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Developing salt tolerant plants in a new century: a molecular biology approach

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Abstract

Soil salinity is a major abiotic stress in plant agriculture strongly, influencing plant productivity world-wide. Classical breeding for salt tolerance in crop plants has been attempted to improve field performance without success. Therefore, an alternative strategy is to generate salt tolerant plants through genetic engineering. Several species and experimental approaches have been used in order to identify those genes that are important for salt tolerance. Due to high level of salt tolerance, halophytes are good candidates to identify salt tolerance genes. However, other species such as yeast and glycophytes have also been employed. Three approaches are commonly used to identify genes important for salt tolerance. The first approach is to identify genes involved in processes known to be critical for salt tolerance (osmolyte synthesis, ion homeostasis, etc.). The second approach is to identify genes whose expression is regulated by salt stress. This is relatively simply and applicable to any plant species. Genetic amenability of some species allows the third approach, which consists in the identification of salt tolerance determinants based on functionality. At the moment, there is a large number of reports in the literature claiming that plants with increased salt tolerance have been obtained. The main problem is that different plant species, stage of development, organs, promoters and salt conditions used it is difficult to compare the degree of salt tolerance conferred by different genes. In this review, we discuss progress made towards understanding the molecular elements involved in salt stress responses that have been used in transgenic approaches to improve salt tolerance.

The complexity of salt stress

More than 40 years of research on salinity have produced an uncountable number of papers and unpublished results that reflect the importance of this problem in agriculture. Salt stress is certainly one of the most serious environmental factors limiting the productivity of crop plants (Ashraf, 1999). However, despite the advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive.

This is due to the fact that salinity affects most aspects of plant physiology. Salinity reduces the ability of plants to absorb water, causing rapid reductions in growth rate, along with as array a suite of metabolic changes identical to those caused by water stress. A comparative study of the physiology of salt and water stress has recently been reviewed by Muuns (2002).

High salt concentration in the external solution of plant cells produces several deleterious consequences. First, salt stress causes an ionic imbalance (Niu et al., 1995; Zhu et al., 1997). When salinity results from an

excess of NaCl, which is by far the most common type of salt stress, the increased intracellular concentration of Na^+ and Cl^- ions is deleterious to cellular systems (Serrano et al., 1999). In addition, the homeostasis of not only Na^+ and Cl^-, but also K^+ and Ca^{+2} ions is disturbed (Serrano et al., 1999; Hasegawa et al., 2000a, b; Rodriguez-Navarro, 2000). As a result, plant survival and growth will depend on adaptations that re-establish ionic homeostasis, thereby reducing the duration of cellular exposure to ionic imbalance. Second, high concentrations of salt impose an hyperosmotic shock by decreasing the chemical activity of water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought. Third, salt-induced water stress reduction of chloroplast stromal volume and generation of reactive oxygen species (ROS) are also thought to play important roles in inhibiting photosynthesis (Price and Hendry, 1991). ROS can be generated in the chloroplast by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at Photosystem I, in the Mehler reaction (Foyer et al., 1994; Allen, 1995).

Plants respond to salt stress at three different levels, i.e., cellular, tissue and whole plant level. Cell-based mechanisms of ion homeostasis and the synthesis of osmoprotectants are essential determinants for salt tolerance. However, as plant cells become specialized during ontogeny, it is clear that the adaptive mechanisms to tolerate salt stress may be different. Integration and coordination of the responses of cells, tissues, and organs responses are required for a proper tolerance to salt stress. However, the separate study of each level of response is the best way to correctly place the pieces to understand the whole picture of salt tolerance. Moreover, because salt tolerance is regulated throughout the plant development and is a tissue-specific phenomenon, plant tolerance responses at one stage of development are not necessarily the same at other stages (Johnson et al., 1992; Lauchli and Epstein, 1990). Therefore, the mechanisms of tolerance at specific stages of plant development must be studied in order to understand the biochemical events that play important roles in the responses to salt stress (Borsani et al., 2001a).

Given all the factors determining the pleitropic deleterious effects of salt stress, it is not surprising that adaptation to salinity may involve the modification of a large number of parameters.

The search for salt tolerance determinants

In tomato plants, sources of salt tolerance have been identified among related wild species and primitive cultivars (Jones, 1986; Cuartero et al., 1992). These genetic resources have been used to improve salt tolerance in modern crop cultivars. However, in spite of considerable effort through breeding programmes, progress to enhance salt tolerance has been slow. Classic genetic studies have demonstrated that the ability of plants to tolerate salt stress is a quantitative trait involving the action of many genes. As a result, it has been difficult to obtain salt tolerance in crop plants by traditional methods (Foolad and Lin, 1997). This situation has been complicated by the fact that the main character selected in crop plants has been productivity, which is also a complex trait. Therefore, the integration of genes required to increase salt tolerance in a specific genotype is difficult without affecting other important multigenic traits like flowering, fruit quality and dry matter production (Flowers et al., 2000).

Previous knowledge of the critical genes for salt tolerance is not needed when using traditional breeding programs. Thus, is not the case with the use of genetic engineering. Therefore, an important objective is to determine the limiting factors and key processes that produce salt tolerance, i.e. salt tolerance determinants. Hence, much effort has been devoted toward understanding the adaptive mechanisms of plant salt tolerance (Bohnert et al., 1995; Hasegawa et al., 2000a, b; Xiong et al., 2002).

The first approach employed to ascertain salt tolerance determinants in plants was to identify the metabolic processes critical to tolerate NaCl. Establishment of ion homeostasis is an essential requirement for plants to survive under salt stress conditions. Plant cells respond to salt stress by increasing Na^+ efflux at the plasma membrane and Na^+ accumulation in the vacuole. Therefore, proteins, and ultimately, genes involved in these processes can be considered as salt tolerance determinants. Salt tolerance requires not only adaptation to Na^+ toxicity but also the acquisition of K^+ whose uptake by the plant cell is affected by high external Na^+ concentration. The uptake of K^+ is affected by Na^+ due to the chemical similarities between both ions. Potassium is an essential nutrient being the major cationic inorganic nutrient in most terrestrial plants. Therefore, K^+ transport systems involving good selectivity of K^+ over Na^+ can also

be considered as an important salt tolerant determinant (Rodriguez-Navarro, 2000).

Another metabolic response to salt stress is the synthesis of compatible osmolytes. These organic compounds are thought to mediate osmotic adjustment, protecting sub-cellular structures and oxidative damage by their free radical scavenging capacity (Hong et al., 1992; Smirnoff, 1993; Hare et al., 1998). Thus, genes regulating the accumulation of these organic compounds can be considered as salt tolerant determinants.

Salt, drought, and to some extent cold stress cause, an increased biosynthesis and accumulation of abscisic acid (ABA) (Koornneef et al., 1998; Taylor et al., 2000). ABA role in osmotic stress tolerance is well known and has been exhaustively reviewed (McCourt, 1999; Rock, 2000; Zhu, 2002).

However, there are some evidences that ABA could be involved in the control of ion homeostasis. For example, ABA content was slightly increased only in the leaves of the salt tolerant rice cultivar *versus* the sensible cultivar. This increased of ABA content was accompanied by an improved K^+/Na^+ ratio (Bhora et al., 1995). Also, the transport and accumulation of K^+ in higher plant roots has been shown to be regulated by ABA (Roberts, 1998). Recent reports indicate that ABA regulates K^+ channel activity in maize and *Arabidopsis* roots, suggesting that ABA regulation of K^+ transport in roots is, at least in part, ion channel-mediated (Roberts and Snowman, 2000).

Many plants respond to high salt levels by sequestering ions within the vacuole. This process is mediated by a vacuolar Na^+/H^+ antiporter that uses the proton-motif force to concentrate ions against a gradient. A recent characterization of five Na^+/H^+ antiporters from *Arabidopsis thaliana* showed that the transcripts of two of them (AtNHX1 and AtNHX2) accumulate in response to ABA. However, this accumulation did not occur in the *aba1-2* mutant indicating that the osmotic stress responsiveness of these genes is ABA-dependent (Yokoi et al., 2002). Several cDNAs encoding membrane-located ion pumps like H^+-ATPases and V-ATPases have been isolated. The accumulation of these transcripts was induced by salt, some of them being also regulated by ABA (Narasimhan et al., 1991; Tsiantis et al., 1996).

Also important to ion homeostasis is the increase of cytoplasmic free Ca^{+2} that is induced by ABA, an event regulated by cyclic ADP-ribose (Wu et al., 1997). The identification of stress- and ABA-inducible mRNAs that code for a Ca^{+2} binding membrane protein in rice (Frandsen et al., 1996) and phosphatidylinositol-specific phospholipase C in *A. thaliana* (Hirayama et al., 1995) respectively, provides indirect evidence for the involvement Ca^{+2} in ABA signalling in vegetative tissues. The isolation of the RD20 gene, a Ca^{+2}-binding protein that is induced by ABA and salt stress, suggests a link between the salt stress, ABA and Ca^{+2} signalling pathways.

With the advent of molecular biology, a common approach used to determine mechanisms of salt tolerance has been the identifications of cellular processes and genes whose activity or expression is affected by salt stress (Bray, 1993; Botella et al., 1994; Zhu et al., 1997; Hasegawa et al., 2000a, b). Identification of these salt-regulated genes has allowed a better understanding of the complexity of salt tolerance in higher plants (Bray, 1993, Zhu et al., 1997, Serrano et al., 1998, Hasegawa et al., 2000a, b). The generalized assumption was that a gene regulated by salt stress would probably be important in tolerance. Many of these salt-regulated genes have been reported but the most direct procedure to demonstrate their importance in salt tolerance is through functional genetic analysis. This approach is not easily accomplished in crop plants.

Mutagenesis is *Arabidopsis* and tomato has been recently employed to identify key genes and cellular processes involved in plant salt tolerance (Zhu, 2000; Xiong et al., 2001; Borsani et al., 2001a). Genes were identified by selecting and characterizing salt-hypersensitive mutants (Ishitani et al., 1997). As a result, four *sos* (for salt overly sensitive) mutants in Arabidopsis and two *tss* (for tomato salt sensitive) mutants in tomato have been identified (Wu et al., 1996; Liu and Zhu, 1997; Zhu et al., 1998; Borsani et al., 2001a; Shi et al., 2002b). Interestingly, mutations in most of these genes rendered the *sos* and *tss1* plants hypersensitive to the ionic component of NaCl stress with no significant effect on osmotic tolerance. The exception was the *tss2* mutant, which is hypersensitive to both ionic and osmotic stresses (Borsani et al., 2001a). The studies have revealed that all three *SOS* and the *TSS1* genes are necessary for K^+ nutrition as well, because the mutants were also hypersensitive to growth at low K^+ concentrations. In addition, the isolation of the *sos3* and *tss1* mutants provided insight into the role of Ca^{+2} in salt tolerance, since both high NaCl hypersensitivity and the inability to grow on low K^+ was abolished by raising

the Ca^{2+} to millimolar levels (Liu and Zhu, 1997; Borsani et al., 2001a). Analysis of *tss2* confirmed that signalling by the plant hormone abscisic acid (ABA) is important for salt and osmotic plant tolerance, because *tss2* was also hypersensitive to growth inhibition caused by ABA (Borsani et al., 2001a).

The molecular nature of all four *SOS* genes was recently determined. The *SOS1* gene encodes a protein that has significant sequence similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi et al., 2000). SOS1 is preferentially expressed in epidermal cells at the root tip and in the parenchyma cells at the xylem/symplast boundary of roots, stem, and leaves (Shi et al., 2002a). The same study demonstrates that a Na^+/H^+ antiporter can play a different role under mild or low salt stress showing different responses under different salt stress situations.

The *SOS2* gene encodes a serine/threonine protein kinase with an N-terminal catalytic domain similar to that of the yeast SNF1 kinase (Liu et al., 2000). The *SOS3* gene encodes a Ca^{+2} binding protein and has its greatest sequence homology with the yeast calcineurin B subunit and a neuronal calcium sensor, both of which are activated by Ca^{+2} (Liu and Zhu, 1998). Double mutant analysis showed that *SOS1*, *SOS2*, and *SOS3* function in a linear pathway (Liu and Zhu, 1998; Zhu et al., 1998). This is further supported by two recent findings. First, the *SOS2* protein kinase interacts physically with and is activated, in the presence of calcium, by the calcium sensor *SOS3* (Halfter et al., 2000). Thus, the *SOS2*/*SOS3* kinase complex represents a regulatory pathway that, along with Ca^{+2}, controls Na^+ and K^+ homeostasis and plant salt tolerance. Second, the up regulation of *SOS1* in response to NaCl is reduced in *sos2* and *sos3* mutant plants, supporting the notion that *SOS1* expression is controlled by the *SOS2*/*SOS3* regulatory pathway (Shi et al., 2000). Recently, reconstitution of Arabidopsis response SOS signaling pathway in a yeast heterologous system indicates that SOS3 activates and directs SOS2 to the plasma membrane for the stimulatory phosphorylation of the Na^+ transporter SOS1 (Quintero et al., 2002). *SOS4* encodes a piridoxal (PL) kinase that is involved in the biosynthesis of PL-5-phosphate, an active form of vitamin B6 (Shi et al., 2002b). Besides being a cofactor for many cellular enzymes, PLP is also known as ligand that regulates the activity of certain ion transporters in animal cells. The authors of this study, propose that this property might be related to the salt tolerance function of SOS4 (Shi et al., 2002b). In addition, SOS4 may also regulate SOS1 because it contains a putative binding sequence for pyridoxal-5-phosphate (Zhu, 2002).

Species employed for the identification of genes involved in salt tolerance

The genetic identification of mechanisms involved in K^+ nutrition as a process that is critical for salt tolerance in *Arabidopsis* and tomato suggests that despite the different degree of salt tolerance among these plant species, they share common mechanisms. These mechanisms include, in addition to K^+ nutrition, synthesis of compatible osmolytes, Na^+ and Cl^- exclusion and compartmentation, increase of toxic radical scavenging capacity and increase in the content of the phytohormone ABA. Besides these common mechanisms, it is becoming clear that glycophytes have the genes necessary to tolerate salt stress but probably the salt tolerance determinants are properly expressed only after adaptation to stress (Hasegawa et al., 1994; Zhu, 2000).

A number of different organisms have been employed to identify those processes or genes required for salt tolerance. These organisms vary from prokaryotic organisms such as *Escherichia coli*, unicellular eucariotic organisms such as *Saccharomyces cerevisiae*, to halophytic land plants such as *Mesembryanthemum crystallinum*, and glycophytic plants such as rice, tomato or *A. thaliana*.

Yeast

In addition to its genetic amenability, *S. cerevisiae* shares basic ion transport mechanisms with plants. Therefore, it can be used as an excellent model system for the study of salt tolerance at a cellular level. Genetic analysis has been very successful in elucidating salt stress tolerance determinants in yeast (Toone and Jones, 1998; Serrano et al., 1999). A number of salt-sensitive yeast mutants have been identified and the cloning of the corresponding genes has shed light on the nature of many genes that are essential for salt tolerance (Brewster et al., 1993; Mendoza et al., 1994; Toone and Jones, 1998; Serrano et al., 1999).

Yeast responds to NaCl by activating several signal transduction pathways. Two of these pathways are

needed to sense the osmotic stress induced by NaCl by different osmosensors. The first osmosensor comprises SLN1, a transmembrane histidine kinase, and SSK1, the sensor and response regulator respectively, of a two-component system (Maeda et al., 1994). The second osmosensor, SHO1, is an independent transmembrane protein containing a SH3 domain (Maeda et al., 1995). Both osmosensors connect to a Mitogen Activated Protein Kinase (MAPK) that modulates the pathways that finally converges at the PBS2 kinase (a MAPKK). PBS2 kinase phosphorilates the HOG1 kinase leading eventually to the induction of several defence genes including the glycerol biosynthetic genes and the *ENA1* (Na^+-efflux pump) (Serrano, 1996; Toone and Jones, 1998).

Plant proteins homologous to those of the HOG pathway in yeast have been identified. A putative MAPK from *Pisum sativum* (PsMAPK), which is 47% identical to Hog1p, functionally complements the salt growth defect of the *hog1* yeast mutant (Popping et al., 1996). Combinations of *Arabidopsis* proteins ATMEKK1 and MEK1, or MAPKKK and MAPKK, can functionally complement the salt growth defect of the *pbs2* yeast mutant (Ichimura et al., 1998). The *Arabidopsis* gene *ATHK1* was identified by its sequence homology to the yeast osmosensor SLN1 (Urao et al., 1999). Over-expression of *ATHK1* suppressed the lethality of the temperature-sensitive osmosensing-defective yeast mutant *sln1-ts*. The transcripts of *ATHK1* are transiently induced after NaCl stress, also suggesting a role of this protein in plant salt tolerance. Therefore, it seems that plants and yeast have similar components for sensing salt stress and transducing the signal. However, the actual function of the signal components in NaCl tolerance of plants remains to be determined through functional genetic analysis.

The third signal transduction pathway for tolerance NaCl in yeast is specific for the ionic component of NaCl stress. It regulates ion homeostasis and involves the protein phosphatase calcineurin (Mendoza et al., 1994). The actual sensor of this pathway is not known. However, it is speculated that it could be a vacuolar cation exchanger that releases Ca^{+2} in exchange with Na^+ (Serrano, 1996). Calcineurin is a Ca^{+2}- and calmodulin-dependent protein phosphatase consisting of a catalytic A subunit (CnA) and a regulatory B subunit (CnB). CnB has four high affinity EF-hand calcium-binding sites and full activation of CnA requires calcium-CnB and calcium-calmodulin dependent complexes (Klee et al., 1988). Calcineurin regulates Na^+, K^+ and Ca^{+2} homeostasis (Nakamura et al., 1993; Mendoza et al., 1994). Mutations in the catalytic and/or the regulatory subunit of the phosphatase calcineurin render yeast strains hypersensitive to Na^+ and Li^+ (Nakamura et al., 1993; Mendoza et al., 1994).

Despite biochemical evidence for a calcineurin-like activity in plants (Luan et al., 1993), the identification of the specific plant gene has been unsuccessful so far. Notwithstanding the identification of CnB-like proteins in plants, none can interact with the yeast CnA, suggesting that they are not functional homologues (Kudla et al., 1999). Functional complementation of the calcineurin yeast mutant with *Arabidopsis* identified *AtGSK1*, which encodes a GSK3/shaggy-like protein kinase (Piao et al., 1999). Interestingly, NaCl and ABA but not KCl induce the expression of AtGSK1 suggesting a role for this gene in salt tolerance. Two *Arabidopsis* transcription factors, *STO* and *STZ* also suppressed the Na^+ and Li^+ hypersensitive phenotypes of the calcineurin deficient mutants (Lippuner et al., 1996). Moreover, overexpression of these genes enhanced the salt tolerance of the wild-type yeast strain. Salt tolerance mediated by *STZ* is dependent on the P-ATPase encoded by *ENA1*, whereas *STO*-mediated salt tolerance is independent of *ENA1* (Lippuner et al., 1996). As occurred with *AtGSK1*, the expression of *STZ* but not *STO* was found to be induced by NaCl. Similar to these transcription factors, the yeast *HAL2* gene was found to increase salt tolerance when overexpressed in *Arabidopsis*. *HAL2* encodes a (2′), 5′-bisphosphate nucleotidase and inositol 1-polyphosphatase enzyme which functions in the catabolism of inositol 1, 4, 5-trisphosphate (IP_3). Its activity was inhibited by Li^+ and Na^+ (Murguia et al., 1995), suggesting that this enzyme is a target for Li^+ and Na^+ toxicity. Evidence of the importance of this mechanism in salt tolerance in other species came from the identification of the *Arabidopsis SAL1* by functional complementation of the yeast *ena1-4* strain, a yeast strain that lacks the major Na^+ and Li^+-extrusion system. *SAL1* is the plant ortholog of the *HAL2* gene from yeast (Quintero et al., 1996). The *SAL1* is identical to the later identified *FRY1* gene (Xiong et al., 2001). Mutation in this gene results in super-induction of ABA- and stress-responsive genes. Seed germination and post-embryonic development of *fry1* are more sensitive to ABA or osmotic stress inhibition. The mutant plants

are also compromised in tolerance to freezing, drought, and salt stresses (Xiong et al., 2001).

Halophytes

The most important characteristic of halophytes plants is their capacity to grow under high concentrations of NaCl. It now seems clear that the halophytes's tolerance to NaCl is not the result of unique adaptive mechanisms or metabolic processes that are unique to these plants (Flowers et al., 1977; Yeo, 1998; Glenn et al., 1999). It seems that the biochemical mechanisms leading to salt tolerance in these plants are regulated in such way that allow a more successful response to salt stress than in other plants (Hasegawa et al., 2000a, b). The question to be addressed is whether or not the mechanisms employed in salt stress tolerance by halophytes could be employed by glycophytes plants without a loss in productivity. The halophytic land plant *M. crystallinum* has been frequently used as model plant in salt tolerance studies. This plant is now being used in the identification of ESTs differentially expressed after plant salt exposition (Bohnert et al., 2001). A problem in the use of most halophytes is the identification of gene by the use of a genetic approach (i.e. searching for salt hypersensitive mutants). For this purpose, the study of *Thellungiella halophila* might be of particular interest in the identification of genes involved in salt tolerance. This halophyte plants can survive at seawater-level salinity and its DNA sequence have a similarity of more than 90% of *Arabidopsis* (Zhu, 2001). This *Arabidopsis* closely related species to can also be easily transformed allowing insertion tag mutagenesis (Bressan et al., 2001).

Glycophytes

All major crop plants are glycophytes and the study of this plants as model organisms may uncover processes related to salt tolerance that are specific to these plant species. Of all the glycophytes, there is no doubt that *Arabidopsis* is becoming very useful in the determination of processes involved in salt tolerance. In fact, genetic analysis using *Arabidopsis* as a model is leading to a deeper knowledge of a key signal transduction pathway in salt tolerance, such as the SOS pathway, critical for salt tolerance (Hasegawa et al., 1994; Zhu, 2000).

Another glycophyte recently employed in genetic analysis using mutagenesis is tomato (Borsani et al., 2001a). Tomato is a widely distributed annual vegetable crop adapted to a large variety of climates. However, in spite of its broad adaptation, production is concentrated in a few warm and rather dry areas (Cuartero and Fernandez-Muñoz, 1999). In these areas with an optimal climate for tomato production, salinity is a serious problem (Szabolcs, 1994). For this reason, a large number of physiological studies of salt stress have been performed using tomato as a model plant (Cuartero and Fernandez-Muñoz, 1999). Unlike *Arabidopsis*, direct studies on salinity, adaptation, and molecular changes in this plant can be assessed also for crop yield.

Increasing plant salt tolerance through genetic engineering

Numerous reports in the literature have shown improvement of salt tolerance via genetic engineering. Genes employed in this studies have been isolated from a number of organisms, ranging from prokaryotic organisms such as *E. coli* to halophytes or glycophytes (Table 1). The improvement of tolerance to salt stress has been analyzed in different plant species, different stages of development and by using different evaluation criteria. These aspects make it extremely difficult to compare the improvement conferred by different genes in salt tolerance. But, the metabolic pathways and mechanisms modified to improve the salt tolerance can be grouped according the genes employed in transgenic experiments. These genes can be classified into five groups according to their functions:

– Synthesis of osmolytes,
– Protection of cell integrity,
– Oxidative stress,
– Ion homeostasis,
– Transcription factors.

Genes involved in the synthesis of osmolytes

In plants, a common response to osmotic stress is the accumulation of compatible osmolytes such as proline (Pro), glycine betaine and sugar alcohols. It has been suggested that compatible osmolytes do not interfere with normal biochemical reactions and act as osmoprotectans during osmotic stress.

Proline is the most common osmoprotectant that accumulates in plants in response to water stress and salinity (Hanson and Hitz, 1982; Yoshiba et al.,

Table 1. Salt tolerance of transgenic plants expressing several genes

Name	Source specie	Gene product	Function	Harbourg species	Stage development expressing tolerance	Parameters studied	Reference
betA	*E. coli*	Choline dehydrogenase	Betaine synthesis	tobacco	seedling	Dry weight	Lilius et al. (1996)
BADH	*E.coli* *S. oleracea*	Betaine dehydrogenase	Betaine synthesis	tobacco	seedling	Increase of biomass protection of photosynthetic apparatus	Holmstrom et al. (2000) Liang et al. (1997)
CodA	*A. globiformis*	Choline oxidase	Betaine synthesis	*Arabidopsis* rice	seedling germination	Inprove of growth and photosynthetic activity	Hayashi et al. (1997) Sakamoto et al. (1998)
COX	*A. pascens*	Choline oxidase	Betaine synthesis	*Arabidopsis* tobacco *Brasssica napus*	seedling	Improve of shoot growth	Huang et al. (2000)
TUR1	*A. polyrriza*	Inositol synthase	InsP3 synthesis	*Arabidopsis*	seedling	Slight allevation of NaCl stress	Smart and Flores (1997)
IMT1	*M.crystalinum*	Myo-inositol *O*-metyltransferase	D-ononitol synthesis	tobacco	seedling	Improve of photosynthetic activity	Sheveleva et al. (1997)
MtlD	*E.coli*	Mannitol 1-phosphate dehydrogenase	Mannitol synthesis	*Arabidopsis* tobacco	seedling germination	Germination and growth	Tarczynski et al. (1993) Thomas et al. (1995)
P5CS	*V. aconitifolia*	Δ^1Pyrroline-5-carboxylate	Proline synthesis	tobacco rice	adult plant	Biomass and flower development	Kavi Kishor et al. (1995)
ProDH	*A. thaliana*	Proline deydrogenase	Proline degradation	*Arabidopsis*	adult plant	Turgency mantainement	Nanjo et al. (1999)
HVA1	*H. vulgare*	LEA protein	Protein protection	rice	seedling	Growth	Xu et al. (1996)
Nt107	*N. tabacum*	glutathione S-transferase/ glutathione peroxidase	GSSG synthesis	tobacco	seedling	Shoot length	Roxas et al. (1997)
Alfin1	*M. sativa*	Transcription factor	Improve gene expression	alfalfa	adult plant	Root growth	Winicov (2000)
GS	*O. sativa*	Glutamine synthetase	Glutamine synthesis	rice	seedling	Photorespiration capacity	Hoshida et al. (2000)

Table 1. (*continued*)

Name	Source specie	Gene product	Function	Harbourg species	Stage development expressing tolerance	Parameters studied	Reference
HAL1	*S. cereviseae*	K^+/Na^+ transport regulation	K^+/Na^+ homeostasis	tomato melon *Arabidopsis*	seedling shoot apex	Sustain of K^+/Na^+ ratio plant growth.	Bordas et al. (1997) Gisbert et al. (2000) Yang et al. (2001)
HAL3	*S. cereviseae*	FMN-binding protein	K^+/Na^+ homeostasis	*Arabidopsis*	seedling		Albert et al. (2000)
AtNHX1	*A. thaliana*	Vacuolar antiporter Na^+/H^+	Na^+ vacuolar secuestration	tomato *Arabidopsis* *B. napus*	adult plant	Biomass fruit and oil production	Apse et al. (1999) Zhang and Blumwald (2001) Zhang et al. (2001)
GlyI	*B. juncea*	Glyoxylase	S-D-Lactoylglutathione	tobacco	detached leaves	Chlorophyll content of detached leaves	Veena Reddy and Sopory (1999)
DnaK	*A. halophytica*	Heat shock protein	Protein stabilization	tobacco	seedling	CO_2 fixation Na^+ content	Sugino et al. (1999)
MnSOD	*S. cereviseae*	Superoxide dismutase	Reduction of O_2^- content	rice	seedling	Oxidative stress	Tanaka et al. (1999)
Apo-Inv	*S. cereviseae*	Apoplastic yeast-derived invertase	Sucrose synthesis	tobacco	seedling	Photosynthetic activity and osmotic pressure	Fukushima et al. (2001)
OsCDPK7	*O. sativa*	Protein kinase	Improve gene expression	rice	seedling	Wilty phenotype	Saijo et al. (2000)
TPX2	*N. tabaccum*	Peroxidase	Change cell wall proprieties	tobacco	seeds	Germination water retention in seed walls	Amaya et al. (1999)
TSP1	*S. cereviseae*	Trehalose synthase	Trehalose synthesis	tobacco	adult plant	Plant growth	Serrano et al. (1999)
DREB1A	*A. thaliana*	Transcription factor	Improve gene expression	*Arabidopsis*	adult plant	Plant growth and survival rate	Kasuga et al. (1999)
CaN	*S. cereviseae*	Calcineurin	Improve Ca^{+2} signaling	tobacco	seedling	Plant growth	Pardo et al. (1998)

1995). Proline is synthesized from glutamic acid via two intermediates, glutamic-γ-semialdehyde and Δ^1-pyrroline-5-carboxylate (P5C). The pyrroline-5-carboxylate synthase (P5CS) synthesizes the first step in the biosynthesis of this amino acid. In salt-treated *Arabidopsis* seedlings, the induction of *AtP5CS* mRNA synthesis follows an exponential curve (Yoshiba et al., 1995). The ABA-induced transcript accumulation of *AtP5CS* is inhibited in the early phase of ABA induction in the *aba1* mutant an ABA-deficient mutant. This indicates that ABA controls the salt-induced expression of *AtP5CS* in the early transcriptional stage (Strizhov et al., 1997). This salt-induced expression is reduced, but not completely inhibited by the *abi1-1* mutation, and unaffected in the *abi2-1* and *abi3-1* mutants. Since *ABI1* encodes phosphatase 2C proteins (Leung et al., 1994; Meyer et al., 1994; Gosti et al., 1999), it is likely that ABA-dependent protein phosphorilation is important in proline accumulation during salt stress. However, analysis of proline accumulation in the *flacca* mutant (deficient in ABA synthesis) showed that proline

accumulation in response to osmotic stress is not dependent on ABA content. This result indicates that ABA control of the proline accumulation is not direct (Stewart and Voetberg, 1987).

Overaccumulation of proline has been achieved in different plant species by either increasing its synthesis or avoiding its degradation, by manipulation of the *P5CS* and *PDH* (proline dehydrogenase) genes, respectively. Overexpression in transgenic tobacco of a gene encoding a *P5CS* from mungbean plants resulted in the accumulation of proline up to 18-fold over control plants resulting in a enhanced biomass production under salt stress (Kishor et al., 1995). On the other hand, antisense suppression of proline degradation improved salt tolerance (Nanjo et al., 1999). Tolerance to salinity was measured as the capacity to maintain leaves turgor under high salt concentration (600 mM) (Nanjo et al., 1999).

Glycine betaine accumulates in cells of a number of halophytes and bacteria as an adaptive response to high salt. Oxidation of choline to betaine aldehyde is the predominant biosynthetic route in all betaine producers (Rhodes and Hanson, 1993). The first step differs among various organisms according to the type of enzyme. This step is catalyzed by a soluble flavoprotein oxidase (COX EC 1.1.3.17) in some bacteria and fungi, by a soluble ferrodoxin-dependent mooxygenase (CMO) in the chloroplast of higher plants or by a poorly characterized membrane-associated choline dehydrogenase (CDH EC 1.1.99.1) (Huang et al., 2000). Transgenic tobacco plants harbouring the *E. coli betA* gene encoding a coline dehydrogenase were more tolerant to salt conditions than wild type plants, the tolerance was measured as difference in the dry weight (Lilius et al., 1996). The *codA* gene, isolated from the soil bacteria *Arthrobacter globiformis*, encodes choline oxidize. This enzyme converts choline into glycinebetaine. Transformation of *A. thaliana* with *codA* enabled the plant to accumulate glycinebetaine and enhanced its tolerance to salt stress (Hayashi et al., 1997). Seeds of the transgenic plants were able to germinate in 300 mM NaCl whereas seeds of wild type did not germinate at all. In addition, transgenic plants sustained Photosystem II activity under salt stress conditions. Transgenic rice plants carrying the *codA* but with the encoded protein directed to the chloroplast were more tolerant than the transgenic plants with the protein localized in the cytosol. This result suggested that the protective function of glycine betaine is more efficient when produced in a photosynthetic organelle (Sakamoto et al., 1998). An *Arthrobacter pascens* gene encoding a COX enzyme was used to generate transgenic plants in three different species: *Arabidopsis*, *Brassica napus* and tobacco (Huang et al., 2000). Salt tolerance varied among the species and the authors could not assign this difference in tolerance to the different glycine betaine accumulation levels. Another step in the glycinebetaine synthesis is catalyzed by the enzyme betaine dehydrogenase (BADH). Transgenic tobacco plants expressing the *BADH* gene accumulated a higher amount of glycine betaine in cytosol and chloroplasts and exhibited increased tolerance to salt stress (Liang et al., 1997; Holmstrom et al., 2000). These transgenic plants also showed decreased photoinhibition during salt stress and this caused an increase in fresh weight relative to wild type. Similar results were obtained in transgenic tobacco plants over-expressing a *BADH* from spinach instead of a *BADH* prokaryote gene (Sakamoto et al., 1998).

Another molecule that can act as osmoprotectant is the polyalcohol mannitol. Tobacco plants transformed with the *mtlD* gene from *E. coli*, which encodes a mannitol 1-phophate dehydrogenase accumulated mannitol and showed increased plant growth under salt stress (Tarczynski et al., 1993). Later studies revealed that the increase in mannitol content was not enough to explain the tolerance solely based on osmotic adjustment and was assigned to this molecule a possible antioxidant function (Karakas et al., 1997). *Arabidopsis* plants transformed with the same gene were able to germinate in the presence of a concentration of salt inhibitory to wild type plants. However, unlike tobacco *mtlD* transformants, *Arabidopsis* transgenic plants did not tolerate prolonged salt stress (Thomas et al., 1995).

Ectopic expression of a gene encoding myo-inositol *O*-methyltransferase (*IMT1*) in tobacco resulted in the accumulation of methylated inositol (D-ononitol), that conferred higher salt tolerance by increasing the photosynthetic activity (Sheveleva et al., 1997). The accumulation of this compound reached up to 600 mM in the cytosol, thus osmotically balancing the high external Na^+ concentration.

Arabidopsis plants expressing the enzyme that catalyzes the first committed step in inositol biosynthesis, the D-*myo*-inositol-3-phosphate (Ins3P) synthase, exhibit an increased level of free inositol (Smart and Flores, 1997). Despite a slight increase in salt tolerance, the authors could not detect significant differences in the phenotype of transgenic plants when a number of characteristics linked to functions

of inositol and inositol-derived metabolites were analyzed. These results suggest that the engineering of inositol metabolism to generate salt tolerance may require the manipulation of several genes.

Improved salt tolerance of transgenic tobacco was obtained expressing a yeast invertase gene in the apoplast (*Apo-Inv*) (Fukushima et al., 2001). These plants maintained constant photosynthetic activities under salt stress and had much higher osmotic pressure in the cell sap than wild type tobacco.

In plants, presence of the disaccharide trehalose is rare (Ingram and Bartels, 1996) but it is common in yeast. Plants overexpressing enzymes related to trehalose synthesis (*otsA*) showed an increased tolerance to salt (Pilon-Smits et al., 1998). The yeast trehalose-6-phosphate synthetase gene (*TPS1*) introduced in tobacco and melon improved the tolerance to salt stress as measured by plant growth. However, several pleiotropic effects were observed in these transgenic plants suggesting that this molecule affects other plant developmental processes (Serrano et al., 1998).

Genes involved in the protection of cell integrity (lea-like genes)

The *HVA1* gene encoding a LEA protein was found to be induced by ABA and several stresses including salt (Hong et al., 1992). Transgenic rice plants expressing *HVA1*, driven by the constitutive promoter from the rice *actin1* gene, showed significant increased tolerance to salt (Xu et al., 1996). The mechanism involved in the action of this gene is not clear but the authors proposed that the improved tolerance could be due to a stabilization of the cell structure.

The DnaK/Hsp70 family of molecular chaperones can bind non-native states of other proteins and assist them to reach a functional conformation, in most cases through the waste of ATP (Boston et al., 1996). Transgenic tobacco plants transformed with *DnaK1* from the halotolerant cyanobacterium *Aphanothece halophytica* overexpressed the protein in the cytosol (Sugino et al., 1999). After 3 days of treatment with 0.6 M NaCl, the CO_2 fixation rate was markedly improved in transgenic plants. The sodium content in the leaves of control plants increased after salt stress whereas it remained constant in the transgenic plants (Sugino et al., 1999).

The cell wall is a fundamental structure of plant cells whose composition can change dramatically in response to environmental stresses. Because cell wall properties like water permeability and elasticity are involved in the maintenance of cell growth during salt stress (Iraki et al., 1989), cell wall alterations may be crucial to stress tolerance. Overexpression in tobacco of a cell wall peroxidase from tomato (TPX2), significantly increased the germination rate under salt stress (Amaya et al., 1999). The higher capacity of transgenic seeds to retain water could result in a higher germination rate in conditions where the availability of water is restricted (Amaya et al., 1999).

Genes involved in oxidative stress

Salinity generates an increase in reactive oxygen species that can induce deleterious effects on cell metabolism (Polle, 1997; Borsani et al., 2001b). Various groups have developed plants that over-express several oxidative-stress-related genes, with varied results, depending on the tests used to evaluate these transgenic plants (Bajaj et al., 1999). The potential role of superoxide dismutase (SOD) in the protection against salt stress was examined using transgenic rice plants (Tanaka et al., 1999). At high salinity, the transgenic plants had an ascorbate peroxidase activity about 1.5-fold higher than control plants. Total SOD activity was maintained at a high level and ascorbate peroxidase increased upon salt stress. It was found that the PS II activity and the electron transport in the chloroplast were higher in the transgenic plants compared to the wild type plants under salt stress. These results suggest that an increased in the levels of ascorbate peroxidase and chloroplastic SOD are important factors for salt resistance in rice (Tanaka et al., 1999).

Transgenic tobacco seedlings overexpressing glutathione S-transferase (GST) and glutathione peroxidase (GPX) have been generated. Transgenic seedlings had GST- and GPX-specific activities approximately two fold higher than wild-type seedlings. The GSH/GSH+GSSG ratios were 0.68 and 0.45 in the control and transgenic plants, respectively. Despite the increase in the oxidized glutathione pool, transgenic seedlings exhibited increased salt tolerance (Roxas et al., 1997). It has been previously reported that overexpression of glutathione reductase in transgenic plants leads to elevated levels of GSH, increasing tolerance to salt and oxidative stress in leaves (Foyer et al., 1991). Therefore, the influence of the oxidation state of glutathione on the tolerance to salt stress in plants is currently unclear.

An alternative strategy to cope with oxidative damage under salt stress would be to suppress the production of active oxygen species. Although a role for photorespiration in stress conditions is still contro-

versial, it may function as a possible route for the dissipation of the excess of light energy or reducing power (Osmond and Grace, 1995). Several studies suggest that the rate-limiting step in photorespiration is the reassimilation of ammonia catalyzed by chloroplastic glutamine synthetase (Wallsgrove et al., 1987). When rice plants were transformed with a chloroplastic glutamine synthetase (*GS2*) gene from rice (Hoshida et al., 2000). They accumulated about 1.5-fold more GS2 than control plants. These transgenic plants also had an increased photorespiration capacity and enhanced tolerance to salt stress (Hoshida et al., 2000).

The glioxalase system has been proposed to be involved in processes such as protection against α-oxaldehyde cytotoxity, regulation of cell division and proliferation, among others (reviewed in Thornalley, 1990, 1993). Induction of the glyoxalase I gene expression in response to salt and osmotic stress was observed in tomato (Espartero et al., 1995). Transgenic plants overexpressing glyoxalase I showed an increased tolerance to high salt, as measured in a detached leaf disc senescence assay (Veena Reddy and Sopory, 1999).

Genes involved in the regulation of ion homeostasis

An alternative approach to generate plant salt tolerant is the introduction of genes that modulate ion transport systems such as *HAL1* (Gisbert et al., 2000; Yang et al., 2001) and *HAL3* (Albert et al., 2000) from *S. cerevisiae*. Overexpression of *HAL1* in tomato improved salt tolerance by maintaining a high internal K^+ concentration and decreasing intracellular Na^+ during salt stress (Gisbert et al., 2000). The results are similar to those already described when *HAL1* was overexpressed in yeast (Serrano and Glaxiola, 1994) or other plants species such as *Arabidopsis* (Yang et al., 2001) and melon (Bordas et al., 1997). This suggests that the mechanisms controlling the positive effects of the *HAL1* gene on salt tolerance are conserved among plants and yeast. The yeast *HAL3* gene encodes a FMN-binding protein. Albert and co-workers have recently reported that overexpression of *AtHal3*, the *A. thaliana HAL3* gene orthologue, in *Arabidopsis* improves salt tolerance (Albert et al., 2000).

Abundant experimental evidence has shown the involvement of cytosolic Ca^{+2} in salt stress signaling (Lynch et al., 1989). The PP2B phosphatase calcineurin (CaN) is a critical component of a Ca^{+2}-dependent signal transduction pathway that mediates Na^+, Li^+ and Mn^{+2} tolerance in *S. cerevisiae*. A truncated activated form of the catalytic subunit and the regulatory subunit of yeast CaN were co-expressed in transgenic tobacco plants to reconstitute an active phosphatase *in vivo* (Pardo et al., 1998). The enhanced capacity to survive NaCl shock of plants expressing this gene was similar when the evaluation was conducted either on seedling in tissue culture raft vessels or plants in hydroponic cultures (Pardo et al., 1998). The importance of calcium in salt tolerance was also determined by the use of transgenic rice overexpressing the calcium-dependent protein kinase *OsCDPK7*. These plants were able to avoid the wilting phenotype observed in control plants exposed to 200 mM NaCl (Saijo et al., 2000). Overexpression of *OsCDPK7* enhanced the induction of *rab16* and *wsi18* genes that encode group 2 and group 3 LEA related proteins respectively (Saijo et al., 2000).

Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles. Overexpression of a vacuolar Na^+/H^+ antiport from *A. thaliana* (*AtNHX1*) in *Arabidopsis* plants promoted sustained growth and development in soil watered with up to 200 mM NaCl (Apse et al., 1999). Tomato plants overexpressing the same gene were able to grow, flower, and produce fruit in the presence of 200 mM NaCl (Zhang and Blumwald, 2001). Surprisingly, these plants showed high accumulation of Na^+ in leaves but low Na^+ content in the fruit. There is also a recent report on transgenic *Brasssica napus* plants overexpressing the *AtNHX1* gene which were able to grow, flower, and produce seeds in the presence of 200 mM of NaCl (Zhang et al., 2001). The seed production and seed oil quality were reported to be not affected by the soil salt content.

Transcription factors

The transcription factor *DREB1A* (Dehydration Response Element Binding) specifically interacts with the DRE (Dehydration Response Element) box promoter sequences and induces expression of stress tolerance genes with DRE elements in their promoters. The overexpression of *DREB1A* cDNA in *Arabidopsis* plants activated the expression of many of these stress tolerance genes under normal growing conditions (Liu et al., 1998). Transgenic plants with *DREB1A* ectopically expressed under the control of

the CaMV 35S promoter showed morphological abnormalities under unstressed conditions (Liu et al., 1998). In contrast, plants expressing *DREB1A* under the control of the salt inducible *rd29A* promoter looked healthy and exhibited high tolerance to salt stress (Kasuga et al., 1999). These results demonstrate that stress-inducible promoters may be more desirable in order to generate plants that are tolerant to stress. However, this may not be always the case since growth was not adversely affected in transgenic alfalfa plants overexpressing the *Alfin1* transcription factor under the control of the 35S promoter (Winicov, 2000). These plants showed both an enhanced expression of the salt-inducible *MsPRP2* gene in roots and an increase in root growth under salt stress.

Conclusions and future prospects

Transgenic approaches for increasing plant salt tolerance are feasible. So far, results obtained with many genes are encouraging, and recent results obtained in transgenic plants harbouring genes encoding an Na^+/H^+ antiporter or a transcription factor show the possibility of increasing the salt tolerance. However, it still needs to be tested whether or not this is the case for all crops since may diverge in the mechanisms of stress responses. Salt tolerance responses are being investigated in several species through large scale gene expression analyses. But, it should be noted that this is only the beginning. Transformation of agronomic important crops and the identification of uncovered tolerance determinants and stress inducible promoters must be further explored to obtain plants with increased tolerance to salt stress. The use of promoters that direct the expression at the proper time and place will maximize salt tolerance. In addition, it will avoid potential undesiderable pleiotropic effects resulting from the ectopic expression of foreign genes.

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