Instituto Juan March
de Estudios e Investigaciones

128 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Molecular Basis of Ionic Homeostasis and Salt Tolerance in Plants

Organized by

E. Blumwald and A. Rodríguez-Navarro

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# Introduction

A. Rodríguez-Navarro and E. Blumwald

**Molecular basis of ionic homeostasis and salt tolerance in plants**

Salting our agricultural lands is a 6,000 year old problem originated by extensive irrigation in semi-arid regions. This process slowly decreases the surface of productive agricultural lands and works against the increase in food production which must keep pace with population growth. Although famine in the world nowadays is originated by complex problems and not only by an insufficient production of food, there is no doubt that the gains in food production provided by the Green Revolution have reached their ceiling while the world population continues to rise. Therefore, increasing the yield of crop plants in normal soils and in less productive lands, including salinised lands, is an absolute requirement for feeding the world.

The excess of sodium chloride in the soil solution has two physicochemical effects. First, it decreases the water potential and, as a consequence, hinders the water flow from the soil solution to the xylem vessels and to the upper part of the plant. Second, the toxic ions, sodium and chloride, are taken up instead of other nutrients, inhibiting sensitive metabolic processes, and producing deficiencies in essential nutrients, mainly potassium. Plants can overcome the first stress by accumulating solutes both in cells and in the xylem sap. This decreases the osmotic potential, and restores the flow of water and the turgor pressure of the cells. The second stress must be overcome by excluding toxic ions from the cytoplasm of the plant cells, either keeping them outside the cells or confined into the vacuole.

Salt tolerance in halophytes is a complex adaptation which includes, vacuolar confinement, low sensitivity of the normally salt-sensitive metabolic processes, and use of the salt for water potential adjustments. Glycophytes are less adapted to grow in salty environments, and their low tolerance is only accounted for by salt exclusion. Most crop plants are glycophytes, many of them sensitive to sodium chloride at concentrations well below those producing an osmotic stress (ca. 200 mM NaCl), for which traditional plant breeding has produced tolerant cultivars in very few cases. Because ionic tolerance can be explained by simple physiological traits, exclusion, confinement, and intrinsic tolerance of metabolic processes, the improvement of salt tolerance of crop plants may be technically difficult but not impossible. For this improvement, genetic engineering offers the best possibilities, because the processes leading to ionic tolerance can be constructed in crop plants using genes of different species.

A major difference between traditional and genetic-engineered plant breeding is that whereas the first approach is empirical, in the second the processes giving rise to tolerance and the involved genes need to be identified at the molecular level. In other words, the second approach needs a stronger biochemical and physiological background. During the last twenty years the scientific understanding of the processes involved in plant salt tolerance has improved substantially. This includes cloning of many genes encoding sodium and potassium transporters, identification of signalling pathways governing the activity of ion transporters, the expression of other stress tolerance genes, and the development of simpler models to understand salt tolerance. The advance of the knowledge at this moment is very intense and it is predictable that a breakthrough leading to the construction of salt tolerant glycophytes may occur soon. In fact, transgenic plants with increased salt tolerance have been constructed already.

Alonso Rodríguez-Navarro and Eduardo Blumwald

# Session 1: Sodium and chloride toxicities
## Chair: Dale Sanders

# Salt tolerance traits in crop plants: multistress interactions

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Mechanisms of salt tolerance and salt toxicity in plants can be broadly categorized into osmotic effects, ion-specific effects, and effects caused by oxidative stress (Pitman and Lauchli, 2001). These effects need to be evaluated at scales from ecosystem through whole-plant responses to membrane processes down to the molecular level. Recent up-to-date reviews have been presented by Hasegawa et al. (2000) and Zhu (2001). With regard to genetic engineering of crop plants for improved salt tolerance, the governing physiological and biochemical markers or traits of salt tolerance should be identified. The most important traits appear to be those that control the processes of K+/Na+ -selectivity at cell membranes, Na+/H+ antiport activity at the tonoplast and Na+ compartmentation, activation of Ca2+ signal transduction, accumulation if compatible solutes in the cytoplasm, and activity of antioxidants in specific compartments (Muhling and Lauchli, 2001).

Plants rarely experience stressful conditions caused by a single environmental constraint. Salinity stress under field conditions is often accompanied by drought, heat, waterlogging, other mineral stresses, and others, causing multistress interactions. We have begun to investigate potential interactions of salinity with other soil-borne mineral stresses, such as heavy metal toxicity, specifically due to cadmium, and excess boron on wheat plants. First results on salinity-cadmium interactions indicate that salinity increased intracellular cadmium concentration and activities of peroxidase and ascorbate peroxidase in the leaves, particularly in a salt-sensitive wheat genotype.

Boron toxicity in crops often co-occurs with salinity buildup in the soil (Nikolaichuk et al. 1988). We found saline conditions to increase significantly the concentration of soluble boron in leaves of wheat, both in the intercellular and intracellular compartments (Wimmer et al. 2001). Boron uptake appears to occur via a combination of passive lipid diffusion and aquaporin-mediated transport (Dordas et al. 2000). Excessive intracellular soluble boron is likely the primary cause for boron toxicity in plants.

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# Physiological basis of salt tolerance and the role of physiologically-based selection traits

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New sources of salt tolerance are needed for crops grown in areas at risk of salinisation. There are two main avenues for improving salt tolerance: (1) searching amongst natural diversity within the species, or closely related and inter-fertile species, and (2) genetic engineering. With both avenues, back-crossing into the current adapted cultivars is required. This requires precise screening of large numbers of progeny.

Screening a large number of plants for salt tolerance is not easy. Salt tolerance is achieved through the control of salt movement into and through the plant, to increase the time taken to accumulate to toxic levels in the oldest leaves (Munns, 1993). For this reason, salt-specific effects on growth are seen only after long periods of time. Early effects on both growth and metabolism are likely due to osmotic effects of the salt, that is, to the salt outside the plant (Munns, 2001). Precise screening of a large number of plants for salt tolerance itself is not feasible, as measuring the effect of salt on biomass or yield requires plants to be grown for lengthy periods of time, in both saline and non-saline conditions. In the glasshouse, this is not feasible for the hundreds of lines necessary in breeding protocols. In the field, the major drawback is the heterogenous nature of salinity across even small areas.

To avoid the necessity of growing plants for long periods of time, practical selection techniques based on physiological traits can be used. This opens the potential for marker-assisted selection based on sound physiological principles. Molecular markers for these traits can provide an efficient selection technique in breeding programs. Molecular markers would be particularly useful for pyramiding different traits for salt tolerance (Flowers et al., 2000) and additionally, for incorporating characters associated with other accompanying stresses, such as drought or waterlogging.

We are therefore attempting to identify molecular markers for salt tolerance in wheat. We illustrate this approach with current work on selection for low-sodium uptake in durum (pasta) wheat, using the rate of sodium accumulation in a given leaf as a quantitative trait. We have explored a wide range of genetic diversity, and identified a new source of sodium exclusion. Low $Na^+$ accumulation (and high $K^+/Na^+$ discrimination) of similar magnitude to that of bread wheat was found in an ancient durum landrace (Munns et al., 2000). We have now confirmed that the trait has a high heritability, and have checked for possible penalties associated with the trait.

Penalties may be associated with the trait of $Na^+$ exclusion, such as inability to accumulate sufficient ions for osmotic adjustment. We tested this by comparing four wheat genotypes with contrasting rates of $Na^+$ accumulation. The results, as described by Rivelli et al. (2001), showed that there was little difference between genotypes in the effect of salinity on water relations, as indicated by their water potential, turgor, and relative water content.

Osmotic adjustment occurred in all genotypes, as the low-$Na^+$ genotypes had higher accumulation of $K^+$. We asked the question: would $Na^+$ exclusion carry a penalty in saline soils with low $K^+$? Studies with the same four genotypes with contrasting rates of $Na^+$ accumulation showed that low $K^+$ supply reduced the uptake of $K^+$ in all genotypes, however it had little effect on the sum of ($K^+$ + $Na^+$) uptake. We conclude that $Na^+$ exclusion, as it is linked with selectivity for $K^+$, will not carry a penalty for plants in saline or non-saline soils.

We are currently searching for molecular markers for the $Na^+$ exclusion trait. The low-$Na^+$ durum landrace was crossed with the current durum cultivar Tamaroi. The $F_1$ progeny were intermediate between the parents in $Na^+$ accumulation, showing that more than one gene was involved, but exclusion was recovered in the $F_2$ progeny. Genetic analysis of the $F_2$ population indicated that exclusion was due to two co-dominant genes of major effect. Using the same cross, construction of a genetic linkage map based on AFLPs and microsatellites has been initiated, to identify the chromosomal regions of major effect on $Na^+$ accumulation. The AFLP markers are being anchored with microsatellite markers. One area of interest is on the group 4 chromosomes, to see if there are regions on chromosomes 4A or 4B in durum wheat that may correspond to the homoeologous region carrying *Kna1* on 4D, which has been identified as the main source of salt tolerance in bread wheat (Dubcovsky et al., 1996).

Long-term experiments with selected lines are proving the concept that low $Na^+$ accumulation will confer salt tolerance. New studies are under way to identify other mechanisms of salt tolerance for which a phenotype can be quantified and a marker identified. This way the traits can be pyramided.

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## The role of cell cycle regulation in plant growth responses to salt stress

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Plant growth, a process that is highly responsive to stresses such as salinity and osmotic stress, is a result of ongoing cell division in the plant's meristems and the subsequent expansion of newly formed cells [1]. The cell division cycle in individual meristem cells is regulated by an extensive network of interacting molecules [3]: The core of this cell cycle control system is formed by a family of Cyclin Dependent serine/threonine Kinases, CDK's. These kinases form active complexes by association with, CKS1, a putative docking factor, and cyclin subunits. Of the latter there are many homologs, subdivided into 3 main groeps (A, B and D types, respectively). By their generally cell cycle phase specific transcription and degradation they determine the activity and substrate specificity of the kinase complex. The activity of the complex is also determined by the fosforylation status of the kinase subunit by a number of kinases and phosphatases. Lastly, a family of small molecular weight inhibitory proteins plays a regulatory role, inhibiting the complex in response to stress signals and in places were division is inappropriate. Substrates for this complex are the Rb protein, that when phosphorylated releases the transcription factor E2F, thereby starting transcription in S-phase, and various M-phase specific proteins such as MAPs which presumably regulate spindel and cell plate formation. This whole network is highly conserved between all eukaryotes, and much of the knowledge initially obtained in model systems such as yeasts is now applied to higher plants.

We have used *Arabidopsis thaliana* seedlings growing on agar-solidified media to investigate the role of cell division in the response of plant growth to saline growth conditions. Kinematic analysis of cell division and cell expansion in the primary root tip, of 9 day old seedlings, at 3 days after transfer from control medium to a solution with 0.5% NaCl, revealed that root elongation rate was reduced by 32%. This reduction was associated with a 24% reduction in mature cortical cell length and an 11% reduction of overall cell production. Interestingly, the reduced cell production was not associated with an inhibition of the rate or duration of individual cells, but by the size of these cells when leaving the meristem. The reduction of cell production at this time was entirely related to a reduction of the number of dividing cells whereas cell cycle duration of individual cells was unaffected. In similar experiments we also found that upon salt stress fewer lateral roots and leaves and shorter hypotocyls and petioles are formed. For the leaves we found that the number of epidermal cells was reduced dramatically, but that cells are much larger [2]. Taken together, these results clearly show that next to cell expansion there is a critical role for the cell cycle regulatory mechanism in the adaptation to salt stress.

In accord with this role, we found that in the root tips kinase activity of the CDK compex is reduced in salt stressed roots. Moreover the extent of the zone where the promoter of the mitotic cyclinB is active corresponded closely to the size of the meristem determined kinematically. When studied over time, using both promoter Gus activity and RT-PCR, we showed that at moderate salt concentrations that enable recovery of growth, the expression of cyclinA and B is initially strongly reduced and then gradually recovers as the plant adapts to the stress. In contrast the expression levels of the CDKA and B subunits is hardly affected by the stress [2].

In other laboratories it has also been shown that in response to water stress CDKA undergoes an inhibitory phosphorylation [4], and that in response to ABA, a hormone generally associated with water stress response, the expression level of the CDK inhibitor ICK1 is significantly upregulated [5]. Thus, it seems likely that the cell cycle regulatory mechanism plays a critical role in the adaptation of plant growth to salt and water stress.

To test this hypothesis, we analyzed the salt stress response of transgenic *Arabidopsis* plants that ectopically express a mutant version of the CDKA that cannot undergo the inhibitory phosphorylation at the threonine 14 and 15 residues. Under control conditions such plants do not show an obvious phenotype. However, under short term (1 week) salinity stress growth of the shoot of transgenic plants was much less inhibited that that of the wild type [2]. Leaves that were actively dividing during the stress treatment, contained a much larger number of cells in the transgenic plants than those of the wild type, which supports the hypothesis that the phosphorylation at these residues plays a role in stress adaptation. Interestingly, these plants appeared hypersensitive when subjected to prolonged stress conditions. This observation leads to the hypothesis that the cell cycle regulatory mechanism not only inhibits cell division and growth, but also induces stress defense mechanisms. It is therefore clear that cell cycle research is critical for our understanding how plants adapt to water and salinity stress.

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## Salt-induced oxidative stress and its relevance to salt tolerance

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Osmotic stress and ion toxicity have long been known to contribute to salt stress, whereas oxidative stress has been only recently acknowledged as an additional major aspect of the stress. The contribution of oxidative stress to the damage caused by salt was reported in several plant systems and the effects of salt on the activity of key enzymes involved in the antioxidant defense mechanisms were demonstrated. In citrus cells, these effects were observed only after a few days of salt stress imposed on the cells. In order to understand the relation between salt and oxidative stress we conducted studies on short-term exposure to salt. We focused on salt-induced increase in the expression of two genes/proteins involved in catalysis of redox reactions and compared their pattern of expression in salt-sensitive and salt-tolerant citrus cells The first is *csa* and its encoded protein phospholipid hydroperoxide glutathione peroxidase (PHGPX), and the second is lipoxygenase (LOX).

The gene *csa* and its encoded protein were isolated in our laboratory from salt-tolerant citrus cells exposed to salt stress. Its enzyme activity was demonstrated and was shown to have a direct relationship to oxidative stress. When the two cell lines were exposed to 0.2 M NaCl, a fast and transient increase in *csa* transcript level was observed in the salt-sensitive cells, whereas no increase was observed in the salt-tolerant cells during this time. Exposure of cells to *tert*-butylhydroperoxide (tBH), a PHGPX substrate, resulted in a faster induction of the gene and abolished the differences between the two cell lines. Loading cells with antioxidant agents abolished the salt-induced increase in the *csa* transcript level and demonstrated the relationship between salt stress, oxidative stress and *csa* transcript level. A model was proposed in which ROS and peroxides are intermediate in the cascade of events leading from exposure to salt stress to the increase of *csa* transcript level. Promoter analysis of *csa* shows that salt and oxidative stresses regulate its expression on the transcriptional level.

Salt-induced increase in the level of LOX was also abolished in the presence of excess of antioxidants, but unlike PHGPX the salt-induced increase in the level of the protein was specific to salt-tolerant cells.

It is well known that salt-stress damages are attenuated by high external calcium ions (3-10 mM). Elimination of $Ca^{+2}$ from the growth medium resulted in a more severe salt-induced oxidative stress both for PHGPX and LOX. We monitored *csa* expression as a marker for oxidative stress. We observed that increase in *csa* transcript level occurred when $Ca^{+2}$ entered the cells from the external medium (A23187), or as a result of its release from internal pools to the cytosol (caffeine), in the absence of salt. Additional studies with EGTA and $La^{+3}$, in the presence and absence of salt led us to suggest that the actual displacement of $Ca^{+2}$ from the surface of the plasma membrane create ROS.

## Metabolic engineering of the glycine betaine synthesis pathway

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One effective mechanism to reduce damage from salinity stress is the accumulation of high intracellular levels of osmoprotectant compounds. These compounds include glycine betaine, proline, and polyols and have evolved in many different organisms. Since some crop plants have low levels of these osmoprotectants or none at all, engineering osmoprotectant biosynthesis pathways is a potential way to improve salt tolerance. First-generation engineering work – much of it with single genes – has successfully introduced osmoprotectant pathways into plants that lack them naturally, and this has often improved salt tolerance. However, the engineered osmoprotectant levels are generally low and the increases in tolerance commensurately small. To get beyond trace levels of osmoprotectants and marginal tolerance increments it is necessary to diagnose what limits metabolic flux in engineered osmoprotectant pathways, and to use additional genes to overcome these limitations by an iterative engineering approach. The utility of such an approach will be illustrated using the plant glycine betaine synthesis pathway.

## Pre-mRNA processing: a target for salt toxicity?

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Soil salinity is (together with drought) the major environmental stress factor which currently reduces crop yields world-wide; indeed, the progressive salinisation of irrigated land has turned the genetic improvement of salt tolerance into an urgent need for the future of agriculture in arid regions (*1, 2*). Genetic engineering provides a rapid and general method for the transfer to crop plants of halotolerance genes, once these have been isolated and characterised. At present, different strategies are being used to improve salt tolerance of (transgenic) plants, including the stimulation of ion transport, metabolic engineering to increase osmolyte synthesis, and transformation with regulatory genes from yeast and plants. A complementary approach could be based on the manipulation of cellular systems most sensitive to the toxic effects of salts, but this has been hampered by our very limited knowledge regarding the nature of the cellular targets of ion toxicity in eukaryotic cells (*3*).

Searching for novel targets of salt toxicity in plants, we have screened an *Arabidopsis thaliana* cDNA library to isolate genes conferring increased tolerance to salt stress when expressed in the yeast *Saccharomyces cerevisiae*. Surprisingly, the only three independent clones isolated in this screening, from ca. 750.000 transformants, all appear to be involved in the process of pre-mRNA processing, since they encode:

- the U1A protein, a previously characterised component of the spliceosomal U1-snRNP (*4*), the complex responsible for recognition of the 5'-splice site in the initial step of pre-mRNA splicing,

- two putative members of the family of SR-like proteins, which are defined by the presence of a so-called RS (or "alternating arginine-rich") domain, with a high content in RS/SR, RE/ER and/or RD/DR dipeptides; all characterised proteins in this family are also splicing factors, components of the spliceosome, or are involved in other steps of messenger RNA processing (*5, 6*).

The expression in yeast of the RS domain of each SR-like protein (which we have named RCY1 and C-SRL1), conferred tolerance to LiCl and NaCl, under different conditions and genetic backgrounds, whereas expression of U1A showed a weaker phenotype of lithium tolerance, but no tolerance to sodium. None of the Arabidopsis proteins protected yeast against unspecific osmotic or oxidative stress, indicating that their effect is specific for the "ion toxicity" component of salt stress. The salt tolerance phenotypes were maintained in a yeast strain deffective in vacuolar transport, and no significant decrease in the intracellular concentration of lithium was observed in yeast cells incubated in the presence of LiCl upon expression of any of the Arabidopsis cDNA clones, as compared to the control strain transformed with the empty plasmid vector; all suggesting that the effects of the plant proteins are not mediated by the stimulation of ion transport. Therefore, the hypothesis was advanced that pre-mRNA splicing may be a target of salt toxicity in yeast; the expression of

heterologous splicing proteins could stimulate this process, probably in a non-specific manner, counteracting the inhibitory effect of the salt.

We have obtained some biochemical evidence supporting this hypothesis, since processing of specific introns appears to be inhibited *in vivo* in yeast cells incubated in the presence of LiCl, while the simultaneous expression of the *C-SRL1* cDNA reduces this inhibition. First, we measured the specific activity of β-galactosidase synthesised in yeast cells from a plasmid expressing the *E. coli lacZ* gene, artificially interrupted by an intron (*7*). Upon addition of LiCl to the medium, we observed a concentration-dependent decrease in the accumulation of β-galactosidase, as compared to that produced in yeast expressing the control construct without intron. These experiments also showed an inhibition by salt of β-galactosidase accumulation from the intronless construct, although weaker than for the intron-containing one. In both cases, simultaneous expression of Arabidopsis *C-SRL1* partially blocked the observed inhibition, but with a stronger relative effect in yeast expressing the intron-containing construct.

As a second approach, we directly measured the inhibition of splicing in the presence of lithium, by the accumulation of the precursor of an endogenous mRNA. On the assumption that a general inhibition of splicing would first affect removal of those introns normally processed with lower efficiency, we chose for these experiments the *SAR1* pre-mRNA, which contains such an intron and is often used as a model in splicing studies in yeast (*8*). In initial experiments, standard RT-PCR using primers designed to specifically amplify the *SAR1* pre-mRNA allowed to detect a concentration- and time-dependent increase of its steady-state levels in yeast cells grown in the presence of LiCl. These assays were repeated decreasing the number of PCR cycles to ensure linearity of the amplification reactions, and labelling the amplified products. Here again, we observed the accumulation of *SAR1* pre-mRNA by incubating yeast cells under salt stress conditions, and how it was partially reversed by expression of *C-SRL1*.

The general significance of our results was supported by the increased LiCl and NaCl tolerance shown by transgenic Arabidopsis plants expressing the *C-SRL1* cDNA under control of the CaMV 35S promoter. Seeds from all obtained transgenic lines germinated and grew better at concentrations of lithium and sodium chloride clearly toxic for the wild-type controls. In addition, treatments of plants grown in pots with 200 mM NaCl strongly inhibited the elongation of the reproductive stem, and production of mature siliques and viable seeds in wild-type controls, whereas they had much milder effects on the transgenic plants. This observations suggest that pre-mRNA splicing is a target of salt toxicity in Arabidopsis as well as in yeast, as may be expected considering the conservation of the splicing machinery and general mechanism in all eukaryotic cells.

Our results point to pre-mRNA splicing, and probably also additional steps of mRNA processing, as novel targets of salt stress, which may be relevant for the understanding of the toxic effects of lithium and sodium in eukaryotes. On the other hand, the fact that SR-like proteins (and perhaps also other splicing factors) appear to have a protective effect against salt stress offers a novel route for the improvement of salt tolerance in crop plants.

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## Session 2: Sodium and potassium transporters
## Chair: Andrew D. Hanson

# Pathways for sodium uptake in plant cells

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An understanding of salt toxicity and salt tolerance depends fundamentally on knowledge of the pathways by which the $Na^+$ ion enters plant cells. Although decades of research have resulted in the kinetic characterisation of $Na^+$ uptake, it is only recently that the molecular mechanisms have begun to become apparent. This talk willreview recent data on the identification of $Na^+$ transport systems at the plasma membrane.

One major pathway for $Na^+$ uptake is likely to be via non-selective cation channels. These channels are present in a wide range of species and tissues, and are relatively voltage-insensitive (Amtmann & Sanders, 1999, Tyerman & Skerrett, 1999). Unlike inward and outward rectifying channels, voltage insensitive channels are freely $Na^+$ permeable, so although they are expressed at lower density, they are responsible for considerably more $Na^+$ uptake than do the other channel types.

Although non-selective channels have been characterised electrophysiologically, their molecular identity has, until very recently, remained unclear. Nevertheless, there are now some clues to the molecular properties. Channel activity is markedly down-regulated by cyclic nucleotides (both by cAMP and cGMP) in a rapid and reversible fashion that suggests direct binding of the ligand (Maathuis & Sanders, 2001). The discovery in 1998 by Schuurink et al of genes that encode putative cyclic nucleotide-gated channels (CNGCs) in plants has raised the possibility that the currents measured in the electrophysiological experiments might be through CNGCs. Indeed, animal CNGCs behave as non-selective cation channels, conducting both $Na^+$ and $Ca^{2+}$ with high efficiency. In accord with the notion that plant CNGCs might mediate $Na^+$ uptake in salt-stress conditions, we found (a) that $Na^+$ uptake by *Arabidopsis* roots is partially inhibited by membrane-permeable cyclic nucleotides and (b) that salt stress in *Arabidopsis* seedlings can be alleviated by membrane-permeant cyclic nucleotides (Maathuis & Sanders, 2001). These findings provide correlative support for a role of CNGCs in mediating $Na^+$ uptake, although they do not, of course, prove the case unequivocally since the physiological effects of artificially modulating cyclic nucleotide levels could be far-reaching.

The CNGC family in *Arabidopsis thaliana* comprises 20 members (Maeser et al., 2001) and the roles in $Na^+$ transport of two isoforms – CNGC1 and CNGC3 – have been explored in more detail. Both CNGC1 and CNGC3 are fairly ubiquitously expressed, with CNGC1 expression specifically high in guard cells. When expressed in yeast cells compromised in their ability to extrude $Na^+$ (strain G19), CNGC3 further inhibits growth at high $Na^+$ concentrations and increases $Na^+$ contents of these cells. A *cngc1* T-DNA insertional mutant has been identified and exhibits some intriguing properties. At moderate NaCl concentrations (80-100 mM) the mutant exhibits a $Na^+$-tolerant phenotype. In contrast, *cngc1* mutants in the early seedling stage are more salt-sensitive than wild type to high concentrations (150 mM) of $Na^+$. These findings suggest that CNGCs might be involved in both $Na^+$ transport and salt stress signalling, with the latter

phenotype emerging, for example, if $Ca^{2+}$ uptake through CNGC1 is required for effective signalling. A role for CNGC1 in uptake of divalent cations is indicated by the increased tolerance of the *cngc1* mutants to $Pb^{2+}$ (Sunkar et al., 2000) and $Zn^{2+}$.

However, it is unlikely that CNGCs form the sole pathway for $Na^+$ uptake into plant cells. The HKT1 transporter from wheat has been identified as a high affinity $Na^+/K^+$ symporter that can mediate low affinity $Na^+$ transport at high concentrations of $Na^+$ (Rubio et al., 1995; Gassmann et al., 1996). The *Arabidopsis* ortholog AtHKT1 appears to behave solely as a $Na^+$ transporter (Uozumi et al., 2000). A further possible pathway for $Na^+$ uptake in wheat might be encoded by the *LCT1* gene. *LCT1*-transformed yeast cells exhibit increased unidirectional influx of a wide range of cations, including not only $Na^+$ but also $K^+$, $Ca^{2+}$ and various heavy metals (Schachtman et al., 1997; Clemens et al., 1998). To investigate the role of LCT1 in salt tolerance, we expressed LCT1 in the yeast strain G19 that displays a salt sensitivity comparable to that of wheat (Amtmann et al., 2001). Transformed cells are hypersensitive to NaCl and exhibit a marked decrease in intracellular $K^+/Na^+$ ratio due to the combined effect of enhanced $Na^+$ accumulation and loss of intracellular $K^+$. $Na^+$ uptake through LCT1 was inhibited by both $K^+$ and $Ca^{2+}$, and addition of these ions at high concentrations rescued growth of transformed cells on saline medium. These findings are compatible with a role of LCT1 in providing a $Ca^{2+}$-sensitive $Na^+$ uptake pathway in wheat.

A complex picture is therefore emerging, in which it is likely that several classes of transport system operate in parallel to admit $Na^+$ to the cytosol. In most cases, the physiological roles of these transport systems can only be guessed at. It seems unlikely that they evolved with the specific function of $Na^+$ uptake, and it is more likely, perhaps, either that they function more generally in cation transport for osmotic purposes, or that a role in $Ca^{2+}$ uptake for signalling is paramount.

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## Cloning and characterization of two sodium/hydrogen antiporters from tomato (*Lycopersicon esculentum* Mill.)

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In plants sequestration of $Na^+$ inside vacuoles serves to overcome osmotic and ionic toxicity of high $Na^+$ concentrations. Recently the first putative vacuolar $Na^+/H^+$ antiporters were cloned from *Arabidopsis thaliana* and *Oryza sativa*, but the exact functional role of these genes in plants remains to be elucidated. We have cloned and characterized two isoforms (LeNHX1 and LeNHX2) from tomato (*Lycopersicon esculentum* Mill. cv Moneymaker). The LeNHX2 protein, which is member of a new subgroup within the family of putative intracellular antiporters more closely related to the yeast NHX1 protein, is abundant in roots and stems, and induced in leaves by short term salt or abscisic acid treatment. LeNHX1 is less abundant and induced in stems and roots, but absent from leaves. LeNHX2, complements the salt and hygromycin sensitive phenotype caused by NHX1 gene disruption in yeast, and mainly affects intracellular $K^+$ concentrations. Yeast cells expressing LeNHX1 accumulate high amounts of $Na^+$, but do not display enhanced resistance to hygromycin B, and only marginally to NaCl. Expression data and yeast complementation thus indicate that both isoforms have different ion specificities and might play distinct roles in salt or osmotolerance through differential function, localization and induction in plant tissues. We suggest that LeNHX1 represents a specific $Na^+/H^+$ antiporter, whilst leNHX2 might represent a non-specific Cation/$H^+$ antiporter.

## Chloride channels and salinity

Steve Tyerman, Martha Skerrett and Wen-Hao Zhang

In general the uptake of $Na^+$ under salinity is charge balanced by the uptake of $Cl^-$ and efflux of $K^+$ Anion uptake across the plasma membrane is normally an active process requiring cotransport with protons. However, at high external $Cl^-$ concentrations it is possible for the membrane potential to be less negative than the $Cl^-$ equilibrium potential allowing for a passive influx (Skerrett and Tyerman, 1994). This depends very much on the concentration of $Cl^-$ in the cytoplasm. Under non-saline conditions, the values normally measured are around 20 mM (e.g. Grabov et al., 1997; Felle, 1994 ). At high salinities, some studies have indicated that passive $Cl^-$ influx into cells and into the xylem of roots can occur (Cram 1973, Kingsbury and Epstein 1986, Binzel et al., 1988).

Plant cells are unlikely to tolerate high cytoplasmic $Cl^-$ concentrations and cells show strong depolarsiation when the $Cl^-$ concentration increases in the cytoplasm; for example *Chara* cells, guard cells, and wheat root protoplasts (Shimmen and Tazawa, 1980; Blatt, 1987; Findlay et al., 1994). In wheat root cells, pump activity was only observed with the whole cell configuration of patch-clamp when the $Cl^-$ concentration on the cytoplasmic side of the membrane was less than about 20 mM (Findlay et al., 1994). There are likely to be mechanisms to maintain low cytosolic $Cl^-$ concentrations either by efflux across the plasma membrane or by efflux across the tonoplast to the vacuole. At the tonoplast either $Cl^-/H^+$ antiport (Pantoja et al., 1989) or ion channels (Matinoia et al., 1986) could be involved. In addition, anion uptake is sensitive to the ratio of internal $NO_3^-/Cl^-$ (e.g. Deanne-Drummond, 1986). When the plasma membrane potential difference (pd) is more negative than the $Cl^-$ equilibrium potential requiring that $Cl^-$ influx to the cell is active ($Cl^-/nH^+$ symport), then the cytoplasmic $Cl^-$ concentration may be regulated by controlled efflux across the plasma membrane through an anion channel. The increased $Cl^-$ permeability of plasma membrane vesicles after salt treatment of plants (Yamashita et al., 1994; Yamashita and Matsumoto, 1996) may be an indication of this. Anion efflux channels in root tip cells are involved in a mechanism of aluminium tolerance (Ryan et al., 1997; Zhang et al 2000), however, very little is known about how anion channels are involved in chloride transport under saline conditions.

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# Engineering salt tolerant crop plants: Role of the vacuolar $Na^+/H^+$ antiport

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Salinization of agricultural land has been an important factor affecting human history. Agricultural productivity is severely affected by soil salinity, and the damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. Much of the research in plant salt tolerance aims to breed crop cultivars with high salt tolerance. This research assumes that salt tolerant cultivars will occur only after pyramiding in a single genotype several characteristics, each of one alone could not confer a significant increase in salt tolerance. Salt tolerance is a complex trait involving a number of genes and a long list of salt stress-responsive genes have been identified. Nonetheless, only recently a cellular mechanism for salt tolerance has been identified[1]. We have produced transgenic tomato[2] and Canola[3] plants overexpressing a vacuolar $Na^+/H^+$ antiport. These plants were able to grow, flower and produce seeds and fruits in the presence of 200 mM sodium chloride. Our results demonstrate the ability of the transgenic plants to utilize salty water for growth. These results clearly demonstrate that the enhanced accumulation of $Na^+$, mediated by the vacuolar $Na^+/H^+$ antiport, allowed the transgenic plants to ameliorate the toxic effects of $Na^+$. While the transgenic leaves accumulated $Na^+$ to almost 6% of their dry weight, the tomato fruits displayed only a marginal increase in $Na^+$ content. Moreover, in the transgenic Canola plants growing in high salinity neither the seed number nor the oil quality was affected.

Worldwide, more than 60 million hectares of irrigated land (representing 25% of the total irrigated acreage in the world) have been damaged by salt. Our findings demonstrate the feasibility of producing salt tolerant transgenic plants that will not only produce edible crops, but could also be used for the reclamation of saline soils for agriculture use.

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# The use of the fungal sodium-ATPase for improving plant salt tolerance

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The first cloning of a gene encoding an eukaryotic, non-animal $Na^+$-pump ATPase was accomplished in yeast ten years ago [1]. Since then, further research has demonstrated that this type of pump ATPase probably exists in all fungi and in some intracellular parasites, as *Leishmania* and *Trypanosoma* [2]. In contrast, it does not exist in plants [3], whose only $Na^+$ efflux systems are $Na^+/H^+$ antiporters. This suggests that fungi can eliminate $Na^+$ from their cytoplasm more efficiently than plants can do it from the symplast. Functional analyses of different types of ATPases in the IID or ENA phylogenetic group, in which fungal $Na^+$-pump ATPases cluster, have shown that the function of these ATPases in fungi is complex and more related to $K^+$ efflux than to $Na^+$ efflux in ancestral fungi. The $Na^+$ pumping capacity of fungi is a function recently acquired, that plants could not acquired because they lacked an ancestral ATPase functionally homologous to the fungal enzyme [2].

The inefficiency of plant $Na^+$ efflux is particularly evident in soils with a high $Na^+$ content and a pH above neutrality, because in these conditions the functions of the plant electroneutral $Na^+/H+$ antiporters are impaired [3] and dependent on root acid excretions. This poses the question of whether the expression of a fungal $Na^+$-pump ATPase could improve plant salt tolerance.

The definitive answer to this question is obviously empirical and in progress. However, the experimental approach is not as simple as transforming the plant with an *ENA* cDNA under the control of a plant strong promoter, analyzing the transformants, and accepting that a negative result is equivalent to a negative answer. The following difficulties can be predicted: (i) the selection of the convenient cDNA is not easy, because possibly all fungal ENA-ATPases have both $Na^+$- and $K^+$-efflux activities and the latter might be deleterious for the plant; (ii) it is difficult to predict the most convenient promoter to be used in the experiment, without considering the Na+ pathways in the plant and the cells in which the ATPase would be most effective; (iii) factors as transpiration rate or medium pH may condition the result. Present knowledge, as is discussed below, suggests that a $Na^+$-pump ATPase may not be necessary in plants grown in glass houses with high humidity and in a medium at pH 5.0-6.0, whereas may be essential in field conditions, at high transpiration rates, and in neutral or slightly alkaline soils.

Considering all the process involved in $Na^+$ tolerance in glycophytes, two of them seem to be of crucial importance, $Na^+$ exclusion from the plant, and Na+ inclusion into the vacuole [4]. The former is the net result of influx and efflux, and recent results with several plants have shown that the rice model [5], in which the transpiration rate determines the $Na^+$ influx, applies to many plants. According to these results, at high transpiration rate, Na+ influx is several fold higher than the maximum capacity of the plant to accumulate $Na^+$ into the vacuole. In these conditions, if the influx is physically linked to transpiration and cannot be decreased, furnishing the plant with an active efflux system is essential to increase $Na^+$ tolerance. The approach could be the enhancement of an intrinsic electroneutral $Na+/H+$

antiporter or the introduction and functional expression of a fungal Na+-pump ATPase. For simple thermodynamic reasons, the former approach would not function if the external pH is higher than the cytoplasmic pH.

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# Function of Shaker $K^+$ channels in the plant and roles in salt tolerance

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Electrophysiological studies *in planta* have revealed a large array of $K^+$ channels, rendering the membrane conductance to $K^+$ to be the dominant one. $K^+$ channels on the plant cell membrane are thought to take part in control of membrane potential and signal transduction (in response to stimuli such as hormones, light or pathogen attack) (Zimmermann *et al.*, 1998) but molecular or genetic evidence in this field is still lacking. On the other hand, characterisation of knock-out mutants has revealed a role that can be looked at as typical of $K^+$ channels in plants, contribution to sustained wholesale $K^+$ transport, *e.g.* $K^+$ uptake from the soil solution (Hirsch *et al.*, 1998) or $K^+$ secretion into the xylem sap towards the aerial parts (Gaymard *et al.*, 1998).

The first plant $K^+$ channels characterised at the molecular level, AKT1 (Sentenac *et al.*, 1992) and KAT1 (Anderson *et al.*, 1992), were cloned by functional complementation of yeast strains defective for $K^+$ transport, and shown to belong to the so-called voltage Shaker family, initially identified in drosophila and common to animals, fungi and plants. Systematic screening and genome sequencing has thereafter revealed that AKT1 and KAT1 belong to a multigene family, with 9 members in *Arabidopsis*, endowed with inward or outward rectification (Zimmermann and Sentenac, 1999). Data regarding the structure-function relationship of these proteins, their expression pattern and roles in $K^+$ transport in the plant will be discussed.

In saline environments, $Na^+$ is thought to enter the cytoplasm passively, down its electrochemical gradient. Kinetic studies of $^{22}Na^+$ transport in roots indicate that sodium influx occurs *via* low affinity $K^+$-uptake mechanisms , supporting the hypothesis that the ionic selectivity of $K^+$ channels contributes to the ability of the root to control the entry of $Na^+$. Contribution of Shaker channels to $K^+$ entry into the cell will be discussed based on data obtained in heterologous systems: yeast cells or *Xenopus* oocytes expressing plant Shaker channels. Northern blot analysis of the effect of salt stress on Shaker channel expression in *Arabidopsis* will also be described. These data suggest that at least one member of the Shaker family plays a crucial role in the plant adaptation to high concentrations of salt in the environment.

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# Proteins governing sodium homeostasis

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Plant adaptation and resumption of growth after exposure to salt stress are dependent, among other concomitant processes, on the re-establishment of proper cellular ion homeostasis. Adequate cytosolic $Na^+$ content is preserved by the concerted interplay of regulated ion uptake, vacuolar compartmentation and efflux to the extracellular milieu. Vacuolar partitioning of $Na^+$ and other ions contributes also to maintenance of cellular water relations in an hypertonic medium [3]. Energy-dependent exclusion of $Na^+$ from the cytosol is coupled to downhill reverse transport of $H^+$ by $Na^+/H^+$ antiporters located in both the plasma membrane and tonoplast [2]. The AtNHX1 protein of *A. thaliana* was the first tonoplast $Na^+/H^+$ exchanger identified mediating vacuolar compartmentation of $Na^+$ and imparting salt tolerance to transgenic plants [1]. We have begun to characterize five additional members of the *AtNHX* gene family of Arabidopsis. Based on primary sequence relatedness, AtNHX proteins can be categorized in two groups comprising AtNHX1-4 and AtNHX5-6. Genes *AtNHX2*, encoding the isoform most similar to AtNHX1, and *AtNHX5*, as representative of the more distant phylogenetic group, have been cloned and expressed in yeast mutants lacking endogenous $Na^+$ transporters. Similarly to AtNHX1, AtNHX2 and AtNHX5 plant exchangers suppressed the cation sensitivities of a null mutant devoid of the single endosomal/vacuolar $Na^+/H^+$ antiporter ScNHX1, demonstrating that all these transporters are functional homologues. Cation resistance afforded by the plant proteins correlated with greater ion content indicative of vacuolar compartmentation. There were however differences in the cation resistance spectrum among isoforms as if each protein had a distinctive ion transport specificity.

*AtNHX1* and *AtNHX2* transcript levels were higher in Arabidopsis seedlings subjected to iso-osmotic NaCl and sorbitol treatments, and in response to exogenous ABA. Salt-dependent gene induction was absent in an *aba2* mutant with little capacity for stress-induced ABA accumulation, but was unaffected in *sos1*, *sos2* and *sos3* mutants. These results suggest that AtNHX1 and AtNHX2 are instrumental in plant adaptation to an osmotic challenge through an ABA-dependent process. In keeping with the probable functional overlapping of AtNHX1 and AtNHX2, an AtNHX2:GFP translational fusion expressed transiently in onion cells was targeted to the tonoplast. Interestingly, *AtNHX5* expression was upregulated by NaCl but not by sorbitol or ABA. Gene induction was not impaired in *aba2* or *sos1,2,3* mutants, suggesting the existence of a novel, NaCl-specific signaling pathway distinct from the ABA and SOS pathways. In fact, *AtNHX5* transcript levels, and to a lesser extent that of *AtNHX1* and *AtNHX2*, were greater in untreated *sos* mutants. Thus, the SOS pathway may negatively affect *AtNHX* gene expression.

AtSOS1 is a putative $Na^+/H^+$ antiporter that functions in $Na^+$ extrusion out of the cell [4]. *SOS1* gene expression is regulated by the protein kinase SOS2 and the associated $Ca^{2+}$

sensor SOS3. To investigate whether the transport activity of SOS1 was also regulated by SOS2/SOS3, we have reconstituted the SOS pathway in yeast cells. Expression of SOS1 increased the $Na^+$ tolerance of yeast mutants lacking endogenous plasma membrane $Na^+$ transporters. Co-expression of SOS2 and SOS3 together with SOS1 dramatically increased SOS1-dependent $Na^+$ tolerance, whereas SOS2 or SOS3 separately had little or no effect. Expression of a constitutively active allele of *SOS2* partially substituted for the requirement of SOS3 for SOS1 activation. SOS2/SOS3 phosphorylated SOS1 both *in vivo* at the plasma membrane and *in vitro* after purification of a SOS1:His6 tagged protein. Because the Arabidopsis *sos* mutants have defective $K^+$ uptake at low external concentration, we investigated whether SOS1 is a $K^+$ transporter. Neither the unmodified nor the SOS2/SOS3-activated SOS1 protein showed $K^+$ transport capacity *in vivo*, as determined by expression of SOS1 in a yeast *trk1,2* mutant lacking the major $K^+$ uptake system, and in a *nha1* mutant with reduced $K^+$ efflux. $K^+$ transport by SOS1 could not be primed by external $Na^+$, suggesting that SOS1 is not a $Na^+/K^+$ co-transporter.

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## Session 3: Signalling pathways governing ion homeostasis and stress responses
## Chair: Ramón Serrano

# Plant MAPK cascade in stress and hormonal signaling

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Mitogen-activated protein kinase (MAPK) cascades have emerged as a universal signal transduction mechanism that connects diverse receptors/sensors to cellular and nuclear responses in eukaryotes. Recent studies in plants indicate that mitogen-activated protein kinase cascades are vital to fundamental physiological functions involved in hormonal responses, cell cycle regulation, abiotic stress signaling, and in defense mechanisms. New findings have revealed the complexity and redundancy of the signaling components, the antagonistic nature of distinct pathways, and the use of both positive and negative regulatory mechanisms.

Plants possess sophisticated protection mechanisms to cope with various environmental stresses such as cold, freezing, heat, drought, ozone, UV, salinity, osmotic shock, and mechanical wounding. Accumulating evidence indicates that plants rapidly activate MAPKs when exposed to multiple abiotic stress stimuli. The identity, roles, and specificity of MAPK cascades in the regulation of diverse abiotic stress responses and their link to defense mechanisms and growth regulation are the focal points of intensive research. Although each stress stimulus may involve a distinct perception process and trigger specific responses, there appears to be some common underlying mechanisms for abiotic stress signaling. The convergent points include the production of second messengers such as calcium and $H_2O_2$ and/or reliance on common signaling cascades and transcription factors. A connection between the activation of a plant MAPK cascade and one common second messenger, $H_2O_2$, generated by diverse stress stimuli has been demonstrated using the *Arabidopsis* protoplast transient expression assay. The study also shows that redundancy may be the nature of plant MAPK activation as found in yeast and mammals. It is further demonstrated that transgenic tobacco plants expressing the constitutively active version of NPK1, a tobacco ortholog of ANP1, exhibit tolerance to multiple stresses such as cold, heat, drought and high salinity. Activation of this MAPK cascade also results in the repression of the auxin-responsive promoter, providing a molecular link between stress and hormone signaling

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## Gene expression and signal transduction in response to osmotic stress

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Plants respond and adapt themselves to water deficit conditions or high salinity conditions through various physiological and biochemical processes. Under dehydration conditions various genes are induced, whose products are thought to function in protecting cells from dehydration. To analyze the function and expression of stress-inducible genes, we cloned many cDNAs for drought-inducible genes in *Arabidopsis thaliana*. We analyzed the expression of the drought-inducible genes and identified at least four independent regulatory systems in drought-responsive gene expression, two are ABA-dependent and two are ABA-independent (1). In one of the ABA-dependent pathways, transcription factors encoding MYC and MYB homologues were shown to be involved in ABA-responsive gene expression of the *rd22* gene under drought stress (2). In another ABA-dependent pathway, we showed two AREB proteins encoding novel bZIP-type proteins specifically bind to ABRE and regulate ABA-dependent gene expression (3).

In one of the ABA-independent pathways, a cis-acting element (DRE/CRT) is involved in dehydration- and cold-inducible gene expression. We isolated five cDNAs for DRE binding proteins (DREB) using the *rd29A* promoter, and classified into two groups, DREB1 and DREB2 (4). The DREB2 gene is induced by dehydration stress whereas the DREB1 gene by cold stress, which suggest that two DREB transcription factors separate two stress signaling pathways, cold and dehydration. Improvement of drought, salt, and freezing tolerance was performed by overexpression of DREB1A in transgenic Arabidopsis (4, 5). Recently, we identified many drought-, cold-, and salinity-inducible genes by using full-length cDNA micorarray. We also identified many DREB1A-target genes and speculated their functions (6). Recently, we showed that PI turnover and PLC function in the osmotic stress responsive expression of *rd29A* using Arabidopsis T87 cultured cells (7).

Under drought stress, various genes are induced and are thought to function in protecting cells from dehydration. Many genes involved in signal transduction are also upregulated by osmotic stress. We have shown that genes for protein kinases involved in the MAP kinase cascade are transcriptionally upregulated by various stresses (8). We demonstrated rapid and transient activation of two MAP kinases, ATMPK4 and ATMPK6, in Arabidopsis plants by high osmolarity, low temperature, and touch (9). These results suggest an important role of MAPK cascade in stress signaling and involvement of redundant MAP kinase cascades in stress responses. ATMPK6 is also activated by reactive oxygen (ROS), which suggests the involvement of ROS in stress response (10). Recently, we have isolated T-DNA insertion mutants of ATMPK6, of which phenotype will be discussed.

We isolated a cDNA (ATHK1) encoding a two component histidine kinase, a yeast osmosensor Sln1 homolog (11). Introduction of ATHK1 into a yeast mutant lacking two osmosensors, SLN1 and SHO1, allowed normal growth and activation of the HOG1 MAPK cascade under high osmolarity. These results suggest that ATHK1 can sense and transduce a signal of external osmolarity to downstream targets. We then generated transgenic Arabidopsis plants that overexpress dominant negative ATHK1 mutant cDNAs. The transgenics showed growth retardation in shoot and roots and accumulation of anthocyanin. Several stress-induced genes were upregulated in the transgenics under unstressed conditions. The role of ATHK1 as an osmosensor in pants will be discussed.

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## Osmotic stress rapidly induces MsK activity in alfalfa roots

Claudia Jonak

Protein phosphorylation plays an important role in cellular signaling when plants encounter high salinity. For example, it is likely that a MAP kinase signaling pathway is involved in mediating salt stress. Recently, we showed that a MAP kinase (SIMK) becomes rapidly activated when alfalfa cells are exposed to hyperosmotic conditions. The salt-induced activation of SIMK seems to be mediated by the dual-specificity MAPK kinase SIMKK.

Now, we present evidence that a novel serine/threonine protein kinase, denoted MsK, is rapidly activated in alfalfa roots exposed to high salt conditions. Immunokinase assays of root protein extracts using a MsK-specific antibody revealed that untreated roots contained only very little MsK activity, but incubation of roots with more than 125 mM NaCl activated the MsK kinase. Exposure of roots to 250 mM NaCl activated MsK within two minutes. Maximal activation was obtained after 10 minutes. MsK activity sustained for at least one hour. The activation pattern of MsK resembles SIMK activity profile.

MsK is not only activated by high concentrations of NaCl but also by KCl and sorbitol indicating that MsK could be involved in mediating a general hyper-osmotic response.

## Delimiting the osmotic component of salt stress

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In many laboratories, salt stress have been used instead of drought in the search for genes critical for osmotic tolerance. In contrast to yeast, where genes essential for osmotic tolerance have been identified by the use of NaCl, mostly genes involved in ionic tolerance have been identified in plant using NaCl. By the use of mannitol instead of NaCl we have isolated a novel mutant in tomato (*tos1*) caused by a single recessive nuclear mutation that is hypersensitive to general osmotic stress. *tos1* is only hypersensitive to high concentrations of NaCl, suggesting that only high concentrations of NaCl exert sufficient osmotic stress to impair root growth. Growth measurements demonstrated that the *tos1* mutant is less sensitive to intracellular abscisic acid (ABA) and this decreased ABA sensitivity of *tos1* is a basic cellular trait expressed by the mutant at all developmental stages. The ABA insensitivity is not caused by a deficiency in the ABA synthesis because the *tos1* seedlings accumulated more ABA than the wild type after osmotic stress. In contrast, the *tss2* tomato mutant, which is also hypersensitive to osmotic stress, is hypersensitive to exogenous ABA. Comparative analysis of *tos1* and *tss2* indicates that appropriate ABA perception and signaling is essential for osmotic tolerance.

## Plant salt tolerance: mutants, genes and signaling pathways

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In *Arabidopsis thaliana*, the *Salt Overly Sensitive 1* (*SOS1*), *Salt Overly Sensitive 2* (*SOS2*), *Salt Overly Sensitive 3* (*SOS3*) and *Salt Overly Sensitive 4* (*SOS4*) genes are required for intracellular $Na^+$ and $K^+$ homeostasis. Mutations in these genes cause $Na^+$ and $K^+$ imbalance and render plants more sensitive toward growth inhibition by salt stress. SOS3 is a myristoylated calcium-binding protein that maybe a sensor for cytosolic calcium signals elicited by salt stress. *SOS2* encodes a serine/threonine protein kinase. SOS2 physically interacts with and is activated by SOS3. Salt stress up-regulation of *SOS1*, which encodes a plasma membrane $Na^+/H^+$ antiporter, is partly under control of the SOS3/SOS2 pathway. SOS2 also directly activates the $Na^+/H^+$ exchanger activity of SOS1. SOS4 encodes a pyridoxal kinase important for the biosynthesis of pyridoxal-5-phosphate, which could regulates SOS1 activity because SOS1 contains a putative pyridoxal-5-phosphate binding motif in its C-terminal cytoplasmic tail. These *SOS* genes define a novel regulatory pathway important for the control of intracellular ion homeostasis and salt tolerance in plants.

SOS2-SOS3 interaction is mediated through the regulatory domain of SOS2. We have delimited within the SOS2 regulatory domain, a 21 amino acid motif (designated as the FISL motif) that is both necessary and sufficient for binding to SOS3. On the SOS3 side, no discrete motif could be identified that is sufficient for mediating the interaction with SOS2. It appears that the central EF-hand as well as the N-terminal and C-terminal regions of SOS3 is required for binding to SOS2. Deletion of the FISL motif or a Thr168-to-Asp mutation results in a constitutively active SOS2 that is independent of SOS3 or $Ca^{2+}$. Expression of the constitutively active SOS2 mutants in yeast or *Arabidopsis* is partially sufficient for SOS1 activation and salt tolerance.

In addition, recent progress on the characterization and cloning of several mutations that affect osmotic stress and ABA regulated gene transcription will be presented.

# Altered abscisic acid (ABA) levels and osmotic sensitivity in the *Arabidopsis sre2* mutant

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During the course of a screening for mutants able to germinate and carry out early growth in medium containing a high NaCl concentration, we identified three complementation groups, named *sre1* to *sre3* for salt resistant in a germination assay. The *sre* mutants were also able to germinate in high osmoticum media, indicating they are osmotolerant in a germination assay. Whereas *sre1* and *sre3* were allelic to *aba2* and *aba1* mutants, respectively, *sre2* was not allelic to previously described *aba* mutants. Additionally, *sre2* was not abscisic acid (ABA) insensitive in a germination assay. Germination in paclobutrazol and transpiration assays indicated that *sre2* could have a reduced ABA level as compared to wild type individuals. Indeed, the ABA content was reduced in sre2 as compared to the wild type, both in turgid rosettes as well as in salt-treated plants or wilty rosettes. Physiological characterization of *sre2* reveals a reduced seed dormancy, hypersensitivity to osmotic stress in vegetative tissues, and impaired ABA-mediated responses, i.e. reduced proline increase in response to osmotic stress and impaired stomatal regulation. We discuss that *sre2* might represent a new locus encoding an ABA-biosynthesis enzimatic activity or, alternatively, a regulatory element controling ABA-biosynthesis in response to osmotic stress.

## Session 4: Genomics and salt tolerance
## Chair: Kazuo Shinozaki

# Evolutionary conservation and uniqueness of salinity stress responses

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High-throughput analysis systems are now replacing the classical gene-by-gene approaches in studies of gene expression and function. Possible are now large or genome-wide analyses of expressed genes and proteins present in cells or tissues or during development and under different external conditions. We have applied these tools to study plant tolerance to abiotic stresses. Several laboratories have pooled their resources to understand plant responses to salinity stress and, more recently, drought through the magnifying glasses provided by cDNA library profiling and microarray analyses. We included Baker's yeast, *Aspergillus*, *Synechocystis*, and *Dunaliella* as fungal and algal halophytic models. Among higher plants, we focussed on the halophytic *Mesembryanthemum crystallinum* and the glycophytic *Arabidopsis thaliana* and *Oryza sativa*, which are excellent molecular genetic models. From yeast to higher plants, salinity and drought affect approximately 8% of all transcripts, i.e., 500 to 3,000 genes. The overlap between drought- and salt- affected transcripts may, however, be as low as 30%. Both stress factors affect very different sets of genes depending on the tissues and, above all, developmental stage and progression and severity of the stress condition. A number of physiological or biochemical responses that have amply been documented in the past as stress-induced can be identified in the expression profiles but their contribution to survival may be of little significance because stress-tolerant as well as stress-sensitive lines of, for example, rice express those transcripts. In rice, where our analysis is most complete, it seems that the early responses to a salt shock, including transcripts in the category of signal transduction, are important. Significant increases of transcript levels for signaling functions are only seen in lines that later show tolerance or resistance to the stress. As a summary, it seems that most or all genes supporting tolerance are present in all species, yet most important is the speed, magnitude and persistence with which these downstream effectors of tolerance are networked to provide a whole-plant response and how fast and how strongly they are engaged.

**References**:
This work has been supported by the National Science Foundation of the USA (plant genome program). It represents a collaboration between laboratories at several universities: Arizona (David Galbraith, Jian-Kang Zhu), Nevada (John Cushman), Oklahoma State (Robert Burnap, Rolf Prade), and Purdue (Ray Bressan, Mike Hasegawa). The work is carried out by too many students, postdoctoral fellows, and visitors to mention all of them. A summary of approaches, and of the names, can be found in:
Hans J. Bohnert, Patricia Ayoubi, Chris Borchert, Ray A. Bressan, Robert L. Burnap, John C. Cushman, Mary Ann Cushman, Michael Deyholos, Robert Fischer, David W. Galbraith, P. Michael Hasegawa, Matt Jenks, Shinji Kawasaki, Hisa Koiwa, Shin Kore-eda, Byeong-Ha Lee, Chris B. Michalowski, Eduardo Misawa, Mika Nomura, Neslihan Ozturk, Bradley Postier, Rolf Prade, Chun-Peng Song, Yuko Tanaka, Hong Wang, Jian-Kang Zhu. A genomics approach towards salt stress tolerance. Plant Physiol. Biochem. 39: 295-311 (2001).

# Genomics scale evaluation of *Arabidopsis* mutants for altered salinity tolerance

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In recent years a considerably improved understanding of the underlying basis of salinity tolerance in plants has been achieved through the use of the model plant *Arabidopsis thaliana*. In particular, the isolation of several salt sensitive mutants of and the identification of the responsible genes by Zhu and co-workers and others has begun to lay a foundation for the genetic basis of salt tolerance. We have begun a genomics scale forward genetics screen for T-DNA insertion tagged mutants of *Arabidopsis* with altered salinity tolerance. Our collection of well over 300,000 tagged lines allows an essentially genome saturated screen for mutations. Three principal types of screens have been initiated. The first involves direct evaluation of salt sensitivity or tolerance by a root bending or seeding growth assay on agar medium plants. From screens of 100,000 mutant lines, over 100 confirmed mutants of this type have been identified. We have also screened about 60,000 lines for altered regulation of the stress-responsive rd29 promoter using an rd29::luc fusion system. About 50 confirmed mutants have been isolated from this screen.

Finally we have also started a number of screens designed to detect extragenic suppressor mutations for other important mutants that affect salinity tolerance such as the SOS3 mutation. From a screen of more than 65,000 SOS3 lines, 14 suppressor mutants have been isolated and confirmed. Using TAIL PCR, the mutated genes responsible for several altered salinity response and tolerance phenotypes from these three types of screens have been tentatively identified. Interestingly, besides expected genes such as those encoding kinases, phosphatases and transcription factors, several other unexpected classes of genes have also been found.

## Session 5: Eukaryotic microorganisms as models for plant salt tolerance
## Chair: Kazuo Shinozaki

# Salt tolerance in *Debaryomyces hansenii*. Many questions and a few answers

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Why is internal sodium toxic for most of the living cells? Why do some organisms require sodium to grow? Yeast cells are useful tools in the study of salt tolerance processes. *Saccharomyces cerevisiae* is a well studied yeast and it has been shown that, in this yeast, salt tolerance is associated with the ability to maintain low levels of $Na^+$ in the cytoplasm. What are the basis of *S. cerevisiae* sensitivity to $Na^+$? This is a question without a definite answer. So far the phosphatase Hal2p is the only proposed target for $Li^+$ (and $Na^+$) toxicity (1). In any case, *S. cerevisiae* may not the best model organism since it shows moderate tolerance to salt stress. From this point of view, *Debaryomyces hansenii* could be a more appropriate yeast since several groups have shown that its growth rate is stimulated by the presence of relatively high $Na^+$ concentrations (0.5 M) (2,3). We have also shown that in the presence of different stress conditions (extreme pHs or high temperatures) the presence of external sodium improves *D. hansenii* performance (4).

To try to understand salt tolerance in *D. hansenii*, we are following two different approaches. On the one hand, we are studying the physiology of this yeast and we are looking for genes that we know are important determinants in salt tolerance in other organisms. On the other hand, we have constructed a genomic *D. hansenii* library in order to identify specific genes from *D. hansenii* involved in halotolerance. By following the first approach we have identified key processes and genes in ion homeostasis (potassium transport, sodium efflux and *ENA* genes (5), sodium sequestration into the vacuole and the *NHX* gene). By using the genomic library, we have transformed *S. cerevisiae* and we have isolated transformants that are more resistant than the wild type recipient strain. Our hypothesis is that halotolerance in *D. hansenii* is due to the functioning of a multicomponent system. Our goal is the identification of every single component and the evaluation of its contribution to the whole process of salt tolerance.

Acknowledgements
Our research is a collaborative work with the group of Dr. Loureiro-Dias (Lisbon). The technical assistance and advice of the group of Dr. Rodríguez-Navarro (Madrid) is highly appreciated.

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# Regulation of $K^+$ transport, membrane potential and pH in yeast by the Ppz protein phosphatase: implications for salt tolerance, cell cycle progression and cell integrity

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The protein phosphatase Ppz, encoded by the redundant *PPZ1* and *PPZ2* catalytic subunit genes and the *HAL3* inhibitory subunit gene, is an important determinant of salt tolerance, cell cycle progression and cell integrity. These disparate phenotypes are actually connected by the fact that Ppz regulates Trk, the high affinity $K^+$ transporter of yeast cells. First, salt tolerance, cell integrity and cell cycle phenotypes of Ppz mutants are mostly dependent on a functional Trk system. Second, Ppz mutants exhibit altered activity of the Trk system as measured by $Rb^+$ uptake. Third, Ppz mutants have altered intracellular $K^+$ and pH, as expected from $H^+$ efflux providing electrical balance during $K^+$ uptake. Our unifying picture of Ppz phenotypes is as follows. $K^+$ transport is a major consumer of electrical potential and therefore activation of Trk by decreased Ppz activity results in plasma membrane depolarization and reduced uptake of toxic cations ($Li^+$, $Na^+$, polyamines, hygromycin B) mediated by unidentified, non-selective cation channels. Activation of Trk results in increased intracellular $K^+$ and therefore increased turgor which compromises cell integrity. Finally, the resulting increase in intracellular pH induces the expression of pH-regulated genes such as *ENA1* (via the calcineurin and the Rim101 pathways) and it seems to promote expression of G1 cyclins and cell cycle progression as measured by improved recovery from α-factor-induced cell cycle block. One identified feed-back mechanism of this regulatory circuit is that increasing pH within the physiological range dissociates the Hal3-Ppz1 complex. Our present and previous results provide strong evidence for a causal relationship between intracellular cation homeostasis and both a potential cell cycle checkpoint and a turgor control system.

# POSTERS

# rd22BP1 (MYC) and ATMYB2 (MYB) act as transcription factors in ABA signaling

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In *Arabidopsis*, the induction of a dehydration-responsive gene, rd22, is mediated by abscisic acid (ABA) and requires protein biosynthesis. Previous experiments have established that MYC and MYB recognition sites in rd22 promoter function as cis-acting elements in the dehydration-induced expression of the rd22 gene. A cDNA encoding a MYC-related protein (rd22BP1) and encoding a MYB-related protein (Atmyb2) have been isolated. We have also shown that both rd22BP1 and Atmyb2 genes are induced in dehydration stress conditions. In a transient experiment using *Arabidopsis* leaf protoplasts, we have demonstrated that both the rd22BP1 and ATMYB2 proteins activate transcription of the rd22 promoter fused to the GUS reporter gene. Co-transfection of rd22BP1 and ATMYB2 has resulted in cooperative transactivation (Abe et al.,The Plant Cell, 9, 1859-1868, 1997). In this workshop, we demonstrated that the transgenic plants overexpressing rd22BP1 cDNA or Atmyb2 cDNA respectively displayed ABA hypersensitivity. Furthermore, transgenic plants overexpressing both rd22BP1 and Atmyb2 cDNAs showed a higher level of ABA hypersensitivity than that of transgenic plants overexpressing either rd22BP1 cDNA or Atmyb2 cDNA alone. ABA induced gene expression of rd22 gene also showed the ABA hypersensitivity. Moreover, a Ds insertion mutant of the rd22BP1 gene (rd22BP1 plant) showed ABA insensitivity. In rd22BP1 plants, ABA induced gene expression of rd22 gene significantly decreased. These results indicate that both the rd22BP1 and ATMYB2 proteins function cooperatively as transcriptional activators in ABA signaling under dehydration stress conditions. This is a novel regulatory system in ABA-responsive gene expression.

## Structural biology of salt tolerance

Albert A, Martinez-Ripoll M., Serrano R.

The product of the yeast HAL2 gene (Hal2p) is an *in vivo* target of sodium and lithium toxicity and its overexpression improves salt tolerance in yeast and plants. Hal2p is a metabolic phosphatase which catalyses the hydrolysis of PAP to AMP. It is the prototype of an evolutionary conserved family of PAP phosphatases and the engineering of sodium insensitive enzymes of this group may contribute to the generation of salt-tolerant crops. The X-ray structure of Hal2p in complex with magnesium, lithium and the two products of the hydrolysis, AMP and Pi, provides a 3D picture for the mechanism of reaction and the molecular basis for the specificity of the inhibitory lithium and sodium cations (1).

The *Arabidopsis thaliana* HAL3 gene product encodes for an FMN binding protein (AtHal3) that it is related to salt and osmotic tolerance and plant growth. AtHal3 shows sequence homology to ScHal3, a regulatory subunit of ScPPz1. It has been proposed that AtHal3 and ScHal3 have similar roles in cellular physiology. The 3D structure of AtHal3 indicates that this protein is designed to interact with another cellular component and to subsequently catalyze the alpha-beta dehydrogenation of a peptidyl cysteine. These structural data, together with the physiological information from ScHal3 allow us to propose a model for the recognition and regulation of AtHal3/ScHal3 cellular partners (2).

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# The *Arabidopsis* Membrane Transport (AMT) chip: A comprehensive tool to Study expression of plant membrane transporters in various nutritional and abiotic stress conditions

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$K^+$ is involved in many metabolic and osmotic processes vital for plant growth. Therefore, distribution of $K^+$ between cellular organelles and tissues requires tight control. Although fifty years of research in the field of $K^+$ nutrition has accumulated a vast amount of physiological and molecular data, we are far from understanding the basic mechanisms underlying $K^+$ homeostasis.

Not only are we lacking a comprehensive picture of tissue localisation and physiological function of all proteins involved in $K^+$ transport, but virtually nothing is known about the regulatory systems underlying $K^+$ homeostasis. Classical approaches had the inherent disadvantage of being time-consuming and providing fragmentary results.

Completion of the *Arabidopsis* sequencing programme has now opened new ways to study $K^+$ homeostasis. Availability of DNA microarrays puts us in a position to study basic questions of plant nutrition on a genome-wide level. This novel and exciting approach not only promises to drastically accelerate progress in this research field but will also provide a more integrative concept of $K^+$ nutrition in plants.

In a joint effort by a consortium of several internationally renown plant scientists and a biotech company we have produced the first "*Arabidopsis* membrane transport" oligonucleotide chip containing probes for more than 1000 known and putative membrane transporters.

RNA has been isolated from plants grown under various $K^+$ regimes, reverse transcribed and labelled for fluorescence ratio analysis after hybridisation with the microarray. Results on the transcriptional regulation of known and novel genes by plant $K^+$ status will be presented on the meeting.

The results from this study are integrated into a larger network of nutritional regulation of gene transcription based on cluster analysis of all data obtained by the consortium including nitrogen and calcium availability, salinity and heavy metal stress.

# Cloning and characterization of two sodium/hydrogen antiporters from tomato (*Lycopersicon esculentum* Mill.)1

Kees Venema, Andrés Belver and Juan Pedro Donaire

In plants sequestration of $Na^+$ inside vacuoles serves to overcome osmotic and ionic toxicity of high $Na^+$ concentrations. Recently the first putative vacuolar $Na^+/H^+$ antiporters were cloned from *Arabidopsis thaliana* and *Oryza sativa*, but the exact functional role of these genes in plants remains to be elucidated. We have cloned and characterized two isoforms (LeNHX1 and LeNHX2) from tomato (*Lycopersicon esculentum* Mill. cv Moneymaker). The LeNHX2 protein, which is member of a new subgroup within the family of putative intracellular antiporters more closely related to the yeast NHX1 protein, is abundant in roots and stems, and induced in leaves by short term salt or abscisic acid treatment. LeNHX1 is less abundant and induced in stems and roots, but absent from leaves. LeNHX2, complements the salt and hygromycin sensitive phenotype caused by NHX1 gene disruption in yeast, and mainly affects intracellular $K^+$ concentrations. Yeast cells expressing LeNHX1 accumulate high amounts of $Na^+$, but do not display enhanced resistance to hygromycin B, and only marginally to NaCl. Expression data and yeast complementation thus indicate that both isoforms have different ion specificities and might play distinct roles in salt or osmotolerance through differential function, localization and induction in plant tissues. We suggest that LeNHX1 represents a specific $Na^+/H^+$ antiporter, whilst leNHX2 might represent a non-specific Cation/$H^+$ antiporter.

# Sodium or potassium efflux ATPases are typical fungal enzymes not present in plants

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All living cells contain relatively high concentrations of $K^+$ and low concentrations of $Na^+$. To maintain $Na^+$ homeostasis, most fungal cells are furnished with a potent $Na^+$-ATPase, which is of crucial importance for $Na^+$ tolerance. However, some of them seem to lack such enzyme. This poses a question about the universal presence of the $Na^+$-ATPases in fungi and plants. Fungi and plants conquered together the emerged lands 500 My ago, facing the same problems of nutrition in an extremely oligotrophic mineral environment and solving the problem with the same $H^+$-ATPase, and the same or similar $K^+$ and $Na^+$ transporters (Rodriguez-Navarro 2000, Biochim.Biophys,Acta, 1469:1-30). To investigate the presence of Na-ATPases in plant and fungi we made a systematic search of mRNAs or genes, which could encode this enzyme. Our results indicate that $Na^+$-ATPases are absent in plants (Garciadeblás et al Plant Soil, in press) but all fungi are furnished with at least one ATPase of a phylogenetic ENA group which include $Na^+$- and $K^+$- efflux ATPases. Our working hypothesis is that the actual fungal $Na^+$-ATPases originated from $K^+$-ATPases, which are necessary for the plant-associated life of fungi. The adaptation of fungi and plants to saline environments has occurred recently, as is demonstrated by the recent common ancestors of most halophytes and glycophytes. For that adaptation, fungi transformed their original $K^+$-ATPases increasing their specificity for $Na^+$, but plants did not follow a similar evolution, because they lack the original enzyme.

## *Arabidopsis* mutation *dry1* identifies a gene essential for drought tolerance

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Osmotic stress caused by drought is one of the most detrimental environmental stress condition limiting plant productivity. One strategy to identify genes essential for osmotic tolerance is to identify mutant plants hypersensitive to osmotic stress.

Because in the search for NaCl hypersensitive mutants, only ionic mutants have been identified, we employed mannitol in our screening in order to identify osmotic hypersensitive mutants. Screening of 45.000 EMS-mutagenized seeds from *Arabidopsis thaliana* genotype Landsberg *erecta* resulted in the isolation of a recessive mutant denominated *dry1* (for drought hypersensitive).

Root growth of *dry1* is specifically hypersensitive to osmotic stress caused by mannitol, sorbitol, choline chloride and proline but is not hypersensitive to NaCl. Analysis of the proline content indicated that *dry1* is not defective in proline accumulation upon osmotic stress. Similarly, root growth of *dry1* at various abscisic acid (ABA) concentrations and the endogenous ABA content were not affected in the mutant.

Adult *dry1* plants were hypersensitive when drought is applied slowly. However no differences in hypersensitivity between WT and *dry1* was found when drought was applied drastically. These data suggest that long-term adaptation to osmotic stress is affected in *dry1*. Northern blot analysis of several osmotic responsive genes showed that *PR5* and *P5CS* were up-regulated in *dry1* respect to wild type after osmotic stress. In contrast *DREB2A* and *RD29A* were down-regulated.

We are currently map-based-cloning *Dry1* in order to gain further insight into the role of this gene in osmotic tolerance.

# Abiotic stress promotes the induction of phosphoenolpyruvate carboxylase expression and the accumulation of malate in roots of wheat seedlings

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Phosphoenolpyruvate carboxylase (PEPC) catalizes the b-carboxylation of PEP in a reaction that yields Pi and oxaloacetate, which is reduced to malate by the action of malate dehydrogenase. This enzyme plays an important role in the primary fixation of CO2 in C4 and CAM plants. In C3 plants, PEPC is found in most organs and, among other roles, it plays an anaplerotic function which consists of the replenishment of oxaloacetate in the tricarboxylic acid cycle whenever the demand of C skeletons for amino acid biosynthesis is high. In wheat seedlings the highest level of PEPC was found in roots. *In situ* hybridisation showed a high accumulation of PEPC transcripts at the vascular tissue, whereas in the root meristem most cells contained PEPC transcripts. The promoter sequence of a PEPC gene that we have isolated from wheat shows elements, which suggest the regulation of PEPC expression by salt, drought and cold stress. We tested the effect of these abiotic stresses on PEPC expression and found that germination of wheat seeds in the presence of NaCl (170 mM) or LiCl (10 mM), promoted the accumulation of PEPC transcripts in seedling roots. Similarly, the incubation of 4-day-old seedlings on dry filter paper promoted the rapid accumulation of PEPC transcripts first in the root meristem and then in the rest of the root, but not in the shoot. In addition, the incubation of wheat seedlings at 4°C also promoted the induction of PEPC expression. Treatments with exogenous ABA and inhibitors of ABA synthesis suggested that the induction of PEPC expression in response to abiotic stress is ABA-dependent. These abiotic stresses promoted the accumulation of malate in seedling roots, probably produced by the higher levels of PEPC expression.

## On the molecular basis of salt tolerance in *S. cerevisiae*

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The baker's yeast *Saccharomyces cerevisiae* is an excellent model system in salt tolerance studies because it shares basic ion transport mechanisms and many signal transduction pathways with crop plants. Genetic analysis has identified in the yeast genome the ENA locus as the major determinant of salt tolerance in yeast. Under salt stress conditions the induction of ENA1 gene, encoding the main $Na^+$ extruding ATPase is a crucial regularoty step which leads the response to changes in intracellular ion homeostasis allowing cell growth to adapt to salinity.

The calcium activated protein phosphatase calcineurin plays an important function in salt tolerance because it up-regulates the expression of ENA1 gene in response to high salt concentrations. We have investigated the molecular mechanisms of this regulation. In a search for yeast genes relevant to halotolerance we isolated four genes (HAL6-9)encoding transcription factors which in multicopy improve cell growth under high external salt conditions. We have characterized HAL8 as the regulatory protein that mediates the effect of calcineurin on ENA1 gene expression. In addition we have identified the regularoty sequences from ENA1 promoter that control the inducible response of ENA1 gene to calcineurin-activating conditions and also constitute target sites for the binding of Hal8p "*in vitro*".

## Altered ABA levels and salt sensitivity in the *Arabidopsis* sre2 mutant

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During the course of a screening for mutants able to germinate and carry out early growth in medium containing a high NaCl concentration we identified four complementation groups, named sre1 to sre4 for salt resistant in a germination assay. The sre mutants were also able to germinate in high osmoticum media, indicating they are osmotolerant in a germination assay. Whereas sre1 and sre3 were allelic to aba2 and aba1 mutants, respectively, sre2 and sre4 were not allelic to previously described aba or abi mutants. Both sre2 and sre4 were not abscisic acid (ABA) insensitive in a germination assay. Germination in paclobutrazol and transpiration assays suggest that sre2 (but not sre4) might have a reduced ABA level as compared to wild-type individuals. Salt-sensitivity assays show that even though sre2 is able to germinate in high NaCl medium, it is salt-hypersensitive in further stages of development. The sre2 mutant was isolated from a T-DNA collection constructed with the activation-tagging vector pSKI15.

A segregation analysis reveals that the sre2 phenotype cosegregates with an in tandem T-DNA insertion. Plant DNA flanking the T-DNA insertion was recovered by plasmid rescue and sequenced. A molecular characterization of the sre2 locus will be presented in the meeting.

# Isolation of potato osmotic stress responsive genes by a functional approach in *E. coli*

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In order to identify key gene functions involved in the potato cellular response to osmotic stress we adopted a functional approach. The methodology used led to the isolation of potato genes whose over-expression enables *E. coli* cells to become NaCl tolerant. A cDNA library from a wild potato species (*Solanum commersonii*) was used to generate a phagemid library by helper phage-mediated *in vivo* excision. After transformation of *E. coli* cells with the rescued library, the IPTG-induced bacterial cells were selected on plates supplemented with different concentration of NaCl (0,5-1,17 M). After repeated selection, 12 salt stress tolerant colonies (N1-N12), containing plasmids with 0.8 - 2.5 kb inserts, were obtained. The inserts were isolated, recloned and introduced into a different E. coli strain. All transformed cells exhibited tolerance in the range 0.5-1.17 M NaCl.

Eight of the cDNA clones were sequenced and partially characterized. Comparative sequence analyses showed that N1 and N8 have significant homology with known plant genes. N1 has high homology (66%) to dehydrin from different plant species, while N8 has 98% identity to *Solanum tuberosum* chaperonin 60 B subunit. The remaining clones, all characterised by sequence domains (myristilation, phosphorylation, Ca++ binding motifs) typical of genes involved in signaling cascades, do not display any significant homology with already described proteins. Homology search identified similar gene sequences of unknown function in the *Arabidopsis* genome. Preliminary Northern analysis revealed that the expression of N1, N2.1, N2.2 and N8 clones were rapidly induced in potato cells upon high salt (NaCl 200mM) e water deficit (PEG 20%) condition, thus suggesting a putative role of the genes in the potato cellular metabolism under stress conditions.

The present data show that the approach utilized is valuable for the identification of plant genes involved in response to stress signals. Further characterization of the corresponding full-length cDNAs will give information about the functional role of the isolated genes in the metabolic and molecular events occurring in the cellular response to environmental stress and in the adaptation process.

# Involvement of a novel *Arabidopsis* phospholipase D, AtPLDdelta, in dehydration-inducible accumulation of phosphatidic acid in stress signaling

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Phospholipid metabolism is involved in plant responses to drought and salinity stress. To investigate the role of phospholipase D (PLD) and its product phosphatidic acid (PtdOH) in stress signaling, we isolated a novel PLD cDNA, designated AtPLDdelta, by screening a cDNA library prepared from dehydrated *Arabidopsis thaliana*. The AtPLDdelta protein, of 868 amino acids, has a putative catalytic domain and a $C^2$ domain that is involved in $Ca^{2+}$ / phospholipid binding. The AtPLDdelta mRNA accumulated in response to dehydration and high salt stress. Histochemical analysis showed that the AtPLDdelta gene is strongly expressed in vascular tissues of cotyledons and leaves under dehydration stress conditions. Under normal growth conditions, AtPLDdelta was expressed in roots, leaves, stems, and flowers but not in siliques. We showed that dehydration stimulates the accumulation of PtdOH. The accumulation of PtdOH in response to dehydration was significantly suppressed in AtPLDdelta antisense transgenic plants. These results suggest that AtPLDdelta may be involved in PtdOH accumulation in dehydration stress response.

# Proton-translocating inorganic pyrophosphatases, wide distribution and tight regulation among phototrophic organisms

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Vacuolar type membrane-bound proton-translocating inorganic pyrophosphatases ($H^+$-PPases) belong to a recently identified category of proton pumps which utilize pyrophosphate hydrolysis as the driving force for $H^+$ movement accross biological membranes. $H^+$-PPases have been identified in a wide variety of organisms such as higher plants, photosynthetic bacteria, archaeas and parasitic and free-living protists. Two different $H^+$-PPases from *Arabidopsis thaliana* have been cloned and expressed in yeast, and shown to have different sensitivities to monovalent cations. We have performed expression studies by Northern blot analysis utilizing specific probes for both isoforms and subjecting the plants to ionic and/or osmotic stress. Transcript levels were checked in roots, stems and leaves. Expression studies have also been carried out in another higher plant, *Antirrhinum majus*, the eukaryotic microalgae *Chlorella fusca* and *Chlamydomonas reinhardtii*, which have different types of carbon metabolisms, and the photosynthetic bacterium *Rhodospirillum rubrum*. We have found that expression levels of genes encoding for $H^+$-PPases exhibit in all these organisms a clear response to environmental ionic and osmotic conditions and to the nutritional cell status. On the other hand, fragments of genes coding for putative $H^+$-PPases have been amplified by PCR from the genome of a range of microorganisms adapted to life in different saline environments. The widespread distribution of these proton pumps among phototrophic organisms as well as their tight regulation by environmental conditions suggest an important role in bioenergetics and ion homeostasis.

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# Cyclic nucleotide signalling and salinity in *Arabidopsis*

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An ever increasing amount of the earth's surface is becoming salinised due to irrigation, posing a major strain on agricultural production. Salt toxicity in plants comprises ionic and osmotic components, both causing diminished growth rates.

Little is known about the mechanism of Na+ entry into the plant symplast and how this pathway is regulated. However, recent reports suggest that voltage independent cation channels (VICs) are involved in Na+ uptake (1,4). Evidence to this effect is mainly based on the observed lack of VIC selectivity amongst cations and the similarity between blocking characteristics of VIC mediated currents and unidirectional Na+ influx by external Ca2+ (2)

In addition to the time dependent, K+ selective ion channels characterised previously in *A. thaliana* root cells (3) we have identified VIC-type channels in these cells. *Arabidopsis* VICs show many of the characteristics described for wheat, barley and maize, such as a lack of selectivity amongst monovalent cations and no or very little voltage dependence.

No information is available regarding gating mechanisms of VIC-type channels. However, patch clamp experiments on *Arabidopsis* root protoplasts showed that some VICs are deactivated by the cyclic nucleotides cAMP and cGMP when added to the cytoplasmic compartment. Channel deactivation occurs within seconds, suggesting that it may result from direct cyclic nucleotide binding to the channel protein .

Further experiments showed that externally applied (membrane permeable) cAMP/cGMP enhances salt tolerance in *Arabidopsis* plants in a dose dependent manner. In addition, externally applied cyclic nucleotides reduce short term unidirectional Na+ influx and ion content analysis revealed significantly less Na+ accumulation in NaCl grown plants. These data suggest that a cyclic nucleotide based signalling pathway plays a role in Arabidopsis salt tolerance and that cyclic nucleotides may directly alter the activity of ion channels that participate in Na+ uptake.

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## Na, Cl and nutrient transport in transgenic tomato plants

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Calcineurin is a Ca and calmodulin-dependent protein phosphatase that, in yeast, is an intermediate of a signal transduction pathway that permits NaCl tolerance through the regulation of Na influx and efflux transport proteins. On the other hand, biochemical and genetic evidence suggests that the system mediating the entrance of sodium into the vacuole is a Na/H antiporter of the NHX family. Tomato transgenic plants transformed with PBTCaN (calceneurin) and AtNHX1 (Na/H antiport) has been grown in a growth chamber with different level of NaCl in the root media. Growth parameters, dry and fresh weights of different plant organs have been determined. Na, Cl and nutrient uptake have been determined by the depletion methods. Distributions of mineral elements in different plant parts (root, stem and leaves) have been calculated. Transport of mineral elements from root to shoot has been determined in order to establish the mechanism of salt tolerance of these tomato transgenic plants.

# Survival in adverse environments: development of freezing drought and salt tolerance in plants

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Plant growth, productivity and distribution are severely limited by environmental stresses including drought, salinity and freezing, all of which disturb the water balance of the cell. Plants have evolved different adaptive strategies to alleviate the adverse effects of these abiotic stresses. Normally exposure to moderate stress or other environmental cues triggers stress acclimation pathways leading to enhanced stress tolerance. This acclimation is associated with expression of a number of stress response genes and subsequent metabolic and physiological alterations required for the enhanced stress tolerance.

Elucidation of the signal pathways controlling the stress response regulons and identifying the target genes required for stress tolerance is one of the key areas of plant stress research. We have characterized a number of target genes upregulated during stress exposure including dehydrins and other proteins such as LTI6/RCA2. Transgenic plants overexpressing some of these genes exhibited enhanced stress tolerance. Furthermore the LTI6 gene was shown to complement the salt-sensitive phenotype of the sna mutant in yeast. Another response to these abiotic stresses is the accumulation of osmoprotectants that help cells to maintain their water balance and, in addition, protect macromolecules in stressed cells. The simplicity of the metabolic pathways leading to osmolyte biosynthesis makes them amenable to genetic engineering. Transgenic plants accumulating glycine betaine and trehalose exhibited enhanced salt tolerance and drought/freezing survival, respectively. Regulon engineering was employed as an alternative strategy to improve stress tolerance including overproduction of transcription factors or antisense silencing of negative regulators of COR/LTI gene expression. The results from our studies as well as by other groups suggest that engineering stress signaling or expression of specific target genes such as those involved in biosynthesis of osmoprotectants may provide an efficient strategies for generation of crop plants with enhanced tolerance to stresses like drought, freezing and high salinity.

Key words: stress acclimation, signal transduction, osmoprotectants, glycine betaine, trehalose, salt tolerance, drought survival, freezing tolerance

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# Transformants of *Saccharomyces cerevisiae* with genes from *Debaryomyces hansenii* display improved salt and $K^+$ transport capacity

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*Debaryomyces hansenii* is an ascomicetous yeast, phylogenetically close to *S. cerevisiae*, usually found in the sea and other environments with high $Na^+$ and low $K^+$ concentrations such as solar saltworks, brines and salted food. In previous works we characterized the phisiology of the resistance to NaCl. We reported that *D. hansenii* can accumulate high intracellular concentrations of $Na^+$ without becoming intoxicated by this cation, even in the presence of low $K^+$ concentrations. It was also observed that, in general, the performance of this yeast is better in the presence of NaCl concentrations near 0.5 M, especially under additional stress conditions. Especially relevant was the stimulation of $K^+$ uptake by $Na^+$, opposite to the effects observed in *S. cerevisiae* where $Na^+$ is a competitive inhibitor of $K^+$ uptake.

We regarded *Debaryomyces hansenii* as a sink of genes that confer salt tolerance and as a model for searching the molecular basis involved in such salt resistance. We have constructed a genomic library from *D. hansenii*. This library was used to transform a wild type strain of *S. cerevisiae* in which the salt tolerance was not changed (W303). The selection of transformants was based on the acquisition of capacity to grow in media with high $Na^+$ and/or low $K^+$, in which *D. hansenii* was able to grow but the receptor *S. cerevisiae* strain did not grow. Using these conditions we were able to obtain several transformants with increased salt tolerance that were able to grow in media with high $Na^+$ and/or low $K^+$ such as 2200mM NaCl or 50mM $K^+$ in the absence of NaCl. Using the several conditions in order to establish different categories of transformants, we tested growth of each transformant in the three extreme conditions previously used for selection. Different patterns were found allowing the distribution of transformants into four classes.

The plasmid from one of the transformants presenting a better performance in high salt and low $K^+$ medium in the absence of NaCl, was used for further characterization. In order to test if it contained a gene related with $K^+$ transport, a trk1*trk2* *S. cerevisiae* strain, strongly defective in $K^+$ uptake and highly sensitive to NaCl, was transformed with the fragment containing the cloned gene. Transformants were selected in mineral medium with 50μM K+. Kinetic analysis of $K^+$ uptake in the trk1*trk2* transformed strain, using a $K^+$ specific electrode, demonstrated that this strain partially recovered the potassium uptake capacity, showing a KM(K+) of 0.2mM in $K^+$ starved cells. The fragment is currently being characterized from a molecular point of view.

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# Differential expression of *Arabidopsis thaliana* NHX $Na^+/H^+$ antiporters in the salt stress response

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The AtNHX1 gene encodes a vacuolar $Na^+/H^+$ antiporter and imparts salt-tolerance to *Arabidopsis* plants and yeast cells (Gaxiola et al, 1999; Apse et al, 1999; Quintero et al, 2000). Five additional AtNHX-like genes were identified in the *Arabidopsis* genome. Based on sequence similarities, the AtNHX gene family can be categorized in two groups, one containing four members (AtNHX1-4) and the other two members (AtNHX5-6). The cDNAs corresponding to isoforms 2, 5 and 6 were isolated and their function was tested in a yeast mutant deficient in the endosomal/vacuolar $Na^+/H^+$ antiporter ScNHX1, confirming that these isoforms are endosomal ion exchangers. Transcription of all six AtNHX genes was detected in *Arabidopsis* seedlings either by northern blot or RT-PCR. The expression of AtNHX1, AtNHX2, and AtNHX5 was increased by NaCl stress. AtNHX1 and AtNHX2 were also upregulated by ABA. To determine which stress signaling pathway is controling the expression of these genes, analysis of AtNHX expression was carried out in mutants defective in ABA production (Shinozaki and Yamaguchi-Zhinozaki, 2000) or components of the SOS signaling pathway (Zhu, 2001). The results obtained indicate that expression of AtNHX1 and AtNHX2 is regulated by the ABA signaling pathway, whereas AtNHX5 is upregulated by an as yet unidentified ABA- and SOS-independent pathway.

# Overexpression of a dehydration inducible novel aldehyde dehydrogenase confers salinity tolerance

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The stress caused by high soil salinity posing a serious threat to agricultural production. Improvement of salinity tolerance of crop plants through genetic engineering requires elucidation of the molecular basis of plant defense mechanisms. In an effort to identify, isolate and characterize the genes involved in stress tolerance substractive hybridization was applied. Using this approach we have isolated a dehydration inducible novel aldehyde dehydrogenase gene from *Craterostigma plantagineum*, a desiccation tolerant plant. Further, we have also isolated the homologous genes from *Arabidopsis*. Aldehyde dehydrogenases (Aldh's) are a family of $NAD(P)^+$- dependent enzymes that catalyze the oxidation of numerous aldehydes (Lindahl, 1992; Yoshida et al., 1998). Aldehydes are highly reactive molecules that are important in numerous physiological and biochemical processes. Various metabolic pathways exist for conversion of aldehydes to less reactive compounds (for e.g., corresponding acids and alcohols). Different types of Aldh's have been distinguished based on their substrate specificity, subcellular and tissue distribution. Under stress conditions, the accumulation of toxic aldehyde molecules is likely to be increased, causing increased cellular damage. Over production of the enzymes involved in detoxification of such aldehydes is likely to open up yet another strategy for the improvement of crop plants tolerance against salinity stress. Towards elucidating the function of Aldh, we generated transgenic lines overexpressing Aldh and subjected for laboratory evaluation. Transgenic plants overexpressing Aldh are tolerant to salinity conditions and suggesting a role for Aldh in conferring stress tolerance.

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## A catalytic subunit of the sugar beet CK2 is induced by salt stress and increases NaCl tolerance in *Saccharomyces cerevisiae*

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Salinity is an important limitant of plant growth and development. We have cloned a catalytic subunit of the sugar beet protein kinase CK2 (BvCKA2) by functional expression in yeast of a NaCl induced cDNA library. BvCKA2 was able to increase the yeast tolerance to NaCl and to functionally complement the cka1 cka2 yeast double mutant upon overexpression. Southern blot analysis indicated that, in sugar beet, the BCKA2 gene is a member of a multigene family. The mRNA levels of BvCKA2 were up regulated in response to NaCl stress which suggests that protein kinase CK2 may be involved in the plant response to salt stress.

# HAK transporters of groups I and II: Identification of amino acids involved in the affinity for $K^+$ and $Na^+$

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Potassium is an essential nutrient for plants that fulfills important functions related to osmoregulation, enzyme activity and movement. Plants take up $K^+$ from the soil solution where the cation concentration is highly variable and several orders of magnitude lower than in the cytoplasm. By contrast, $Na^+$ may be toxic when is present at high concentrations and most plants excude it. Once $K^+$ is taken up by the roots, it is redistributed through out the plant via xylem and phloem to make it available to all plant cells. Several $K^+$ transport systems have been characterized that mediate the movement of $K^+$ through the different membranes and recently genes encoding some of the $K^+$ transporters have been identified. It has been suggested that $K^+$ transporters represent an important pathway for $Na^+$ entry in the plant cell. Thus, $K^+$ transporters are important for plant nutrition and salt tolerance.

The HAK/KUP/KT genes comprise a large gene family present in many plant, fungi and bacterial species. In barley, two cDNAs HvHAK1 and HvHAK2 were isolated that encoded putative $K^+$ transporters. HvHAK1 expressed in yeast mediates high-affinity $K^+$ transport and low-affinity $Na^+$ transport but HvHAK2 did not produce functional complementation in yeast. Several amino acids are conserved between HvHAK1 and HvHAK2 and others are exclusive of the phylogenetic groups to which both transporters belong. Here we report that when amino acid residues characteristic of HvHAK2 are substituted for the corresponding HvHAK1 residues, HvHAK1 affinity for $K^+$ decreases. Moreover, expression of HvHAK2 in bacteria suggests a low affinity of the transporter for K+. Physiological implications of these results for ion homeostasis will be presented.

# AtHKT1 Is a salt tolerance determinant that controls $Na^+$ entry into plant roots

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Two *Arabidopsis thaliana* extragenic mutations that suppress NaCl hypersensitivity of the sos3-1 mutant were identified in a screen of a T-DNA insertion population in the genetic background of Col-0 gl1 sos3-1. Analysis of the genome sequence in the region flanking the T-DNA left border indicated that sos3-1 hkt1-1 and sos3-1 hkt1-2 plants have allelic mutations in AtHKT1. AtHKT1 mRNA is more abundant in roots than shoots of wild type plants but is not detected in plants of either mutant indicating that this gene is inactivated by the mutations. hkt1-1 and hkt1-2 mutations can suppress to an equivalent extent the $Na^+$ sensitivity of sos3-1 seedlings and reduce the intracellular accumulation of this cytotoxic ion. Moreover, sos3-1 hkt1-1 and sos3-1 hkt1-2 seedlings are able to maintain $[K^+]$int in medium supplemented with NaCl and exhibit substantially higher $K^+/Na^+$ selectivity than the sos3-1 mutant. Furthermore, the hkt1 mutations abrogate growth inhibition of the sos3-1 mutant that is caused by $K^+$ nutrient deficiency on culture medium with low $Ca^{2+}$ (0.15 mM) and <200 mM $K^+$. Interestingly, the capacity of hkt1 mutations to suppress the $Na^+$ hypersensitivity of the sos3-1 mutant is abrogated when seedlings are grown in medium with low $Ca^{2+}$ (0.15 mM) and 20 mM KCl. These results indicate that AtHKT1 is an entry system for $Na^+$ and may function in the modulation of high affinity $K^+$ uptake. The hkt1 mutations have revealed the existence of another $Na^+$ influx system(s) whose activity is reduced by high $[Ca^{2+}]$ext.

# Expression and localisation of *Arabidopsis* calcineurin B-like proteins in plant cells

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Until recently the protein phosphatase calcineurin known to be involved in NaCl tolerance in yeast remained elusive in plant cells. The identification of a role for calcineurin in plant salt tolerance (Pardo et al., 1998) and the identification of calcineurin B-like proteins (AtCBLs) in *Arabidopsis* (Kulda et al., 1999) has increased interest in this class of proteins. Although their role, interacting with a family of protein kinases (Albrecht et al., 2001), is becoming clearer, their sub-cellular location remains uncertain.

Eight known CBLs have been identified and four contain the known Myristic Acid binding motif (MGXXXSK) Additionally, one contains a putative ER signal peptide. Microscopical and biochemical techniques are being used to determine if these motifs affect sub-cellular localisation and function.

Constructs to overexpress AtCBL1 fused to GFP have been generated, as well as constructs containing either a mutated or truncated myristic acid binding site. This will enable us to examine the effects of mis-localisation of the protein within the cell. Transient expression experiments have already indicated that the myristic acid site when fused to the N-terminus of the neutral carrier protein phosphinothricin acetyl transferase (PAT) is not sufficient for membrane targeting.

In addition to this work, constructs containing sequences upstream of the AtCBL1 gene, fused to luciferase are being been generated. These will enable us to test these sequences for promoter activity, thus enabling a study of the transcriptional regulation of stress responses.

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# LIST OF INVITED SPEAKERS

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