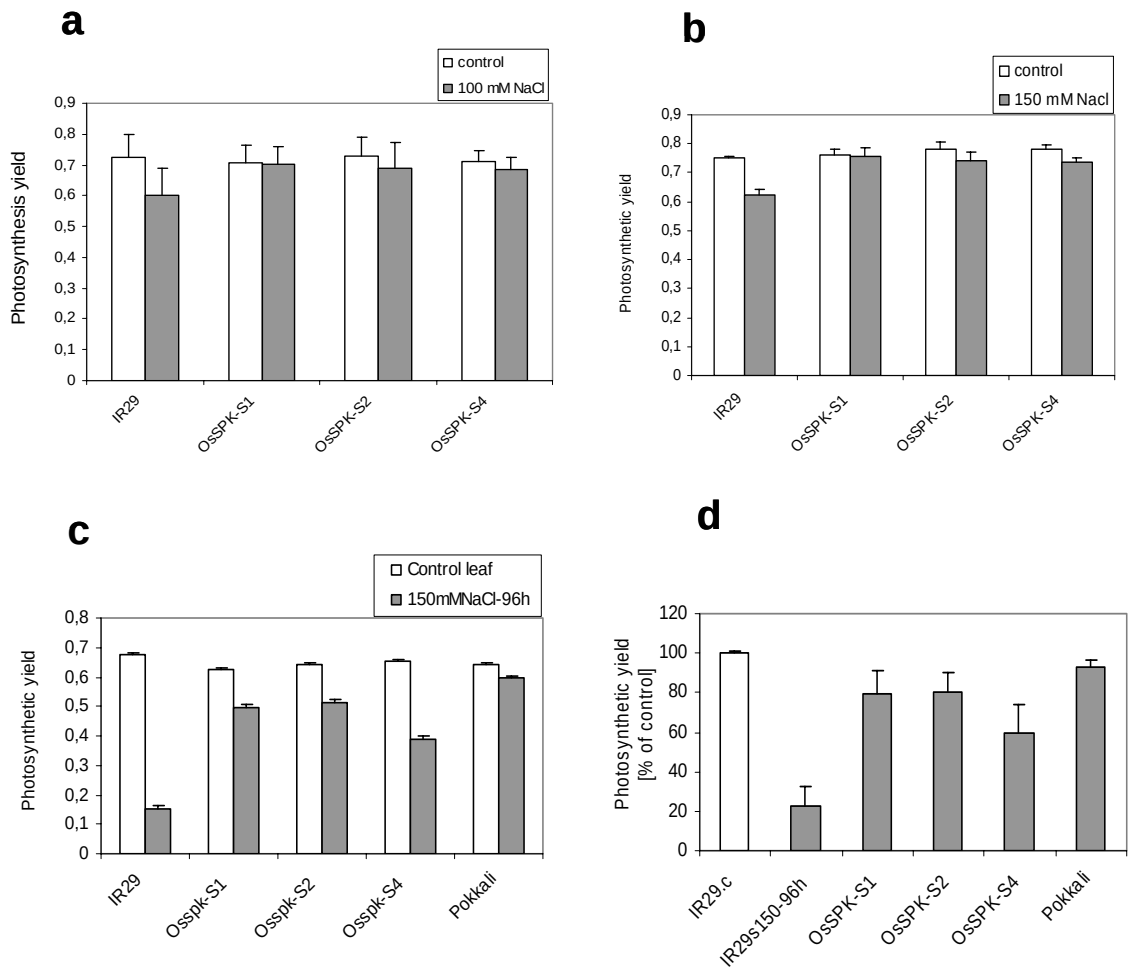


Fig. 3-6.9 Photosynthetic yield of wild-type and *OsSPK3* overexpressing lines under control and 100 mM NaCl conditions 48 h (**a**), control and 150 mM NaCl 48 h (**b**), control and 150 mM NaCl 96 h (**c**) and percentage of control (**d**). Data represent means ± SD, n=3.

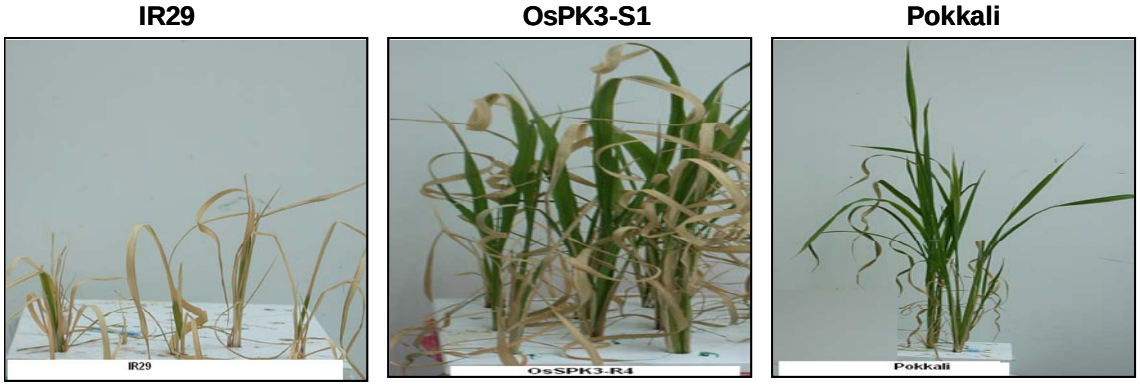


Fig. 3-6.10 Morphological aspect of leaf wild-type, transgenic plants and Pokkali after 17 days in 150 mM NaCl.

3-6.4 Ion uptake in transgenic plants

The capacity of salt tolerance by rice could be related to the ability of a reduced accumulation of Cl^- and Na^+ in cells (Diedhiou and Golldack, 2005; Golldack et al., 2002). In reference to these works, ion accumulation was analysed. Strong accumulation of Cl^- and Na^+ was shown in the wild type grown at 100 or 150 mM NaCl during 48 h, whereas transgenic plants excluded Cl^- and Na^+ (Fig. 3-6.11). The Ca^{2+} content in transgenic and wild-type remained very low and showed no difference of uptake in both plants. K^+ content however was very high in transgenic and wild-type. The highest level could be seen in wild-type plants.

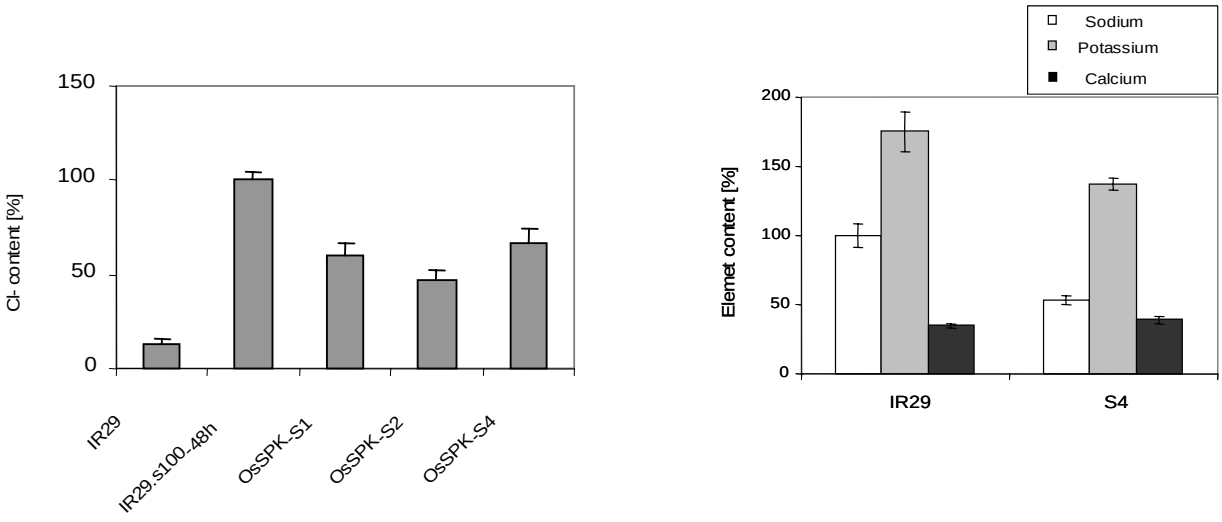


Fig. 3-6.11 Ion accumulation in leaves of wild-type (IR29) and transgenic plant lines grown 48 h under 100 mM NaCl (Cl^-) or 150 mM NaCl (Na^+, $K^+$and Ca^{2+}). Data represent means ± SD, n=7.

4-1 Salt-dependent regulation of chloride channel transcripts in rice

In the present study, expression of the rice CLC-type channel homologue *OsCLC1* was compared in two rice lines with different salt tolerance to determine the role of Cl^- homeostasis in plant salt adaptation. Both rice lines showed significant differences in Cl^- accumulation and transcriptional regulation of *OsCLC-1* which were correlated with expressional changes in *OsPIP2;1* aquaporin, V-ATPase subunit B and the Na^+/H^+ antiporter *OsNHX1*. Analysis of cell-specificity was performed by *in situ* PCR focusing on salt–dependent differences of transcript abundance. The results presented here indicate a correlation of gene expression controlling cation and anion homeostasis in salt stressed plants and suggest a role of *OsCLC1* in adaptation to salinity.

4-1.1 Consequences of salt stress in IR29 and Pokkali

Cultivated in a medium containing 150 mM NaCl, IR29 leaves have shown a precocious leaf necrosis following complete necrosis and death after one week (Fig. 3-1.1). Analysis of the Cl^- accumulation demonstrated that the salt sensitive rice line IR29 accumulated more Cl^- than the salt tolerant one (Pokkali). Golldack et al. (2002a) reported similar results by studying the Na^+ accumulation. The toxicity observed in leaves of the sensitive variety could be related to Cl^- and Na^+ ions and its indirect effects. The specific effect of each ion not well understood. Although most studies have reported toxic effects of Na^+, Cl^- also contributes in a large part to the toxicity of plant salt stress. Tester and Davenport (2003) found that the concentration in which Cl^- becomes toxic is not well defined. Regarding Cl^- accumulation, the strategy used by the tolerant varieties would be to limit the accumulation of Cl^-, thus Pokkali accumulated less Cl^- than IR29. This relationship between Cl^- accumulation and tolerance to salt may give Cl^- the most important role in the process of leaf necrosis in IR29. It seems to confirm studies by Levitt (1980) considering Cl^- accumulation in the aerial parts of rice to be the main reason of toxicity of the NaCl. Accumulation of Cl^- in Pokkali leaf tissues is very low in comparison with IR29. This observation suggests that the tolerant roots exclude more Cl^-, and absorb lower amounts of Cl^- per volume. To keep leaves green over one week, tolerant varieties like Pokkali resist Cl^- toxicity, by limiting the presence of this anion in leaves.

4-1.2 Transcriptional regulation of *OsCL1*

When grown under the control condition, IR29 and Pokkali showed an uptake of Cl^- in root as well as in leaf. Cl^- is not essential for growth for most higher plants. Deficiency symptoms reported by Xu et al. (2000) mentioned some physiological consequences of Cl^- deficiency. Among these the reduction of leaf size followed by necrosis and suppression of lateral development can be noted. Size and the number of fruits decreased as well. Although the Cl^- content at which deficiency symptoms are seen, remain very low (0.1 to 5.7 mg g^{-1} dwt), high Cl^- concentration can be toxic.

Submitted to a salt stress of 150 mM NaCl, IR29 accumulated more Cl^- in leaves than Pokkali. The capability of one cultivar to withstand Cl^- toxicity is frequently related to the ability to restrict Cl^- transport to the shoot. This observation related to the fact that IR29 is NaCl sensitive whereas Pokkali is tolerant. In *Citrus* an interesting approach has been undertaken based on the selection of chemically generated mutants with decreased shoot Cl^- concentration (Garcia Augustin and Primo-Millo, 1995). This classification would be restricted to the monocotyledons because most of the halophytic dicotyledons generate turgor by accumulating high Cl^- concentrations in their tissues. Greenway and Munns (1980) reported a concentration of 340 to 475 mM Cl^- in leaves of *Populus euphratica.*

To reach leaf tissues, Cl^- anions are transported via Cl^- channels. 7 channels have been identified in rice with different localization in subcellular compartments (Table 3-1.1). Hedrich (1994), Schroeder (1995), Allen and Sanders (1997), Barbier-Brygoo et al. (2000), Tyerman and Skerrett (1999) and Krol and Trebacz (2000) described the presence of these proteins in all plant membranes including plasma membrane, tonoplast, endoplasmic reticulum, mitochondrial and chloroplast membranes. Since the plasma membrane constitutes the entrance way to the cell, control of Cl^- to this level may play an important role in the transport of Cl^- in other cell compartments and parts of the plant. In our study *OsCLC1* which for example shares 66% identity to *Arabidopsis AtCLC*-c was predicted to be localized in the plasma membrane and the thylakoid. Expression of this rice *OsCLC1* was compared in the salt sensitive line IR29 and the salt tolerant line Pokkali for investigating function of this voltage-gated Cl^- channel homologue in the regulation of Cl^- homeostasis under conditions of high salinity. The results indicate expression of *OsCLC1* in leaf and in root tissue of both lines with approximately similar signal strength. This confirms the fact that in non saline conditions, chloride channels catalyse the rapid release of anions in the majority of cells and supports the idea that they are implicated in turgor and osmoregulation as described by White and Broadley (2001). Barbier-Brygroo et al. (2000) reported that the expression in control

conditions may be related to the role of Cl^- channels in triggering membrane depolarization and to mediate anion efflux due to the highly negative plasma membrane potential that is established by the plasma membrane H^+-ATPase. This amplification of the depolarisation and the high anion efflux are required for the closure of stomata.

Salt induced strong repression of *OsCLC1* transcript accumulation occurred in leaves of both lines. However, in roots slight decrease after 48 h salt stress has been seen in IR29, while Pokkali showed an increase of *OsCLC1* transcripts.

The high expression of *OsCLC1* in Pokkali roots indicates the importance of the Cl^- channel for maintaining the membrane potential at a similar level as under control conditions while controlling the excess of extra cellular Cl^-. This control may be limited by the salt sensitive line whose *OsCLC1*expression decreased slightly in conditions of stress. According to Lin (1981), root Cl^- channels control the regulation of membrane potential and play a significant role in anion uptake in some conditions (supply of NO_3^- or in salinity) i.e. when the membrane potential becomes more positive than the equilibrium. Under these conditions of osmotic imbalances, salt stress or excess of Cl^- accumulation, anion channels however, may, also be involved in cellular anion uptake. But this work indicates that although the concentration of Cl^- increased in roots of both lines, expression of *OsCLC1* decreases in the salt sensitive line IR29. It might be postulated that *OsCLC1* is not directly linked to the uptake of Cl^-.

If Cl^- entrance in the cytoplasm is related to the expression of *OsCLC1*, its expression would be increased in the sensitive line IR29 which accumulated more Cl^- under salt conditions than the tolerant line Pokkali whose *OsCLC1* transcript amount increase in root. Alternatively the role of *OsCLC1* might be to amplify or stabilize the trans-plasma membrane electrical potential. This hypothesis may be the reason for the tolerance of Pokkali whose *OsCLC1* Cl^- channel transcript amounts increased in the presence of salt whereas IR29 revealed a slight decrease in comparison with the control plants. Many studies reported by Barbier-Brygoo et al. (2000) confirm the excitation of some Cl^- channels under repolarization or depolarization conditions of the plasma membrane. Despite its role in the selectivity of Cl^-, *OsCLC1* might not be the only channel for Cl^- influx. In excess of nitrate, *Arabidopsis* transgenic plants carrying the *CLC*-a gene reported by Geelen et al. (2000) showed a reduction of 50% of their capacity to accumulate nitrate while Cl^-, sulphate and phosphate were kept at the same level as in the wild-type. This fact indicates an interference with other channels or ion transporter proteins. The confirmation of this supposed idea may come from the discovery of Ryan et al.

(1997) who described in root Al^{3+} tolerant plants a novel channel which is activated by Al^{3+} and allows Cl^- efflux.

After being taken up, the root Cl^- is transported to the shoot through the xylem sap. According to the here presented results, IR29 and Pokkali leaves showed a progressive increase of Cl^- contents on the one hand and a progressive decrease of expression of *OsCLC1* on the other hand. These results seem to be in agreement with the difference of salt tolerance in both lines. The salt tolerant line Pokkali, whose expression is higher than the expression in IR29, showed a strong signal of *OsCLC1* transcript as soon as the salt treatments began. The expression was maintained until 24 h at the same level of the control and finally decreased at 48 h. In contrast IR29 showed a slight increase at 3 h followed by a strong decrease from 24 h. The high Cl^- uptake seemed to be linked to the incapability to improve the expression of its Cl^- channel which becomes rapidly saturated by salt. This earlier response permitted Pokkali to limit the Cl^- uptake protecting in the same time its photosynthetic organs. This operating mode is in agreement with the conclusions of Barbier-Brygoo et al. (2000) according to which anion channels play a predominant role in the early steps of signalling pathways leading to developmental as well as adaptative plant responses. Control of Cl^- fluxes across the plasma membrane by regulation of anion permeable channel is likely to have a role in cellular osmoregulation and maintenance of turgor under conditions of salt stress.

Exposed to hyperosmotic mannitol stress *Vicia faba* mesophyll cells increase the uptake of K^+ and Cl^- as reported by Shabala et al. (2000). The authors proposed that the hyperosmolarity induced by K^+ and Cl^- accumulation in plant cells could be sufficient for osmotic adjustment without additional accumulation of organic solutes. Cl^- efflux from seed coats of *Phaseolus vulgaris* was stimulated by hypoosmotic conditions and a role of Cl^- channels in turgor regulation has been hypothesized (Zhang et al., 2000).

Exposition to NaCl caused more Cl^- uptake in leaves of IR29 than in Pokkali. In previous works Golldack et al. (2002a, 2003) detected similar strategies of Na^+ exclusion and accumulation in Pokkali and IR29. This similar operating strategy demonstrates coordinated Na^+ and Cl^- fluxes where Cl^- might be taken up as a counter ion of Na^+ for reason of electroneutrality.

Under NaCl treatment conditions, Na^+ induces metabolic toxicity which is largely a result of its ability to compete with K^+ for essential binding sites that affects for cellular functions. Considering the role of K^+ in the activation of enzymes, we can imagine the control

mechanism used to export Na^+ outward the cytoplasm. Active control of Na^+ influx by HKT1-type transporters has been shown in several plant species (Uozumi et al., 2000; Horie et al., 2001; Rus et al., 2001). Reduced transcript levels of *TaHKT1* correlate with decreases of Na^+ uptake in wheat and enhance the salt tolerance whereas in *Arabidopsis* expression *AtHKT1* under control condition of the *AtHKT1* promoter increases salt sensitivity e.g. in the wild type seedlings (Laurie et al. 2002, Rus et al., 2004). The homologous rice gene *OsHKT1* encodes a Na^+ transporter and its expression in yeast caused hypersensitivity to Na^+ (Horie et al., 2001; Golldack et al., 2002a). Salt stress reduces *OsHKT1* transcript levels in the rice lines IR29 and Pokkali with a more pronounced repression in Pokkali suggesting involvement of the transporter in regulation of the different Na^+ uptake behaviour of both rice lines (Golldack et al., 1997, 2002a). In addition, correlation with the maintenance of K^+/Na^+ homeostasis could also be shown for the inward rectifying K^+ channel homologue *OsAKT1* that was downregulated in roots in the exodermis of salt treated Pokkali but was not significantly affected in IR29 plants (Golldack et al., 2003).

4-1.3 Correlated expression changes of *OsCLC1* and genes involved in maintenance of cellular Na^+ homeosthesis.

To compare salt-dependent expression of *OsCLC1* with the regulation of gene products involved in Na^+ homeostasis, the expression of V-ATPase subunit *VHA-B* and the Na^+/H^+ antiporter *OsNHX1* was compared in salt treated IR29 and Pokkali. Similar alterations in expression of the transcript levels were found in leaf tissue (Fig. 3-1.5). Transcript levels of *OsVHA-B* as well as *OsCLC1* increased in Pokkali as the constraint started and decreased slowly and progressively with time of the constraint maintaining similar levels with the control and future decreased at 48 h. While no changes were seen at 3 h, IR29 showed early decrease at 6 h salt stress. Many studies have reported the role of both genes in the vacuolar compartmentalization of Na^+ the vacuole. Vacuolar Na^+ sequestration in yeast and plants is energized by the tonoplast H^+ gradient that drives the secondary active NHX-type Na^+/H^+ antiporter (Ratajczack, 2000). According to the review of Hasegawa et al. (2000) and Maeshima (2000), salinization induces activity of both vacuolar primary H^+ pumps in tolerant and sensitive species. In their work with salt–tolerant plant species as *M. crystallium*, and *Sueda salsa*, Golldack and Dietz (2001) as well as Wang et al. (2001) reported a transcriptional up-regulation of V-ATPase transcripts in conditions of salt stress. In contrast in the sensitive *A. thaliana*, expression of VHA-D did not respond to salinity (Kluge et al., 1999) indicating that adaptational regulation of V-ATPase for vacuolar Na^+

detoxification occurs in salt tolerant plants but may be missing in salt sensitive species. These findings are corroborated with our results that indicate no increase in the salt sensitive line IR29 while the salt tolerant line Pokkali showed an over-expression in the first hours of the constraint. As shown in many studies, *VHA-B* is implicated in the process of Na^+ sequestration in the vacuole. Expression of *OsVHA-B* follows similar expression of *OsCLC1* and implies a role of the Cl^- channel in the Na^+ sequestration. These results reinforce the conclusion of Gaxiola et al.(1999) according to which the yeast CLC Cl^- channel is required as an anion channel to provide the counterbalancing charge that permits cation compartmentalization into organelles or vesicles. Salt–sensitivity of the yeast mutant *gef1* could be repressed by expression of for example *AtCLC*-c that is homologous to *OsCLC1*.

The tonoplast Na^+/H^+ transporters of the NHX-type mediate vacuolar Na^+ import and have an essential function in maintenance of the cytoplasmic cation homeostasis (Apse et al., 1999, Gaxiola et al., 1999; Quintero et al., 2000). Overexpression of *AtNHX1* in *Arabidopsis*, tomato, and *Brassica napus* as well as of *OsNHX1* in rice improved salt tolerance, and Na^+ accumulation in the transgenic plants (Zhang and Blumwald 2001; Zhang et al., 2001; Fukuda et al., 2004). Interestingly, Apse et al. (1999) and Chauhan et al. (2000) did not detect increased transcript levels of *AtNHX* in salt treated *Arabidopsis* whereas Gaxiola et al. (1999) and Fukuda et al. (1999, 2004) reported salt induced expression in *Arabidopsis* and *Oryza sativa* ssp *japonica* respectively. Considering the first 24 h of salt stress, the results presented in this study seem to be in agreement with those reported in *Arabidopsis* and *Oryza sativa* ssp *japonica* by Fukuada et al. (1999, 2004). In fact Pokkali and IR29 show an over-expression of *OsNHX1* after 6 h of stress by IR29 a response that was delayed to 24 h by Pokkali. The maintenance of the over-expression at 24h in Pokkali might explain its aptitude to tolerate salt stress more than IR29. After long salt stress the ability of vacuolar sequestration is obviously missing in the rice lines IR29 and Pokkali. The ability to tolerate increased salt concentrations depends on species and the duration of the constraint. If plants cannot indefinitely tolerate the stress, tolerance for some species like the rice line Pokkali would be to delay the overflow of Na^+ in to the leaf. As this ability is quickly lost by IR29, Na^+ and Cl^- overflow to all cells and IR29 responds more sensitive. Thus down regulation of Cl^- channels as *OsCLC1* might allow to maintain a turgor and an intracellular osmotic potential by restricting Cl^- fluxes across the plasma membrane.

Our results show a coordinated expression of *OsCLC1* with *OsVHA-B* and *OsNHX1* genes in both lines indicating a co-regulation of Na^+ and Cl^- in condition of stress. While *OsVHA-B* and *OsNHX1* regulate the sequestration of Na^+ in the vacuole, *OsCLC1* might be needed to

provide the counterbalancing charge that will permit cation compartmentalization (Gaxiola at al., 1989).

4-1.4 *OsCLC1* and water channel *OsPIP2;1*

The first constraint of salt stress is related to the difficulty to take up water. Plants submitted to this constraint have to regulate their osmotic potential in order to absorb water. Many strategies are used according to the plant species. Among these, the accumulation of Cl^- in the vacuole through the regulation of passive channels plays an important role. In their work, Moya et al. (2003) show that Cl^- uptake, and hence salt tolerance, depends on water use. In relation with this affirmation the analysis of transcript amounts of a rice water channel (*OsPIP2;1*) showed a similar expressional changes as the Cl^- channel. Submitted to 150 mM NaCl during 48h, the sensitive rice line IR29 and the tolerant rice line Pokkali increased transcript levels of the water channel *OsPIP2;*1 as well as the Cl^- channel *OsCLC1* in the first 6 h of stress and then decline later on (Fig. 3-1.6). Both channels were repressed at 24 and 48 h salt treatment, however the decrease was higher in the Cl^- channel than in the water channel where a recovery could be seen at 48h. This difference in the expression of both channels seems to confirm the hypothesis of Loudet et al. (2003) claiming that anion would results from water status. Moya et al. (2003) noted that this parallelism between water and Cl^- uptake is linked to the transpiration. They make the observation that transpiration under saline stress decreased faster in the tolerant *Citrus* line, indicating that some tolerance responses to salt stress (i.e. readjustments to progressive reductions of Cl^- uptake) took place more rapidly in the tolerant rootstock. Transpiration reductions observed under saline conditions (Behboudian et al., 1986) may be due to the stomatal regulation (Walker et al., 1993; Banfuls and Primo-Millo, 1992, 1995)

4-1.5 Tissue-specificity of *OsCLC1* expression is regulated in response to salinity

In situ PCR analyses were performed with leaf sections of IR29 and Pokkali and similar cell-specificity was observed in non-stressed plants of both rice lines. Strongest signals were detected in mesophyll cells. Besides, expression was seen in stomata and in the vascular tissue in xylem parenchyma cells and in the phloem. A similar expression pattern has been reported for the inward-rectifying K^+ channel homologue *OsAKT1* that was expressed in leaf mesophyll cells, xylem parenchyma and in the phloem (Golddack et al., 2003). These data suggest coordinated transcription of K^+ and Cl^- mediated by *OsAKT1* and *OsCLC1* in rice

leaves for balancing the charge of K^+ by Cl^- flux. Translocations of K^+ and Cl^- at the plasmamembrane participate in osmoregulation, pH homeostasis and movement of the stomatal guards cells (Shabala et al., 2000; Barbier-Brygoo et al., 2000). In addition, expression of *OsCLC1* in cells adjacent to the xylem and in the phloem suggests a function of this Cl^- channel homologue in long distance Cl^- fluxes. Besides, a role in elongation of developing cells in the xylem parenchyma also seems likely. As an example in tobacco pollen tubes Cl^- efflux was disrupted by application of three different Cl^- channel blockers and cell volume increased demonstrating necessity of Cl^- flux for pollen tube growth and maintenance of cell volume and turgor (Zonia at al., 2002).

Differences in cell-specificity between the rice varieties IR29 and Pokkali were observed under salt stress. Expression of *OsCLC1* was down-regulated in xylem parenchyma cells in salt stressed Pokkali but disappeared in the Cl^- accumulating IR29. In both lines however signals declined in mesophyll cells indicating reduced partitioning of Cl^- within the leaf that may participate in maintenance of the cellular osmotic potential under the hyperionic stress conditions. In conclusion the results presented here reveal a role of the CLC-type Cl^- channel homologue *OsCLC1* in adaptation to salt stress and suggest function in cellular osmoregulation in rice.

Na^+ and Cl^- induce both osmotic and ionic effects when administered at high concentrations. Although we cannot completely differentiate between the effect caused by each ion, many studies concentrated on the effect of Na^+, however in some species like *Citrus*, barley and grappe, Cl^- is more effective by inducing toxicity than Na^+ (Moya et al., 2003). The predominance of research on Na^+ uptake and transport rather than Cl^- can be explained by the fact that the control of Na^+ regulation in condition of salt stress is more difficult and costs more energy than Cl^- transport. Because of the negative electrical potential of cells, Cl^- has a passive diffusion and its role is to stabilize the depolarisation of the membrane caused by the presence of a constraint like NaCl. Prevention of Na^+ uptake is not only energetically costly but causes a problem related to ion selectivity. For instance the competition with K^+ may be damaging to species that cannot control Na^+ entry. Since the important role of K^+ in osmotic regulation and in activation of many enzymes in the cytoplasm, the control of the competition plays the key role of salt tolerance. Studies of the expression of *OsVHA-B* and *OsNHX1*, genes implicated in the sequestration of Na^+ in the vacuole show a parallel expression of *OsCLC1* Cl^- channel. This similar expression supports the view that the regulation of Cl^- transport may play an important role in salt tolerance improvement. While our research about

OsCLC1 alone doesn't permit us to explain Cl^- uptake, exploration of the other Cl^- channels already identified in rice would allow to broaden our understanding of the regulation of the Cl^- homeostasis particularly under conditions of stress.

4-2 Salt stress regulates expression of the HAK-type K^+-transporter *OsHAK7* in rice

4-2.1 Photosynthesis and osmotic stress

To assure their survival, plants under stress conditions must be able to control their photosynthetic capacity as well as their water absorption. 150 mM NaCl treatment induced a strong decrease of the photosynthetic capability accompanied by a high increase of osmotic potential of the salt sensitive line IR29. In contrast, the salt tolerant line Pokkali controlled its osmotic potential (Fig. 3-2.2) and maintained its photosynthetic efficiency similar to the control conditions (Fig. 3-2.1). As reported by Cramer et al. (1989), Wright et al., (1993) and Riwalli et al. (2002), salt stress reduces photosynthetic ability. According to Munns (2002) this decrease might be related to the osmotic component of salt stress. The difficulty for plants to absorb water in saline soil reduces the transpiration as well as CO_2 assimilation rates and affects tissue growth. With several fold higher than osmotic potentials under control conditions after 24 and 48 h salt stress, IR29 could not adapt its ionic homeostasis. As a consequence of ion accumulation, early necrosis occurred in IR29 leaves submitted to salt stress. Chlorosis or leaf degradation negatively influenced the photosynthetic efficiency and finally caused the death of the plant. Although we do not know whether chlorophyll degradation is the primary cause of photosynthetic inhibition or the consequence of growth reduction, studies have reported the decrease of chlorophyll under conditions of salt stress (Shabala et al., 2005). In Pokkali, the first decrease of the photosynthetic yield was followed by a recovery of about 10 %. The photosynthetic yield of Pokkali at 72 h of salt stress remained near to the control plants indicating a very ”healthy” plant with optimal photochemistry. Previously, we showed that Pokkali kept leaves green over a week when submitted to 150 mM NaCl whereas IR29 leaves indicated necrosis already after 24 h. One of the mechanisms of salt tolerance in Pokkali could be related to the capacity to maintain the photosynthetic yield under salt stress. Shabala et al. (2005) reported a high photosynthetic

activity when barley plants cultivated in 100 mM NaCl became supplemented with a Ca^{2+}. Not only Ca^{2+} but also other divalent cations (e.g. Ba^{2+}, Mg^{2+}, and Zn^{2+}) significantly affected Na^+ transport across the plasma membrane of salinized leaves. This positive effect of divalent cations was related to K^+ through the mechanism of maintaining an optimal K^+/Na^+ ratio in the cytosol by regulating K^+ transport across the plasma membrane of mesophyll cells. This important role of K^+ in the cytosolic homeostasis was the base of protection and maintenance of optimal photosynthetic activity of mesophyll cells (Shabala, 2003).

4-2.2 Importance and localisation of K^+ and control of Na^+ uptake in IR29 and Pokkali

K^+ is one the most abundant cations in cells. Under saline conditions, the K^+ contents increased in leaves of the rice lines IR29 and Pokkali and decreased in roots. Compared to Na^+, we noted more Na^+ in roots of the salt tolerant line, whereas the leaves of the sensitive line accumulated more Na^+. To maintain low Na^+ content in leaf tissue, Shabala et al. (2005) postulated that the beneficial effects of supplemental Ca^{2+} on leaf photosynthesis may be a result of much improved K^+/Na^+ ratios in the leaf mesophyll cells due to the regulation of leaf K^+ balance. Regulation of K^+ is the central system for the control of salt tolerance. The ability of plants to retain K^+ in order to maintain K^+/Na^+ homeostasis has always been considered a key feature of salt tolerance (Maathuis and Amtmann, 1999; Munns, 2002; Tester and Davenport, 2003). This remark was confirmed by our results. Under 150 mM NaCl, Pokkali maintained moderate concentrations of K^+ in root tissue and reduced the leaf concentration of Na^+. Pokkali tended to maintain a higher photosynthetic activity by limiting Na^+ uptake in the roots. Compared with IR29, Pokkali accumulated more Na^+ in roots. This property seems to be contradictory to the notion of Munns (2002) in relation to salt tolerance who considered the ability of plants to minimize Na^+ uptake by roots as the main feature conferring salt tolerance. Considering his conclusion, IR29 would be more tolerant than Pokkali. Although the salt sensitive line IR29 accumulated less Na^+ in roots, it takes up a lot of Na^+ in the leaves. The results might indicate the ability to minimize the transport of Na^+ to leaves. This seems to be accepted by many researches. Rascio et al. (2001) verified that a wheat mutant with enhanced capacity for K^+ accumulation in leaves was more salt tolerant than the wild type. Then maintaining high concentrations of K^+ in leaf tissue under salt stress conditions could be a result of K^+ transport and selectivity.

4-1.3 Regulation of K^+ transport in the mechanism of salt tolerance by rice

HAK-type transporters have been assumed to function as a major high and low K^+ affinity uptake and transport system in plants, other members of this transporter family play a role in cell expansion and development as it was demonstrated for the *Arabidopsis trh1* and *shy3* mutants that showed defects in root hair elongation in hypocotyl and leaf cell growth (Rigas et al., 2001; Elumalai et al., 2002). Here the involvement of HAK-type transporters in maintaining K^+ influx and *in planta* K^+ distribution appear important under conditions of cation imbalances in high salinity. This research was focussed on the expression of *OsHAK7* in the salt sensitive IR29 and the salt tolerant Pokkali lines. Comparing the transcript abundance as well as the tissue specific localisation of this gene product under control and salt stress (150 mM NaCl) conditions, *OsHAK7* has a predicted localization in the plasma membrane and in the thylakoid membrane. By transcript analysis, expression was found both in leaf and root tissue in both rice lines.

Cell specific expression was analysed by *in situ PCR* in leaf sections and showed strongest signals in mesophyll cells surrounding the vasculature, in the phloem and in the xylem in both lines IR29 and Pokkali after salt treatment. The presence in the vasculature suggests a role in remobilisation of K^+ in the presence of salt to maintain the same level as in the control condition. In addition *OsHAK7* may control the constraint caused by NaCl. Importance of K^+ influx in the vasculature and maintenance of osmotic pressure would play a crucial role in developing cells as in regulation of cell elongation (Su et al., 2002). *OsHAK7* was also strongly expressed in mesophyll cells in control and stress conditions and signals were detected in the epidermis when submitted to salt stress. Since cytosolic K^+ homeostasis is the characteristic feature of mesophyll cells (Cuin et al., 2003), mobilisation of K^+ through *OsHAK7* activity may enable the plant to improve protection and maintenance of optimal photosynthetic activity of mesophyll cells. In addition expression of *OsHAK7* in epidermis cells agreed with the conclusion of Fricke et al. (1994) who stated that the content of K^+ in epidermal cells is used as a buffer for osmotic adjustment. In summary, when exposed to salt stress, IR29 and Pokkali showed the same tissue specificity of *OsHAK7* expression with strongest signals in mesophyll cells neighbouring the vascular tissue, in xylem parenchyma cells and in phloem. Taking together these data strongly suggest a function of *OsHAK7* in long distance K^+ transport and distribution as well as recirculation of K^+ via the phloem in salt stressed rice.

Transcript abundance of *OsHAK7* in roots showed a similar expression in both IR29 and Pokkali. Under 150 mM NaCl during 24h Pokkali and IR29 increased the activation of the K^+ transporter gene in roots whereas in leaves the up-regulation was brief and limited to 6h after salt treatment. In contrast to many K^+ channels or transporters, *OsHAK7* transcript amounts increased upon salt stress. HKT1-type transporters have been shown to function as K^+, Na^+ symporter transporter, respectively and are supposed to mediate Na^+ influx in plants (Horie et al., 2001, Golldack et al., 2002). Under salt stress conditions, *OsHKT1* transcription was down-regulated in roots of IR29. The repression was more pronounced in Pokkali, possibly indicating a genetic control of Na^+ influx in rice by this transporter (Golldcak et al., 2002). As in the salt sensitive rice line IR29 (Fig. 3-2.5), in *Arabidopsis*, mutation of *AtHKT*1 led to increased sensitivity to NaCl accompanied by enhanced accumulation of Na^+ in shoots and reduced Na^+ contents in roots (Berthomieu et al., 2003). In contrast HKT1 in wheat increased the tolerance to NaCl and reduced Na^+ accumulation in roots (Laurie et al 2002). Expressed in wild-type, *AtHKT1* increased salt sensitivity. In the same way, salt adapted plants of the halophyte *M. crystallinum* showed a transcriptional decline of *McHKT1* (Su et al., 2003). Based on the study, *HKT1* has been suggested to control Na^+ influx whereas it is likely to have a significant role in K^+ uptake and distribution in salt stressed plants. Regulation of inward-rectifying K^+ channels of *AKT1* in many species showed the same decrease of transcript amount under salt treatment. Growth of *akt1* mutant seedlings was inhibited by NaCl but could be rescued by supply of external K^+. In addition, *AKT1*–mediated inward currents were impaired by increased cytoplasmic Na^+ (Qui and Salding 2004). In *M. crystallinum* the AKT1-homologue *McAKT1* was downregulated in epidermal and cortical cells of roots (Su et al., 2001). In the salt sensitive rice IR29, *OsAKT1* transcripts were not significantly modified by salt treatment whereas in the salt tolerant line Pokkali, down-regulation could be observed in root exodermis and endodermis (Golldack et al., 2003).

Concerning the regulation of the third group of HAK-type K^+ transporters to which *OsHAK7* belongs, detailed studies in *M. crystallinum* indicated stimulation of K^+ mobilisation. By heterologous expression in yeast, *McHAK1* and *McHAK4* were shown to specifically transport K^+ but not to mediate Na^+ uptake. *McHAK1*, *McHAK2* and *McHAK3* that share homology with *OsHAK7* ranging from 53 to 69% showed transient transcriptional up-regulation in roots, but in salt adapted plants transcript levels were restored. In leaves expression of the three genes was stimulated in response to high salinity (Su et al., 2002). As *OsHAK7* in this study, expression of *McHAK4* increased in the phloem and adjacent mesophyll. The regulation of *McHAK* under salt treatment as well as *OsHAK7* under salt

tress and K^+ starvation (data non shown) indicated an upregulation of the K^+ transporter. This may act as major K^+ transport system in salt exposed *M. crystallinum* as well as in rice notably in Pokkali were the expression was more pronounced. Both rice lines have the capability to regulate K^+ accumulation and transport, however, differences in salt tolerance may be related to variety-specific regulation of Na^+ accumulation by HKT- and AKT-type transporter systems. Interestingly, as shown by Diedhiou and Golldack (2005), the rice lines also showed different uptake characteristic for Cl^- with IR29 accumulating the anion and Pokkali excluding Cl^- at high salinity. In leaves, expression of the CLC-type Cl^- channel *OsCLC1* was transiently up-regulated followed by repression in salt-adapted plants that was more pronounced in IR29 than in Pokkali. Transcriptional down-regulation of *OsCLC1* was located to mesophyll cells (Diedhiou and Golldack, 2005) that showed also repressed transcription of *OsHAK7* in this study. These data suggest coordinated regulation of K^+ and Cl^- transport on the transcriptional levels in salt stressed rice leaves. K^+ and Cl^- fluxes at the plasmamembrane have a role in maintenance of the cellular osmotic potential (Bargier-Brygoo et al., 2000). Shabala et al. (2000) suggested that osmoregulation in plant cells might be achieved by K^+ and Cl^- translocation without additional accumulation of organic solutes. Analysis of divalent cation-induced net K^+ and Cl^- fluxes showed a K^+:Cl^- stoichiometry of 1:1. The increase of Cl^- uptake in relation of K^+ may contribute to balance charge disequilibring when supplemented Ca^{2+} mediates K^+ influx (Shabala et al., 2005). Accordingly, next to function in K^+ distribution and long distance transport, *OsHAK7* might also participate in osmotic adjustment in salt stressed rice.

4-3 Wheat and a salt-tolerant relative, *Festuca rubra* ssp. *litoralis*, regulate a plasma membrane aquaporin, the vacuolar H^+- ATPase and Na^+/H^+ antiporter differently

4-3.1 Two different modes of ion regulation in response to salinity in *Festuca* and *Triticum aestivum*.

Important knowledge on basic mechanisms of plant salt adaptation has emerged particularly by investigation of plant models as *A. thaliana* and the halophyte *M. crystallinum* (Hasegawa et al., 2000). In addition to these studies, comparative analyses of crops and their close relatives with natural tolerance to salt however, will be a powerful tool to dissect networks of salt adaptation and to identify key mechanisms that are suitable to confer improved salt tolerance to crops. Salinity, in addition to osmotic stress, induces an ionic stress due to the presence of Na^+ and Cl^- and causes ion imbalances. Thus, in addition to their toxic effect in cells, Na^+ and Cl^- limit and create an imbalance in the absorption of other ions and micronutrients. While Na competes with K, Mg, Mn and Ca, Cl^- restricts the uptake of the oxidized forms of N, P and S. Therefore, the ability to limit the effect of Na^+ and Cl^- might be an important trait for salt tolerance. Ion uptake and the tolerance to salt were investigated in the present work to determine strategies of salt stress response in the major crop plant wheat that is salt sensitive and the closely related species *Festuca* that is a facultative halophyte and a suitable model for research on molecular mechanisms of salt adaptation. Grown under control conditions, pronounced differences in accumulation of Ca, Mg and Fe were detected with higher contents in *Festuca*. In the light of their important roles in cells, this feature of *Festuca* to acquire higher amounts of Ca, Mg and Fe might be a prerequisite for extreme salt tolerance in *Festuca*. Although both Mg and Fe are essential nutrients that have key functions for example in photosynthesis and enzyme activation, protein synthesis and proliferation control, respectively (Conolly et al., 2003; Shabala and Hariadi, 2005; Rubin, 2005), evidence still has to be provided for the molecular involvement of these elements in plant salt adaptation. In species as *Arabidopsis* and *M. crystallinum*, salt induced transient changes in cytosolic Ca have been shown to activate intracellular signalling by activating of Ca dependent protein kinase (Xiong et al., 2002; Patharkar and Cushman 2000; Chehab et al., 2004). In response to salt stress, the contents of Ca, Mg and Fe was considerably higher in *Festuca* than in wheat thus further supporting a correlating of enhanced accumulation of these ions in salt adaptation. In comparison to *Festuca* that accumulated Na^+ in both tissues,

the Na^+ content increased in roots and slightly in leaves of wheat indicating Na^+ exclusion from leaves as it has been reported for certain salt tolerant rice lines as well (Golldack et al., 2002; Golldack et al., 2003). Interestingly, even when grown under non stressed conditions, considerable amounts of Na^+ were detected in root of *Festuca*. These data indicate a function of high affinity Na^+ uptake systems in roots of non-stressed *Festuca* that might be presented by HKT1-type Na^+ transporter homologues (Golldack et al., 2002). These profiles of ion accumulation in *Festuca* and wheat grown under the same conditions indicate two different strategies for ion uptake that might interfere with the mechanism of ion homeostasis under conditions of salt stress. While wheat excludes Na^+, *Festuca* abundantly accumulates Na^+ and could grow under salinity of almost 500 mM NaCl. The affinity of *Festuca* to incorporate high Na^+ contents in roots and in leaves suggests the existence of an efficient regulation that would reduce the toxicity effect of Na^+ in the cells, whereas in wheat this regulatory system seems to be missing. As a consequence the crop species became quickly affected by the presence of Na^+.

4-3.2 Expression of *PIP2;1* aquaporins under salt stress in *Festuca* and wheat

Salinity is major abiotic stress acting primarily as an osmotic stresses or through establishing a water deficit. Control of water uptake in conditions of salinity might be determinant in the tolerance of species to salt stress. Expression of *Festuca* water channel *PIP2;1* (Plasma membrane intrinsic protein) sharing 90 to 95% EST sequence identity with wheat, rice and barley *PIP2;1*, was compared with the expression in the salt sensitive crop wheat. Regulation of water flux across the plasma membrane is an essential requirement for cytoplasmic adjustment of water homeostasis under conditions of high salinity. Salt induced stimulated expression has been shown in leaves of *Arabidopsis* for *AtPIP2;3*, in radish seedlings, for *RsPIP2;1*, and in leaves of barley for *HvPIP2;1* whereas in salt-stressed maize transcript amounts of PIP-type aquaporin decreased (Jang et al., 2004; Suga et al., 2002; Katsuhara et al., 2002; Wang et al., 2003). In roots of salt-stressed barley, *HvPIP2;1* was down regulated but in *M. crystallinum*, the PIP2 homologue MIP-C showed increased protein levels in roots under high salinity (Katsuhara et al., 2002; Kirch et al., 2000). Expression of the water channel *PIP2;1* was reduced in leaves and roots of wheat under 125 mM NaCl during 48h, indicating down regulation of this aquaporin in the salt sensitive crop wheat. Same behaviour was observed in the salt sensitive and salt tolerant rice lines IR29 and Pokkali when treated for 48 h with 150 mM NaCl (Diedhiou and Golldack, 2005). In contrast, in the halophyte *Festuca* the expression of *FrPIP2;1* under 125 mM NaCl strongly increased in roots and was

not changed in leaves. In wheat that excludes Na^+ from leaves, the reduced expression of *TaPIP2;1*, might have a role in restriction of water loss. In contrast, the stimulated expression in roots of the Na^+ accumulating *Festuca* is likely to facilitate water flux that is driven by the increased intracellular osmotic potential and may maintain the cytoplasm water status. The non changes in the expression of *FrPIP2;1* in leaves at 500 mM NaCl might respond to the stomatal regulation.

4-3.3 Expression of VHA-B and NHX1 is coordinated to the regulation of water and Na^+ homeostasis.

Salinity causes disruption of ion homeostasis and distribution in the cell. In most species tolerant to salt, the sequestration of Na^+ into the vacuole constitutes one of the major strategies to limit the toxicity effects of this ion and to maintain a favourable homeostasis. The V-ATPase mediates electrogenic H^+ translocation at endomembranes of plant cells and energizes salt-induced secondary activated Na^+ transport via NHX-type Na^+/H^+ antiporters at the tonoplast (Ratajczack, 2000; Dietz et al., 2001). The V-ATPases consist of 14 different subunits that showed coordinated transcriptional changes in response to salt stress in the halophyte *M. crystallinum* (Golldack and Dietz, 2001). Accordingly, the regulatory subunit B was selected as representative subunit to analyse for expressional changes of the V-ATPase complex. In wheat, the transcriptional levels of *VHA-B* were more reduced in salt-stressed roots than leaves. In *Festuca*, transcript amounts of *VHA-B* decreased in leaves of salt-stressed plants as well, but increased in roots treated with 125 mM NaCl. No salt-induced transcription of V-ATPase subunits has been described from other salt sensitive species such as *Arabidopsis*, however, stimulated transcription, translation and enzyme activity respectively were reported from the halophytes *M. crystallinum* and *Sueda salsa* (Kluge et al., 1999; Ratajczack, 2000; Wang et al., 2001). In the salt tolerant *M. crystallinum* and *Beta vulgaris*, V-ATPase transcript level increased specifically in leaves but not in roots reflecting accumulation and thus vacuolar sequestration of Na^+ preferentially in leaf tissue of these species (Kirsch et al., 1996; Lehr et al., 1999; Golldack and Dietz, 2001). In *Festuca*, another tissue specificity of *VHA-B* regulation was observed, suggesting the use of different molecular strategies of Na^+ accumulation and compartmentalized detoxification in dicotyledoneous and monocotyledoneous halophytes.

Maintenance of Na^+ homeostasis further depends on NHX-mediated Na^+ transport across the tonoplast. In the salt sensitive *Plumbago media*, no vacuolar Na^+/H^+ antiport activity could be detected, but increased expression of NHX was observed in the closely related salt tolerant

P. maritima (Staal et al., 1991). Employing isolated tonoplast membrane vesicles from halophytes *M. crystallinum* and S*alicornia bigelovii* a Na^+/H^+ antiporter activity was shown to be induced by increased salt concentration (Barkla et al., 1995, Parks et al., 2002). In the halophyte *Atriplex gmelini*, expression of *AgNHX1* was induced by salinity (Ohta et al., 2002). Overexpression of NHX-type transporters in transgenic plants improved salt tolerance in sensitive species as *A. thaliana*, tomato and rice (Hamada et al., 2001; Zhang and Blumwald, 2001; Zhang et al., 2001; Fukada et al., 2004). In wheat, an opposite expressional change of a NHX-type transporter was found here that was similar to the transcription of *VHA-B* indicating specifically coordinated regulation of both genes. *VHA-B* and *NHX1* were expressed in wheat roots and leaves under control condition suggesting the role of these genes in other cells functions as well. According to Apse et al. (2003) and Sottosanto et al. (2004) the vacuolar Na^+/H^+ antiporter is also active for K^+ exchange. Under salt stress *NHX1* decreased in both leaves and roots of wheat. This suggests the reduction of the activation of *NHX1* in presence of salt. Although both *Festuca* and wheat accumulated Na^+ when exposed to 125 mM NaCl, only the halotolerant *Festuca* showed transcriptional stimulation of the key response mechanisms *NHX* and *VHA-B*. Accordingly, the sensitivity of wheat is most likely due to the missing ability of vacuolar Na^+ detoxification.

To gain further insight into the mechanisms involved in tolerance to the extreme salt stress of 500 mM NaCl, *in situ PCR* were performed. Expression of *FrPIP2;1*, *FrVHA-B* and *FrNHX1* could be detected in immature roots of *Festuca*. A similar expression pattern has been reported for MIP-C and for *McVHA-E* in roots of *M. crystallinum* (Kirsch et al., 2000; Golldack and Dietz 2001). Under salt stress, expression of *FrPIP2;1*, *FrVHA-B* and *FrNHX1* was repressed in the epidermis and outer cortex but increased in inner cortex cells, in the endodermis and in vascular tissue. The very high transcript abundance in endodermis and vasculature suggests particular increased Na^+ uptake in these cell types for long distance transport in xylem. These data demonstrate that salt dependent transcriptional regulation of *FrPIP2;1*, *FrVHA-B* and *FrNHX1* is coordinated on the cellular level and is required for cytoplasmic Na^+ exclusion and maintenance of water status in salt tolerant plants.

In conclusion, the results presented in this study demonstrate different transcriptional control of three key determinants of plant salt adaptation in wheat and in *Festuca* and indicate coordinate activation of *PIP2;1* aquaporins, V-ATPase and NHX transporters in salt tolerant but not in salt-sensitive plants to maintain Na^+ and water homeostasis. *Festuca* will be a suitable halophyte model for further comparative studies on plant salt adaptation due to its high sequence identity with wheat and other cereal crops like rice. Novel and regulatory

elements and mechanisms that are differently stress responsive in *Festuca* and wheat may be further elucidated by approaches as transcriptome analyses that will help to identify additional regulatory genes suitable to confer increased salinity tolerance to crop species.

4-4 Salt-responsive genes in rice and *Festuca rubra ssp* litoralis and induction of salt tolerance in the line IR29, rice sensitive

4-4.1 Molecular mechanisms of salt stress in the rice lines IR29 and Pokkali

Generation of cDNA- arrays containing 192 ESTs and comparing two rice lines IR29 and Pokkali, submitted to 150 mM NaCl during different times of stress identified no significant differences between the salt sensitive rice line IR29 and the salt tolerant rice line Pokkali. At 6 h of salt stress, Pokkali revealed more upregulated genes than IR29 in leaf tissue. However, the proportion of upregulated genes subsequently decreased in Pokkali to half at 24 and 48 h, this rate was increased in IR29. After 48 h of salt stress, up-and downregulated genes were more abundant in IR29 than in Pokkali. Kawasaki et al. (2001) studied the expression profiles of both lines and demonstrated that Pokkali achieved salt tolerance by rapidly expressing mechanisms that resist salt stress more efficiently than those available to IR29. They noted significant expressional changes right after 15 min and at 1h in Pokkali, whereas the induced genes in IR29 were only noted at 3 h after treatment. After 1 h, Pokkali tended to recover by reducing its expression, however, IR29 increased the rate of induced genes. Here the same behaviour was detected with more genes induced at 6 h in Pokkali than in IR29. Later IR29 recovered from its delay by inducing more genes between 24 to 48 h of salt stress. These results confirm those performed by Kawasaki et al. (2001) and might confirm the fact that early gene regulation induces Pokkali to be more tolerant than IR29. If NaCl permits to classify rice lines as tolerant or sensitive, LiCl with almost the same induced expression profile does not give enough information for screening both lines because the expression under LiCl was similar. Regulation under salt without K^+ improved the proportion of upregulated genes in Pokkali leaf tissue whereas in IR29 the number of repressed genes was increased. Taking into consideration the role of K^+ in many cellular processes as in photosynthesis, one can assume that this reaction re-established its function. Some plants tolerant to salt stress like Pokkali improve the capacity of sink tissues like leaves as a consequence of gene expression. Thus, gene expression analysis of the hybrid rice strain

LYP9 showed that a large number of genes involved in photosynthesis was upregulated in this hybrid (Bao et al., 2005). At 6, 24, 48 h salt stress no significant differences in the expression of genes were noted between both species however reduction of the proportion of expressed genes and maintaining the transcriptional activation of the activation of only some gene seem to be determinants for salt tolerance in Pokkali. These determinant genes might be constituted by the subset of transcription factors and protein kinases. Chao et al. (2005) noted in this group the highest upregulation after 20 min in the rice tolerant Nona. According to Kawasaki et al. (2001), the salt tolerant line Pokkali achieves tolerance by rapidly expressing tolerance mechanisms that resist more efficiently than those mobilised by the salt sensitive rice line IR29.

4-4.2 *Festuca* cDNA-arrays

With a cDNA-array of 480 genes, the molecular mechanisms of salt tolerance were compared between the halophyte *Festuca* and the salt sensitive rice line IR29. The similarity of actin base between *Festuca* and IR29 is bigger than 88%. The functional categories of transcripts on the *Festuca*-cDNA-arrray were expected to comprise gene members with significant homology to rice sequences. From the genes identified on the array, 30 % were concerning the metabolism, 11.7 % of genes were related to stress response and defence and 25.8 % constituted a gene group with unknown function. Although they share a high sequence similarity, *Festuca* and IR29 showed different behaviour when exposed to salt stress. The strategy used by each species was investigated by comparison of gene expression profiles obtained after different stress periods and different salt concentrations. First analysis of cDNA hybridization indicated less genes being regulated by IR29 than in *Festuca* when treated at 125 mM NaCl for 1 and 6 h. In *Festuca,* induced genes in leaf tissue increased in dependence on the concentration and reached the maximum at 500 mM NaCl. In contrast to these findings, Taji et al. (2004) identified only 6 genes strongly induced in response to high salinity by the halophyte salt cress, whereas 40 genes were identified as salt stress inducible genes in *Arabidopsis*. However the same relative number of genes induced seems to be the same in both the salt sensitive species IR29 and *Arabidopsis*, the two halophytes showed different strategies.

Functional classification allowed to establish that most induced genes belong to the category “metabolism”. In this group, a set of genes related to the NADP-dependent oxidoreductase

that is a component of the antioxidative systems were identified. Among these, a strong regulation of dehydrogenase and oxidoreductase was noted: 4 from 5 induced oxidoreductase enzymes showed transcript abundances that were higher in IR29 than in *Festuca*. The dehydrogenase indicated the same tendency. Transcript amounts of the dehydrogenase increased at 250 mM NaCl and recovered at 500 mM NaCl. Although the salt sensitive line IR29 tends to increase its transcript abundance, *Festuca*, except for some genes did not immediately and strongly respond to the constraint at low salt concentration, but adapted by expressing genes with importance in defence and survival machinery. The halophyte salt cress uses the same strategy with only 6 genes that played the determinant role in tolerance to biotic and abiotic stresses (Taji et al., 2004). With a transcript amount two times more than that in IR29, the aldehyde dehydrogenase may be linked to a survival function required by *Festuca*. *Arabidopsis* transgenic lines overexpressing this gene improve their expression as well as the tolerance to dehydration, NaCl, heavy metals (Cu^{2+} and Cd^{2+}), and H_2O_2. Tolerance of transgenic plants correlates with decreased accumulation of lipid peroxidation-derived reactive aldehydes (Ramanjulu et al., 2003). The oxidoreductase, aldo/keto reductase family was one of the genes that responded atypically, i.e. with transcript amount higher in *Festuca* than in IR29. Importance of this enzyme in the biosynthesis of secondary metabolites indispensable for plant growth and development and ecological functions including osmotic stress resistance to desiccation and salt stress have been reported for *Digitalis purpurea* (Gavidia et al. 2002). As in *Festuca*, leaves of *D. purpurea* subjected to elevated NaCl (250 mM) responds with increased levels of this enzyme. Other strategies consist in responding moderately by increasing progressively the expression with the increasing salt concentration. Thus while IR29 showed an induction factor of 1.05 at 125 mM NaCl 6 h, concerning the enzyme alpha/keto dehydrogenase, in *Festuca* the expression started by 0.95 and reached 5,11 at 500 mM NaCl 6 h. Another criterium of salt tolerance consists in inducing the stimulated production of important biomass indispensable for salt resistance. Depending on enzyme activity, this property is largely associated with CO_2 fixation and NO_3^- assimilation. In addition to the genes linked to the oxidoreduction activity, genes were identified such as cytochrome P450, AMP desaminase, glutamine-dependent asparagine synthetase and delta pyroline carboxylate synthetase with an induction factor higher than 2 in *Festuca*. Activity of these genes might be related to the photosynthetic system and plant growth which suggests that *Festuca* increased its photosynthetic and photorespiratory activity for improving growth development and for a better resistance to the salt. For example some rice varieties tolerant to NaCl increased strongly their biomass production, while Na^+ and Cl^- are stored in the lateral

and older leaves protecting young leaves which maintain a high photosynthetic activity. The process of increasing photosynthesis and respiration in the rice hybrid LYP9 which maintains high growth and yield production, enhanced the production of reactive oxygen species (ROS) (Bao et al., 2005). In this work, many genes related to stress response were upregulated in rice and in *Festuca* with almost a similar expression at 125 mM NaCl. Although IR29 could not grow at the concentration of 500 mM NaCl, *Festuca* increased strongly the transcript amounts of proteins as a senescence related protein, similar to ERD7 protein thaumathin like protein, and dehydrin rab 16b at 500 mM NaCl. The improvement of expression of these enzymes agrees with the observation by Bowler et al., (1992) under drought stress conditions, the activity of enzymes that participate in ROS scavenging increases and a higher scavenging activity may correlate with enhanced drought tolerance of the plants.

Other clearly upregulated genes appeared in the category of transcription factors, translation and signal transduction. In contrast to the other groups, the induction factor did not exceeded 2 and no big difference was seen between both species. However, if IR29 tended to respond with only low levels of upregulation between 1 and 6 h, *Festuca* caught up the delay at increased salt concentrations. According to Munns (2005), most of the transcription factors described so far are induced by rapidly imposed and severe stress, so their function in adapting the plant to a long-term salinity stress is not yet known. According to this observation, it might be better to differentiate IR29 and *Festuca* by expression occurring during the first minutes of salt stress. Chao et al. (2005) identified a group of transcription factors and kinases with highest level after 20 min at 250 mM NaCl in the salt tolerant rice Nona. Despite their transient and discrete expression observed at 6 h salt stress, transcription and signal transduction were shown to play determinant functions in the mechanisms of stress tolerance notably to NaCl. For example pea DNA helicase 45, a homologue to the eukaryotic translation initiation factor eIF-4A, has been reported to be induced in response to abiotic stresses. Its overexpression in tobacco plants conferred tolerance to salinity, indicating its potential capacity to improve salt tolerance (Sanan-Mishra et al., 2005). The *Arabidopsis* mitogen-activated protein kinase 2 (MKK2) has been defined as a key signal transducer for cold and salt stress. Biochemical and genetic analysis revealed that MKK2 modulates cold and salt tolerance through expression of a set of 152 genes. Taken together, these results indicate that the overexpression of one transcription factor or protein kinase might improve tolerance to salinity (Teige et al., 2004). The expression of the translation initiation factor SUI1 as well as the signalling transduction protein kinase SPK3 was not strongly increased in *Festuca*-cDNA-array. However Northern blot expression of *OsTIF* (rice

translation initiation factor) and *OsSPK3* (rice serine-threonine proteine kinase) showed a clear increase in the halophyte *Festuca* at 500 mM NaCl. According to Xiong and Zhu (2002), glycophytic and halophytic plants appear to have similar signalling and tolerance machineries and the difference between salt tolerance and salt sensitivity may be the result of changes in the threshold of some regulatory switches or mutation in some key determinant. To verify this hypothesis and to better understand the role of these determinant genes *OsTIF* and *OsSPK3*, their overexpression in the salt sensitive rice IR29 might be important for establishing the salt tolerance of this line.

4-5 Transgenic plants carrying a rice translation initiation Factor (*OsTIF*)

This study approached an understanding of the functions of the translation initiation factor *OsTIF* in transgenic rice plants. Generated transgenic rice plants overexpressing rice *OsTIF* were examined in relation to the stress tolerance. Insertion of this gene into embryos using *Agrobacterium* mediated transformation, opened up the way to use this method for the insertion of genes with economic interests. Under control growth conditions a slight increase of the expression of *OsTIF* in transgenic plants was detected compared to wild-type. This result indicated that supplemented genes increase the expression even in control condition. Expression of *OsTIF* in the wild-type plants suggests the presence of the factor in wild-type and its expression at low level. Under salt stress conditions, the expression increased when compared to the wild type in control and stress conditions. These results suggest that salt stress induces the expression of *OsTIF* or its post transcriptional stability. Overexpression of the eukaryotic translation initiation factor *BveIF1A* from the halophyte *Beta vulgaris* in *Arabidopsis* increased the expression of the gene as well as the tolerance to salt. Expressed in a NaCl sensitive yeast strain, *BveIF1A* increased the NaCl and LiCl tolerance and improved translation in the presence of NaCl (Rausell et al., 2003). These findings confirm that *OsTIF* showing higher expression under salt stress treatment might play a determinant role in NaCl tolerance in rice.

To analyse the tolerance to NaCl, seeds of T2 transgenic plants were grown in 50 mM NaCl. The results indicated an improvement of the germination rate and as well as an improved growth of young seedlings, whereas the wild-type showed a high inhibition in the germination phase and a delayed growth of young seedlings. In other works, a transcription

factor *OsISAP1* (*Oryza sativa* subspecies *indica* stress-associated protein) was also identified. *OsISAP1* encodes a zinc-finger protein that is induced after different types of stresses namely, cold, desiccation, salt, submergence and heavy metals as well as injury. Overexpression of this gene in transgenic tobacco conferred tolerance to cold, dehydration and salt stress at the seed-germination and seedling stage (Mukhopadhyay et al., 2004). According to these results, one can propose the presence of proteins in rice and other sensitive species with a positive effect in conditions of stress but the implication of these in the mechanisms of tolerance may depend on species, varieties or lines and on the capability to regulate correctly mRNA-levels for overcoming the stress. Thus, in the same group some tolerant and sensitive lines could be found e.g. Pokkali and IR29 in rice. In contrast to these results, the transcription factor dehydrated-responsive element binding protein (DREB) interacts with other proteins and controls the expression of many stress induced genes in *Arabidopsis*. Overexpression of *OsDREB* in rice and in *Arabidopsis* induced not only growth retardation under normal growth conditions but improved tolerance to high salt, drought and cold stresses (Ito et al., 2005). Another group of cold inducible transcription factors is the glycine-rich RNA-binding protein4 (GR-RBP4), one of the eight GR-RBP family members in *Arabidopsis* that showed germination retardation when overexpressed in *Arabidopsis* plants (Kwak et al., 2005). These results suggest different roles of transcription factors in the organism submitted to salt stress, while some are beneficial for one cell function, others support other functions. For example, the germination was altered under salt stress whereas biogenesis and metabolism were improved under overexpression from GR-RBP4 as reported by Kwak et al. (2005).

To survive under salt stress, plants must develop strategies allowing them to maintain the photosynthetic activity. One of these strategies depends on ion accumulation. Our results indicate that transgenic plants carrying *OsTIF* under salt stress accumulate less Na^+ and Cl^- in leaves than the wild-type. It was demonstrated in rice that the capacity to limit the uptake of Na^+ and Cl^- in cells improves the tolerance to NaCl (Golldack et al., 2002; Diedhiou and Golldack, 2005). These studies, in agreement with our results,indicate that *OsTIF* induced salt tolerance when expressed in the salt sensitive rice line IR29. In addition to their toxic effect, Na^+ and Cl^- induce a nutritional imbalance against K^+, $NO3^-$, $PO4^-$, Ca^{2+} and micro-elements (Lutts, 1996b). Thus, K^+ and Ca^{2+} uptake in *OsTIF* transgenic plants showed that Ca^{2+} levels remain identical in both transgenic and wild-type plants, while K^+, despite its high level is slightly more abundant in the wild-type than in the transgenic plants. Looking at its low and constant level in transgenic and wild-type plants, a Ca^{2+} imbalance seems not to

interfere in the process of salt tolerance. However, to maintain low Na^+ content in leaf tissue, Shabala et al. (2005) postulated that the beneficial effects of supplemental Ca^{2+} on leaf photosynthesis may be a result of much improved K^+/Na^+ ratios in the leaf mesophyll cells due to the regulation of leaf K^+ balance. Regulation of K^+ is the central system for the control of salt tolerance. Comparing K^+ accumulation under 150 mM NaCl, the sensitive rice line IR29 accumulated a little bit more K^+ than the salt tolerant rice line Pokkali (Diedhiou and Golldack, unpublished). In relation to these finding, transgenic plants showed the same behaviour as the salt tolerant rice line Pokkali concerning K^+ uptake. The small reduction of K^+ content in comparison to wild-type might be related to its role to balance Na^+ entry. The wild-type, despite its high K^+ content, accumulated more Na^+ in leaves. Thus, Maathuis and Amtmann (1999), Munns (2002) and Tester and Davenport (2003) considered that the ability of plants to retain K^+ and maintaining K^+/Na^+ selectivity has always been considered a key feature of salt tolerance. Taken together these results confirm that transgenic plants carrying *OsTIF* gene are more tolerant to salt stress.

Maintaining an improved regulation of K^+/Na^+ homeostasis in leaves, transgenic plants might maintain a good photosynthetic activity under salt stress conditions. Analysis of the photosynthetic yields confirmed the improved photosynthetic activity of the transgenic plants in comparison to wild-type. Morphological aspects after 2 weeks of salt stress showed transgenic plants with some leaves kept green whereas the wild-type was dead. In comparison with the salt tolerant rice Pokkali grown in the same conditions, transgenic plants maintained a photosynthetic yield almost comparable with Pokkali after 96 h salt treatment. Differences in phenotype between Pokkali and *OsTIF* overexpression were seen just after 2 weeks of salt stress when transgenic plants became more wilted. *OsTIF* induced tolerance to salt but the mechanism regulating the tolerance by Pokkali seems to be more important or more efficient than in *OsTIF* transgenic plants.

4-5.1 Interactions of inserted gene *OsTIF* with other genes.

Hybridization of *OsTIF* transgenic plants with *Festuca* cDNA-arrays identified some induced genes with high or low induction factors (Table 3-5.1). Among these the highest level was seen in the group of signal transduction. The second group with more genes induced was linked to metabolism. Protein turnover, transcription factors, photosynthesis and stress constituted and other distinctly regulated genes. These results suggest that *OsTIF* might exert an influence on the expression of other genes even under control conditions. As a consequence of this influence, the increased expression of the protein kinase could be

explained by the activation from *OsTIF* because it is postulated that stress is perceived and transduced through a chain of signalling molecules that ultimately affect regulatory elements of stress-inducible genes which initiate the synthesis of proteins.

Altered transcript accumulation of genes like Rubisco activase, chaperones and protein turnover could be important to improve photosynthesis. The protein turnover ATP-dependent OsClp protease encodes an ATP-binding protein subunit Clp protease has the activity of a chaperone binding to specific target polypeptide substrates and is expressed in chloroplast (Zheng et al., 2002). The abundant expression of these genes correlated to the photosynthesis system and might be an important factor in optimizing photosynthesis and biomass production, which is an important factor in salt tolerance (Lutt, 1995). Thus, some tolerant rice lines improved their biomass production in conditions of salt stress. Here, photosynthetic measurement and morphological aspects showed that the transgenic plants generated more biomass and higher photosynthetic yield than the salt sensitive wild-type.

The presence of genes related to ROS scavenging (glycine dehydrogenase, oxidoreductase and glutaredoxin) was also noticed. Reactive oxygen species increase with oxidative stress. Activation of ROS scavenging enzymes under control conditions could be a prerequiste to eliminate efficiently ROS or toxic products like aldehydes arising from reactions of ROS with lipids and proteins also under stress. These genes have been identified with high expression in the halophyte salt cress and in transgenic *Arabidopsis* plants carrying an aldehyde dehydrogenase (ALDHs) (Taji et al., 2004; Ramanjulu et al, 2003). These results allow to conclude that overexpression of the rice translation initiation factor *OsTIF* indirectly activates genes responsible for the synthesis of antioxidative enzymes necessary for cellular ROS detoxification.

The experiments with cDNA-arrays, demonstrated the influence of the stable *OsTIF* insertion on the activation of other genes with vital functions (Table 3-5.1). Verification of the gene expression was performed by RT-PCR. Plants with *OsTIF* transgene showed an important improvement of the expression of *OsVHA-B* after 3 h 200 of mM NaCl treatment. In previous work, Diedhiou and Golldack (2005) studied the expression of the same gene in the salt sensitive rice line IR29 and the salt tolerant Pokkali and found increased expression of *OsVHA-B* in Pokkali after 3 h 150 mM NaCl, whereas in the salt sensitive line IR29 expressional regulation of *OsVHA-B* seemed to be missing. In their work with salt–tolerant plant species *M. crystallinum* and *Sueda salsa*, Golldack and Dietz (2001) and Wang et al. (2001) reported a transcriptional up-regulation of V-ATPase in conditions of salt stress. In

contrast, in the sensitive *A. thaliana,* Kluge et al. (1999) noted that *AtVHA-D* did not respond to salinity. All these findings suggest that only salt tolerant plants increased the expression of *OsVHA-B* (subunit B of V-ATPase). According to its role in the sequestration of Na^+ into the vacuole, plants with higher expression of *OsVHA-B* under salt stress might tentatively be considered as salt tolerant. In accordance to this hypothesis, transgenic plants with *OsTIF* insert showed the same behaviour and were more salt tolerant. Therefore, the *OsTIF* translation initiation factor activates the induction of *OsVHA-B* which energizes Na^+ compartmentalization.

The expression of *OsNHX1* vacuolar Na^+/H^+ antiporter which regulates the sequestration of Na^+ into the vacuole showed an improvement of the expression in both wild-type and transgenic plants under 200 mM NaCl for 3 h. Taken together, wild-type plants showed the highest increase. These results seem to be opposed to many studies in which salt tolerant varieties induced more strongly the expression of NHX1-type genes than the wild-type (Zhang and Blumwald, 2001; Zhang et al., 2001; Shi and Zhu, 2002; Fukuda et al., 2004). Expression of the *OsNHX1* in the salt sensitive rice line IR29 and in the salt tolerant line Pokkali under salt stress indicates significant increase of *OsNHX1* in the first hours after salt treatment in both lines. A slightly higher responsiveness advance could be noted in Pokkali at 3 h salt stress, however, at 6 h, IR29 had the most abundant mRNA (Diedhiou and Golldack 2005). Taken together, these reports as well as the findings of this work suggest that *OsNHX1* is not activated only for Na^+ sequestration. Because most of the NHX1 identified belong to salt sensitive species (Aharon et al., 2003), NHX1-type transporters might play a determinant role in the sensitive species in control and in salt stress conditions. Its transcript level was higher in IR29 in control conditions and almost comparable to the expression of Pokkali under salt stress. The high expression of *OsNHX1* in the wild-type could be explained by the finding of Aspe et al. (2003), whose studies established *AtNHX1* as the dominant K^+ and Na^+/H^+ antiporter in leaf vacuoles in *Arabidopsis*. They also suggested that the contribution of *AtNHX1* to ion homeostasis is important not only for salinity tolerance but development as well. According to the ion accumulation pattern under salt treatment, wild-type contained more K^+ than the transgenic line S4. On the one hand, this finding reinforces the hypothesis that the activation of the vacuolar Na^+/H^+ antiporter (*OsNHX1*) is due to K^+ exchange in the wild type accumulating more Na^+ and on the other hand, considering the low Na^+ uptake in the transgenic plants, *OsNHX1* might be considered to be more active for Na^+ exchanges.

The oxidoreductase (Osoxreds) is a NADP-dependent oxidoreductase enzyme which is considered as a component of the antioxidative system (Gavidia et al., 2002; Orberschall et al., 2000). Under control conditions, *OsTIF* transgenic plants showed an upregulation of the expression of Osoxreds. Under conditions of salt stress, transgenic plants also increased the expression of this gene, whereas in wild-type the expression was strongly reduced. The increase in the expression of the oxidoreductase enzyme could be related to the scavenging activity of ROS whose concentrations increase in the presence of environmental stress (Ramanjulu et al., 2003). In tobacco, Oberschall et al. (2000) identified the alfalfa gene encoding a novel plant NADPH-dependent aldose/aldehyde reductase and showed that plants over-producing the alfalfa enzyme had lower concentrations of reactive aldehydes and showed tolerance against oxidative agents and drought stress. Under salt stress, *Arabidopsis* transgenic plants overexpressing the enzyme aldehyde dehydrogenase showed an intense activity as well as a decrease in the accumulation of aldehyde. According to Ramanjulu et al. (2003) this feature is correlated to the detoxification of aldehydes and to the amelioration of oxidative stress. These data, in agreement with the role of the oxidoreductase, suggest that the elimination of reactive oxygen species constitutes one of the mechanisms for salt stress tolerance. Thus, insertion of *OsTIF* in sensitive rice somehow activates the oxidoreductase by a yet unknown mechanism. In contrast to these studies, Parida et al. (2004) showed in the mangrove species (*Bruguiera parviflora)* that the protection against activated oxygen species is the result of the induction of certain antioxidative enzymes however lipid peroxidation was avoidable during salt exposure.

This study showed that the translation initiation factor *OsTIF* inserted in the sensitive rice line IR29 increased the efficiency of mechanisms for reducing cellular damage by eliminating toxic reactants and by improving Na^+ accumulation in the vacuole. Therefore, and for the time being *OsTIF* could be considered as a central gene product improving the tolerance to salt. The augmented response of *OsTIF* transgenic plants to salt stress followed by the enhanced activity of *OsVHA-B* and of the antioxidant activity (oxidoreductase) as well as the improvement of germination, growth and photosynthetic yield tolerance under salt stress, suggests that *OsTIF* translation initiation factor could potentially be used to enhance the tolerance of different plants and crops to salt stress.

4-6 Transgenic plants carrying the protein kinase *OsSPK3*

This part of the study analysed the functions of the signal transduction element *OsSPK3* in transgenic rice plants. Transgenic rice plants generated overexpressing the rice gene *OsSPK3* and examined for their stress tolerance. *OsSPK3* sense lines appeared to express slightly more *OsSPK3* than the wild-type under control growth conditions. These results indicated that the reinforcement of the gene *OsSPK3* by inserting an additional gene copy of *OsSPK3* into the genome slightly increased the expression. In conditions of salt stress, treatment with 150 mM NaCl for 7 days increased significantly the expression of *OsSPK3* in sense lines and decreased it in the wild-type. The mitogen-activated protein Kinase (MAPK) are a key components of the evolutionary conserved signal transduction cascade that eukaryotic organism use to regulate various aspects of cellular functions under environmental stress. Expression of the gene EhHOG (high-osmolarity glycerol response) a *MAPK* homolog playing a central role in *HOG* signal transduction, from the Dead Sea fungus *Eurotium herbariorum* in the wild type *Saccharomyces cerevisiae*, enhanced the resistance against multiple stresses such as salt, LiCl, H_2O_2, low and high temperature (Jin et al., 2005). Guo et al. (2004) found by overexpression, a slight upregulation of the threonine/serine protein kinase SOS2 (salt overly sensitive) under salt stress, in *Brassica napus*, and *Arabidopsis*. The increased active SOS2 levels in *planta* improved significantly the tolerance to salt when compared to wild type. In agreement with these data, *OsSPK3* whose expression strongly increased under salt treatment could play an important role in establishing salt tolerance by activation of mechanisms related to the tolerance. SOS2, a threonine/serine protein kinase represents a novel family of proteins only found in plants. Molecular and biochemical analyses have established a possible signal pathway used by the SOS complex to overcome salt, drought and cold stress (Zhu, 2002). According to the author (Fig. 4-6.1), the ionic aspect of salt stress is signaled via the SOS pathway where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters for ions such as Na^+ K^+ and H^+ (Zhu, 2000). Compared to this system *OsSPK3* seemed to influence the transcriptional expression of some genes implicated in ion exchanges and which play a determinant role in ion homeostasis and in term in the tolerance to salt.

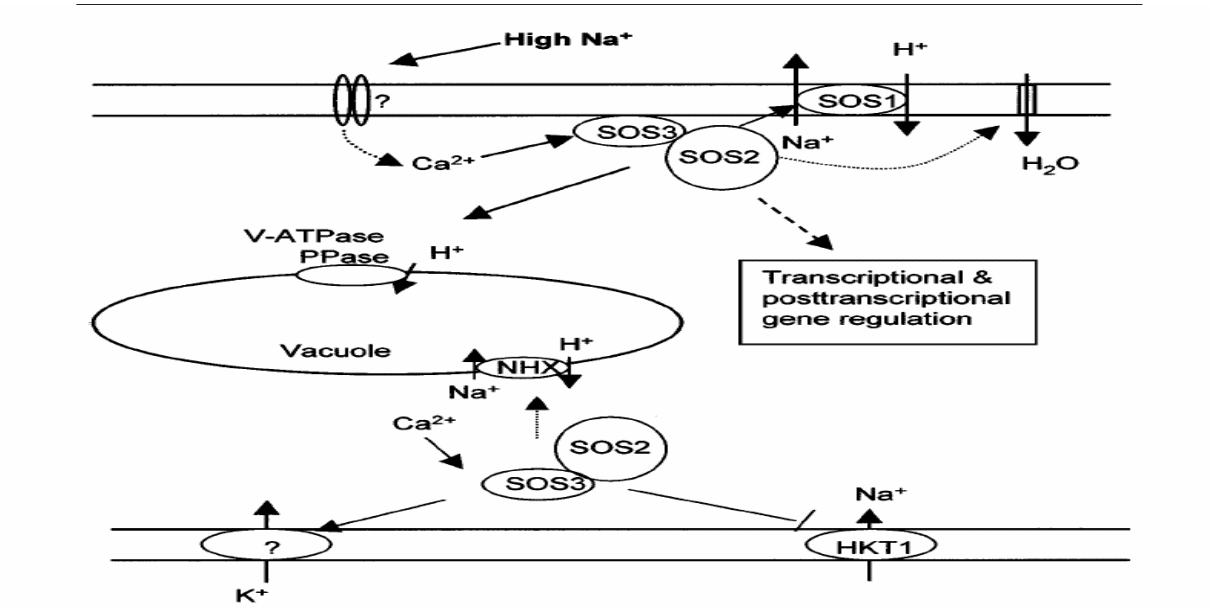


Fig. 4-6.1 Regulation of ion (e.g., Na^+ and K^+) homeostasis by the SOS pathway. High NaCl stress initiates a calcium signal that activates the SOS3-SOS2 protein kinase complex, which then stimulates the Na^+/H^+ exchange activity of SOS1 and regulates transcriptionally and posttranscriptionally the expression of some genes. SOS3-SOS2 may also stimulate or suppress the activities of other transporters involved in ion homeostasis under salt stress, such as vacuolar H^+-ATPases and pyrophosphatases (PPase), vacuolar Na^+/H^+ exchanger (NHX), and plasma membrane K^+ and Na^+ transporters (Zhu, 2002).

Treated for 7 days with 150 mM NaCl, *OsSPK3* transgenic plants showed higher expression of *OsVHA-B* (rice subunit B of V-ATPase), energizing the Na^+ sequestration into the vacuole. The ability of some plants to tolerate extended periods of salt stress is correlated with their capacity to limit the presence of Na^+ in the cytoplasm by accumulating it in the vacuole. Na^+ entry into the vacuole is energy dependent; enhanced *OsVHA-B* levels under salt stress might contribute to the sequestration of Na^+ into the vacuole and might therefore improve the tolerance to salt. Despite the intense activation of *OsVHA-B* in transgenic plants overexpressing *OsSPK3*, the activity of *OsNHX1* (rice Na^+/H^+ antiporter) catalyzing the transport of Na^+ into the vacuole remained low in transgenic plants and high in wild-type plants. This result is in contradiction with many other publications reporting increased expression of NHX1-type transporters in *Arabidopsis* (Apse et al., 1999), *Brassica* (Zhang et al. 2001) and tomato (Zhan and Blumwald 2001) in the presence of elevated salt concentration. The higher expression of *OsNHX1* in wild-type could not be attributed to the capacity of the wild-type to exclude Na^+ from the cytoplasm because the concentration of Na^+ and Cl^- remained strongly higher compared to the transgenic plants however, these ions were related to salt sensitivity. Thus, the wild-type plants showed an early necrosis of their leaves, a low germination rate and growth inhibition as well as a strong decrease of their photosynthetic yield under salt stress. Apse et al. (2003) and Sottosanto et al. (2004)

demonstrated a role of the vacuolar Na^+/H^+ antiporter in K^+ exchange, ion homeostasis and in other cellular functions. For example, the developing flower buds of morning glory change from purple to blue as the petals open. This morphological change is attributed to the activity of an *NHX1* homologue in *Ipomoea* (Yamaguchi et al., 2001). Ion uptake analysis under salt treatment indicated more K^+ uptake in wild type compared to sense transgenic plants. This might establish a correlation between K^+ uptake and the activity of *OsNHX1*. Taken together these results suggest that *OsNHX1* induced in rice wild-type is more involved in K^+ transport than in Na^+ transport. Other reasons for the reduction of the expression in transgenic plants might be related to the influence of *OsSPK3*.

In order to determine the influence of excess *OsSPK3* protein kinase in the regulation of other genes, cDNA from the sense transgenic plants was hybridized to the *Festuca* cDNA-array. The results showed 10 induced genes with an induction factor higher than 1.4. Among these, some genes are suggested to be involved in protein turnover, transcription as well as antioxidant activity. Presence of a chaperon and an ATP-dependent Clp protease was related to protein synthesis (Zheng et al., 2002). The intense expression in control conditions of oxidoreductase and catalase, both enzymes related to cellular ROS detoxification, suggests the upregulation of defence genes against ROS which significance under salt stress. The halophyte salt cress showed the same disposition (Taji et al., 2004). All these results indicate different functions influenced by *OsSPK3*. Detailed studies under stress conditions showed that wild-type plants had the highest transcript amounts of the enzyme oxidoreductase, whereas the catalase expression was more intense in transgenic plants. The true mangrove, *Bruguiera parviflora*, subjected to varying levels of NaCl revealed a decrease of total ascorbate and glutathione contents as well as decreased catalase activity while enhancing the content of H_2O_2 as well as the activities of ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Parida et al., 2004). Catalase expression in this work confirmed these findings. Interestingly despite its tolerance to high salt concentration, the mangrove *Bruguiera parviflora* in conditions of salt treatment showed strong decrease of the activity of catalase (37%), while in control conditions, this activity remained high. Lee et al. (2001) observed the same decrease in rice and suggested that the strong decrease of catalase activity observed in the wild type might be due to an enhancement of H_2O_2. Thus Feierabend et al. (1992) demonstrated that the inactivation of catalase under salt stress was linked to H_2O_2 accumulation. Like the mangrove *Bruguiera parviflora*, transgenic plants overexpressing *OsSPK3* maintained a certain expression level of catalase. The reduction of antioxidant

activity by sense plants and its increase by the wild-type plants suggest a partial inhibition induced by excess *OsSPK3*. Therefore, *OsSPK3* could negatively act through an indirect way and modulate the expression of oxidoreductase.

When submitted to 150 mM NaCl for 7 days, *OsSPK3* transgenic plants significantly increased the expression of *OsSPK3* and increased the transcript level of some genes related to the salinity tolerance. During experimental verification of the tolerance the sense plants had higher germination rates, increased growth, lower Cl^- and Na^+ uptake as well as stimulated photosynthetic activity under salt stress. This improvement suggests that *OsSPK3* (threonine/serine protein kinase) might improve the tolerance to salt of the sensitive rice line IR29 by regulating the ions movement in cells and by suppressing the germination and growth inhibitors activated under salt stress. According to Schuppler et al. (1998) and Burssens et al. (2000), slower cell division under water stress was probably a result of reduced cyclin-dependent kinase (CDK) activity. The reduction of CDK activity may be a result of combined effects of transcription suppression of cyclins and CDKs and induction of CDK inhibitors

Comparison with Pokkali a more tolerant rice line indicated some similarities. For example, the photosynthetic yield evaluated at 96 h showed the same lower decrease upon salting between transgenic and Pokkali plants, whereas the wild type showed a reduction of more than 90 %. By analysing ion uptake, it was found that Ca^{2+}contents did not show changes under salt stress between wild-type and the S4 transgenic plants. Despite its important role in cells namely in membrane structure, Ca^{2+} constitutes a cofactor for many proteins and activates the process of Ca^{2+}-dependent signal transduction. In *Arabidopsis*, for example, the calcium binding protein Salt Overly Sensitive 3 (SOS3) interacts with and activates the protein kinase SOS2, which in turn activates the plasma membrane Na^+/H^+ antiporter SOS1 to bring about sodium ion homeostasis and salt tolerance (Guo et al., 2004). As a cofactor of protein kinase, Ca^{2+} might, in correlation with protein kinase, operate rapidly to induce signal required for the response to salt stress. In *Arabidopsis*, Knight et al. (1997) reported the highest content of free cytosolic Ca^{2+} just 11 seconds after beginning of high salt treatment. This event correlates with the expression of the gene *p5cs* which encodes a protein with a known protective role in the response to drought. Later on, for example at 48 h or even more at one week salt stress, it might be too late to know the real concentration of Ca^{2+} because Ca^{2+} is a transient signal and to speculate about its concentration correlated to tolerance to salinity in the transgenic plants. However addition of Ca^{2+} ameliorated the tolerance to salt

by limiting Na^+ uptake in the leaves (Shabala et al., 2005). The concentration of Cl^- and Na^+ in sense transgenic plants under salt treatment was strongly reduced, whereas the wild-type showed the highest uptake. Toxicity induced by these ions caused complete bleaching of the leaves of wild-type plants, completely faded after one week salt stress, whereas transgenic plants retained their leaves green. The ability to limit Cl^- and Na^+ uptake in leaf tissue improved the photosynthetic activity. Thus at 96 h salt treatment photosynthetic yield of Pokkali and transgenic plants was more than 80%, whereas the decrease reached 80% in the wild-type. Another factor of photosynthetic activity is correlated to the regulation of K^+. In both wild-type and transgenic plants, K^+ content in leaf tissue was high, but higher K^+ levels were detected in the wild-type. Regulation of K^+ is the central system for the control of salt tolerance. Thus, Maathuis and Amtmann (1999), Munns (2002) and Tester and Davenport (2003) considered that the ability of plants to retain K^+ and to maintain K^+/Na^+ selectivity is a key feature of salt tolerance. According to this report, transgenic plants seemed to be more efficient in photosynthetic activity, thus more tolerant to salt stress than the wild type. Selection of plants with enhanced tolerance to salt stress is essential for sustained agricultural production worldwide. Therefore *OsSPK3* improving the tolerance to salt of the sensitive rice line IR29 can be integrated in programs of selection.

In this work, the mechanisms of salt tolerance were investigated in rice. In addition two genes, the translation initiation factor *OsTIF* and the threonine/serine protein kinase *OsSPK3* were analysed in detail. The results obtained from overexpressing these genes in the salt sensitive rice line IR29 increased our knowledge about molecular mechanisms of tolerance to salt stress and provided more evidence for involvement of both genes in conferring salt tolerance to the salt sensitive rice line IR29. However, the understanding of the exact modes of action of *OsTIF* and *OsSPK3* awaits future studies. Suppression of these genes using antisense constructs might elucidate the pathway of regulation for instance in modifying the oxidoreductase and the vacuolar Na^+/H^+ antiporter which are likely to contribute to the phenotype of better growth performance under salt treatment.

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Table 3-4.2 Categories of transcripts regulated in salt-stressed IR29 and *Festuca*

		OsLc125_6	FrLc125_6	FrLc250_6	FrLc500_6
10-unknown	CHY zinc finger, putative	1,03	0,37	1,07	1,07
10-unknown	expressed protein	1,44	2,88	1,61	1,05
10-unknown	hypothetical protein	0,98	0,60	1,19	1,48
10-unknown	tubby-like protein 7	1,82	2,02	1,15	0,58
10-unknown	expressed protein	0,74	0,54	1,40	2,27
10-unknown	expressed protein	0,82	0,27	0,72	1,60
10-unknown	expressed protein	1,25	1,74	0,91	1,25
10-unknown	hypothetical protein	1,15	1,03	0,70	0,78
10-unknown	Zinc finger, C3HC4 type (RING finger), putative	0,59	0,68	1,20	1,33
10-unknown	hypothetical protein	0,71	0,27	1,14	1,17
10-unknown	expressed protein	1,18	2,12	1,33	1,21
10-unknown	hypothetical protein	0,86	0,72	1,05	0,53
10-unknown	expressed protein	1,16	0,63	1,40	1,64
10-unknown	expressed protein	1,32	0,48	1,27	1,31
10-unknown	expressed protein	0,67	0,79	1,05	1,88
10-unknown	expressed protein	0,94	0,53	1,27	1,55
10-unknown	expressed protein	1,04	0,32	1,67	0,83
10-unknown	hypothetical protein	1,08	0,87	0,82	0,65
10-unknown	expressed protein	1,12	0,74	0,99	0,63
10-unknown	hypothetical protein	1,18	0,64	0,36	1,63
10-unknown	expressed protein	1,21	0,32	1,38	0,66
10-unknown	hypothetical protein	1,23	0,26	0,86	0,38
10-unknown	hypothetical protein	1,45	0,55	1,06	0,86
10-unknown	expressed protein	0,27	0,34	1,42	1,94
10-unknown	expressed protein	0,55	0,68	1,47	2,08
10-unknown	dual specificity phosphatase, catalytic domain, putative	0,46	1,00	1,47	3,74
10-unknown	syntaxin 71	0,39	0,64	1,19	2,09
10-unknown	expressed protein	0,20	1,01	1,09	4,38
10-unknown	expressed protein	4,47	2,74	2,06	0,76
10-unknown	Triticum aestivum clone wlm1.pk0017.b7:fis	0,39	0,66	0,46	2,20
10-unknown	tubby-like protein 7	1,71	0,99	0,52	0,59
10-unknown	expressed protein	2,35	1,34	0,92	0,85
5-metabolism	**aldehyde dehydrogenase**	**0,86**	**1,94**	**1,11**	1,67
5-metabolism	Phosphomannose isomerase type I, putative	1,09	1,25	0,79	0,62
5-metabolism	glyceraldehyde-3-phosphate dehydrogenase, type I	0,67	0,44	1,67	0,92
5-metabolism	oxidoreductase, short chain dehydrogenase/reductase family, putative	1,22	0,77	0,92	1,12
5-metabolism	**asparagine synthase**	**0,93**	**0,74**	**5,78**	10,80
5-metabolism	delta l-pyrroline-5-carboxylate synthetase	1,20	0,24	1,76	2,74
5-metabolism	indole-3-glycerol phosphate synthase, putative	1,41	0,35	1,21	1,21
5-metabolism	maleylacetoacetate isomerase	1,33	0,45	1,28	0,88
5-metabolism	glycine dehydrogenase	1,12	1,17	1,47	1,04
5-metabolism	lactoylglutathione lyase, putative	0,80	0,99	1,43	1,17

5-metabolism	**oxidoreductase, zinc-binding dehydrogenase**	**0,96**	**0,72**	**1,16**	**0,92**
5-metabolism	delta l-pyrroline-5-carboxylate synthetase	1,28	2,67	1,60	2,30
5-metabolism	Glycosyl hydrolases family 17	0,93	0,27	0,59	1,07
5-metabolism	Plant neutral invertase, putative	0,82	0,62	0,92	1,37
5-metabolism	putative phosphatidylglycerolphosphate synthase	0,94	1,06	1,00	0,84
5-metabolism	oxidoreductase, aldo/keto reductase family	0,89	1,15	0,92	0,90
5-metabolism	isovaleryl-coa dehydrogenase 2, mitochondrial precursor	1,03	1,31	1,03	0,90
5-metabolism	choline kinase, putative	1,18	2,92	0,85	1,29
5-metabolism	oxidoreductase, aldo/keto reductase family	1,95	1,09	0,91	0,89
5-metabolism	glutamine-dependent asparagine synthetase 1	0,89	0,78	1,42	2,50
5-metabolism	beta-alanine-pyruvate aminotransferase	0,97	0,53	1,62	1,40
5-metabolism	aldehyde dehydrogenase (NAD) family protein	0,74	0,65	1,71	0,64
5-metabolism	glycosyl hydrolases family 32, putative	0,86	0,52	0,94	0,70
5-metabolism		1,11	1,29	1,11	1,07
5-metabolism	fumarylacetoacetase	1,29	0,85	0,83	0,89
5-metabolism	succinyl-coa ligase [gdp-forming] beta-chain	0,52	0,87	1,08	0,68
5-metabolism	putative deoxycytidine deaminase	0,56	0,43	1,80	1,45
5-metabolism	carbonic anhydrase, putative	0,58	1,08	1,06	0,45
5-metabolism	glyceraldehyde-3-phosphate dehydrogenase	1,03	0,94	0,95	0,84
5-metabolism	branched-chain alpha keto-acid dehydrogenase E1 alpha subunit-like protein	1,07	0,95	1,86	5,11
5-metabolism	phosphoethanolamine cytidylyltransferase	1,40	0,39	0,93	0,78
5-metabolism	AMP deaminase, putative	0,37	1,34	0,92	5,85
5-metabolism	putative pyruvate kinase	0,85	1,31	1,28	0,91
5-metabolism	aminotransferase, classes I and II, putative	1,03	0,56	1,03	1,20
5-metabolism	carbonate dehydratase, putative	3,37	0,53	0,83	1,00
5-metabolism	cytochrome P450	0,81	1,71	0,62	2,53
5-metabolism	oxidoreductase, short chain dehydrogenase/reductase family	2,07	0,74	0,51	0,51
5-metabolism	asparagine synthase	2,70	0,52	0,71	2,13
1-transcription	WRKY DNA -binding domain, putative	0,95	0,64	0,81	1,47
1-transcription	RNA-binding protein 3	1,05	0,30	0,77	0,49
1-transcription	RNA recognition motif, putative	0,60	0,26	1,30	1,36
1-transcription	exonuclease, putative	1,02	0,20	1,12	1,51
1-transcription	PAP fibrillin, putative	1,16	1,88	1,43	1,30
2-translation	translation initiation factor SUI1	0,94	1,21	0,77	0,85
2-translation	Ribosomal L18p/L5e family, putative	1,13	1,14	0,66	0,58
2-translation	putative ribosomal protein L13a	1,22	0,22	0,49	0,74
2-translation	peptide chain release factor eRF/aRF, subunit 1	1,13	1,01	0,82	1,11
3-signal transduction	Protein kinase domain, putative protein kinase SPK-3	0,98	0,80	0,99	0,76
3-signal transduction	protein kinase domain, putative	1,52	0,84	1,09	0,99
3-signal transduction	cyclic nucleotide-binding transporter 1	1,26	0,99	1,50	1,28

3-signal transduction	protein kinase domain, putative OSK4 serine/threonine specific PK AP004164	0,95	0,76	0,52	1,84
3-signal transduction	putative wall-associated protein kinase	0,82	1,22	0,46	0,71
4-protein turnover	cysteine proteinase 1 precursor	1,04	1,29	1,24	1,24
4-protein turnover	X-Pro dipeptidase-like protein	0,94	0,26	1,55	1,29
4-protein turnover	Proteasome A-type and B-type, putative	0,85	1,24	0,91	0,84
4-protein turnover	Similar to endopeptidase Clp	1,09	0,85	0,93	0,87
4-protein turnover	cysteine proteinase (EC 3.4.22.)	0,84	1,10	1,22	1,06
4-protein turnover	calreticulin family, putative	1,03	0,71	0,66	0,46
4-protein turnover	26S proteasome subunit P45 family	1,14	0,34	0,90	0,80
4-protein turnover	chaperonin, 10 kDa, putative	0,72	0,33	0,92	0,57
4-protein turnover	ATP-dependent Clp protease	1,04	1,20	0,83	0,84
6-channels and transporters	putative Vacuolar ATP synthase subunit d	1,08	1,61	1,56	0,99
6-channels and transporters	ABC transporter, putative	1,28	1,54	0,85	0,88
6-channels and transporters	Mitochondrial carrier protein, putative	1,01	1,11	1,06	0,79
6-channels and transporters	ATP synthase (E/31 kDa) subunit	0,92	0,64	1,00	0,66
6-channels and transporters	HvnHX1 sodium/proton antiporter	0,80	0,49	2,17	0,90
6-channels and transporters	ABC transporter, putative	2,92	0,84	0,34	1,17
6-channels and transporters	ADP/ATP carrier protein family	0,31	1,09	1,31	1,72
6-channels and transporters	ATP synthase (C/AC39) subunit	0,66	0,91	1,05	3,64
6-channels and transporters	V-type ATPase, A subunit	1,02	0,80	0,55	0,56
6-channels and transporters	nucleoside transporter, putative	1,19	1,28	0,69	1,23
7- stress response and defense	Copper/zinc superoxide dismutase, putative	1,00	0,85	1,16	0,63
7- stress response and defense	catalase, putative	1,33	0,93	1,19	0,77
7- stress response and defense	putative antifungal zeamatin-like protein	1,10	1,98	1,30	1,56
7- stress response and defense	ubiquitin-activating enzyme e1 2	0,87	1,54	1,30	0,93
7- stress response and defense	dehydrin rab 16c	1,78	0,33	1,13	1,79
7- stress response and defense	catalase (EC 1.11.1.6) catA	1,01	0,97	0,44	2,11
7- stress response and defense	glutaredoxin	1,07	0,45	0,86	0,72
7- stress response and defense	thaumatin-like protein TLP7	1,99	0,88	0,27	2,48
7- stress response and defense	similar to glycine-rich RNA-binding protein 2 -rice	0,87	0,88	1,46	1,90
7- stress response and defense	senescence-related protein	0,39	0,42	1,07	2,43
7- stress response and defense	**dehydrin rab 16b**	**1,31**	**0,98**	**1,37**	**2,35**
7- stress response and defense	similar to ERD7 protein	0,31	0,61	1,30	2,04

7- stress response and defense	similar to heat shock protein 82	0,86	1,35	0,74	0,64
7- stress response and defense	lethal leaf-spot 1	1,05	1,09	1,80	1,59

°C	Degree Celcius
µg	Microgram
ABA	Absicic acid
Bp	Base pair
BSA	Bovine serum albumin
cDNA	complementary DNA
Cl^-	Chloride
DEPC	Diethylpyrocarbonate
Dig.	Digoxigenin
dwt	Dry weight
E. coli	*Escherischia coli*
EST	Expressed sequence tag
Fig.	Figure
fwt	Fresh weight
GFP	green fluorescence protein
h	hour
H_2O_2	Hydrogene peroxide
K^+	Potassium
min	Minute
mM	Millimolar
mm	Millimeter
mRNA	Messenger-RNA
Na^+	Sodium
NADP	Nicotinamid adenine dinucleotid phosphate
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyle sulfate

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Erklärung

Hiermit erkläre ich, dass ich diese Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Alle aus der Literatur ganz oder annähernd entnommenen Stellen habe ich als solche kenntlich gemacht.
Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

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