Chapter 16
Molecular Tools for Enhancing Salinity Tolerance in Plants

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Abstract Salinity is nowadays considered one of the main factors that limit crop productivity and a threat to world's food production. Hence, to breed salt tolerant varieties of crops and horticultural species is necessary to increase or at least maintain food production in order to feed the growing world's population. Plant tolerance to salinity is a complex phenomenon at both cellular and plant level. Since salt causes several types of stresses, plants face salinity using different strategies, whose relative importance depends on the species and the growing conditions. Here we present an overview of the salt tolerance mechanisms to counteract osmotic, ionic and oxidative stress, as well as an update of the knowledge on the processes involved in salt tolerance gained in part through the new genomic approaches. To breed new cultivars able to grow and maintain crop productivity on saline conditions requires variability for some of the traits related to salinity tolerance, the discovery of quantitative trait loci (QTL) regulating those traits, a deep understanding of QTL interaction with other QTL and with the environment, and the transfer of QTL from donors to elite lines using phenotypic and marker assisted selection. We have summarised part of the information related to these four issues and some guidance is given to maximize the efficiency of the selection processes. Genetic transformation has become a powerful tool in plant breeding programs since it allows the introduction of gene(s) controlling traits without affecting the rest of the characteristics of an elite genotype. In this chapter we have reviewed the available information on several topics such as: salt tolerance improvement aided by genetic transformation, functional analysis of genes related salt-tolerance, the complexity of the trait and its evaluation method, the number of genes to be introduced, and the sources of genetic variability. Finally, the use of genomic tools like transcriptomic analysis, post-transcriptional gene silencing, insertional mutagenesis and gene traps, to perform the genetic dissection of this complex trait is discussed.

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16.1 Introduction

Over 6% of the world's total land area (840 million hectares) is affected by salinity or sodicity (FAO 2005). This enormous area has only had small consequences for agriculture because there was enough good land for rain-fed crops: of the 1,500 million hectares dedicated to rain-fed agriculture only 32 millions were affected by secondary salinisation, the salinisation caused by agricultural practices (FAO 2005). This scenario is completely different when irrigated land is considered. The 230 million hectares of irrigated land produce about one third of world's food and over 20% of this productive land is affected by secondary salinisation (FAO 2005) because salts dissolved in the irrigation water are deposited in the soil following evapotranspiration. And the greatest danger comes from the continuously increasing secondary salinisation in irrigated areas. In California for example, where irrigation started about 130 years ago, half of the irrigated area was affected by salinity more than 20 years ago (Lewis 1984). The risk of secondary salinisation is proportional to the amount of salts in the irrigation water. This risk will increase as population increases because cities (including gardens and sport premises) and industry will pay for the best quality water leaving the worst to agriculture.

Salinity is nowadays considered one of the main factors that limit crop productivity and a threat to world's food production. In addition, world's population is expected to increase about 50% by 2050. Hence, it is necessary to sustain agricultural productivity on the increasingly saline irrigated land by salt tolerant cultivars. However, it should be pointed out that salt-tolerant cultivars will only be part of the solution. Salinity in agricultural systems tends to increase rather than remain stable, and may rise in the 8–10 years period that takes to release a new cultivar. For this reason, plant breeding cannot be the full answer to the problem of salinisation but part of an integrated programme that would also include irrigation and drainage management (Flowers and Yeo 1995).

Higher plants do not have a salt-tolerant metabolism even if the plant itself thrives in seawater (Flowers 1972a, b). Salt tolerance in organisms other than the halobacteria do not depend on salt-tolerant proteins, but on keeping a defined micro-environment in the cytoplasm, regulated for the quantity and quality of inorganic ions. Single-celled aquatic organisms or cell cultures can excrete into the medium any dangerous substance. Animals have their cells designed to excrete into the bloodstream and special organs to deal with these toxic compounds. The plant root can only excrete into the solution being drawn towards it by transpiration, but the aboveground cells have nowhere to excrete apart from the small volume of the apoplast. The large central vacuole is the limited place where plant cells can store toxic materials, hence its importance in saline conditions. Glicophytes, where most of the crops are included, are not affected by the external salinity per se, but by growth fall below net ion import leading to ever-increasing internal concentrations and, eventually, to catastrophic failure (Munns and Termaat 1986). Contrarily, in halophytes, growth and net salt uptake are coupled, even if we do not know what controls what (Yeo and Flowers 1986). The targets to produce a

plant tolerant to salinity are ion transport and compartmentalization and the synthesis of compatible solutes (osmolytes or osmoprotectants) to counteract osmotic and ionic stresses, oxidative protection, and the maintenance of ion homeostasis. But plant responses to salinity are even more complex at both the whole plant level and cellular level, and research efforts have been focused on understanding the physiological and molecular basis of salt tolerance in higher plants (Mansour and Salama 2004; Sairam and Tyagi 2004; Bartels and Dunkar 2005; Munns and Tester 2008).

In the first section of this chapter, we will describe the mechanisms developed by plants to cope with salinity and the relative importance of each one of them, which vary not only with the species but also with other factors such as stress level, exposure time to salinity, environmental conditions, etc. Advances in our knowledge of the physiological and molecular basis of salt tolerance so far achieved, with special mention to the emerging progress thanks to the new genomic approaches, are summarised. The achievement of salt tolerant cultivars is reviewed in the second part of the chapter, with special attention to the search for natural variation in phenotypic and physiological characters, the discovery of quantitative trait loci regulating salt tolerance, the interaction of these loci with the environment and other loci, and to design efficient breeding programmes based in marker assisted selection coupled with phenotypic selection. In the next section, the transfer and expression of genes related to salt tolerance into elite cultivars or parents of modern F_1 hybrids through genetic transformation is a very attractive approach because, this way, susceptible but productive cultivars could be transformed into tolerant ones, while maintaining all the valuable traits today's cultivars possess. Here we will review the progress in this field, raise the problems associated with the functional analysis of salt tolerance-related genes and discuss the adequacy of genomic tools to perform the genetic dissection of this complex trait.

16.2 Salt Tolerance Mechanisms at Physiological and Molecular Levels

Salt causes several types of plant stress including osmotic stress and ionic stress due to the accumulation of toxic saline ions, nutritional stress due to the altered nutrient uptake, especially of ions such as K^+ and Ca^{2+}, and oxidative stress. However, the main physiological mechanism responsible of plant growth reduction and how environment and genotype modulate plant salinity response are not yet known.

To understand the physiological mechanisms responsible for salinity tolerance, it is important to bear in mind that plants respond to salinity by using two main tolerance mechanisms: mechanisms of osmotic tolerance to avoid the osmotic effect of the salt outside the roots, which occurs normally at low stress levels and over short periods of salt stress, and mechanisms of ionic tolerance to avoid the

toxic effect of the salt within the plant, the main effect induced by long-term or high stress levels. However, the timescale over which ion-specific damage is manifested depends on the salt sensitivity of the species and the stress level. Thus, the ion-specific effect will start earlier in plants with a low ability to regulate the transport of saline ions to the shoot, or when high salt levels are applied. Although the two phases are generally separated in time for most plants, it is also possible for ion toxicity to take effect during the first phase itself and for osmotic effects to persist in the second phase (De Costa et al. 2007; Muñoz-Mayor et al. 2008).

16.2.1 Plant Response to Osmotic Stress Induced by Salinity

The osmotic effect starts immediately after the imposition of salt stress, before the saline ions are taken up by the roots. The salt concentration around the roots reduces the osmotic potential of the growing medium and thus the plant's ability to take up water and nutrients by the roots, similar to the effect induced by drought. (Munns 2002). The plant needs to accumulate solutes to maintain cell volume and turgor, so the response to turgor reduction is osmotic adjustment, which is a major component of salt stress tolerance. Osmotic potential may be reduced either by the simple effect of solute concentration due to reduced water uptake by the plant or by the active solute accumulation. Osmotic adjustment is defined as the lowering of osmotic potential in plant tissue due to net accumulation of solute (Blum et al. 1996).

The main solutes contributing to osmotic adjustment are the inorganic solutes, which are taken up from the substrate and transported to the shoot, and the organic solutes, which are synthesised by the plant. Na^+ and Cl^- are energetically efficient osmolytes for osmotic adjustment, but must be compartmentalised into the vacuole to minimise cytotoxicity. Within the cytoplasm, osmotic adjustment is achieved by accumulation of compatible osmolytes. Some compatible osmolytes are essential elemental ions, such as K^+, but the majority of these are organic solutes, especially sugars (mainly fructose and glucose) and organic acids. Other osmolytes are sugar alcohols (glycerol and methylated inositols), complex sugars (trehalose, raffinose and fructans), quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines and sulfonium compounds (Zhu 2001). Another function of compatible osmolytes that may occur at lower concentrations is osmoprotection, which includes protection of thylakoid and plasma membrane integrity, stabilising proteins, a sink for energy or reducing power, a source of carbon and nitrogen for recovery, or scavenging of reactive oxygen species generated by salt stress (Bartels and Dunkar 2005). These beneficial impacts have been shown by overexpression of different genes involved in osmolyte accumulation (Ge et al. 2008; Yang et al. 2008). However, consensus has not been reached on the effectiveness of accumulation of osmolytes by genetic modification, as occurs in the case of proline. Therefore, further studies are required to give an insight into the role of the osmolytes and their effective utilisation for stress tolerance.

The use of organic solutes for osmotic adjustment is energetically much more expensive than the use of the saline ions proceeding from the substrate (Yeo 1983). Thus, the ATP requirement for the synthesis or accumulation of solutes in leaves was reported by Ravens (1985) as 3.5 for Na^+, 34 for mannitol, 41 for proline, 50 for glycine betaine and about 52 for sucrose. In this respect, salt tolerance may not always be associated with low Na^+ concentration in the leaves. While the more tolerant genotypes of many species are those better able to prevent excessive ion accumulation, the leaves of halophytes do contain high salt concentrations (Santa-Cruz et al. 1999), which are necessary to adjust the leaf water relations to low external potentials and plants use the cheapest solutes from an energetic point of view (Neumann 1997). Such a situation also seems to occur in species that are not very sensitive to salt, such as tomato grown at low-mid salinity levels, as the higher the fruit yield, the higher the contribution of inorganic solutes, including the saline ions, to the osmotic potential (Alarcon et al. 1993; Estañ et al. 2005). Thus, breeding for Na^+ accumulation, rather than exclusion, could be a more effective strategy for improving salt tolerance of conventional crop plants.

16.2.2 Plant Response to Ionic Stress Induced by Salinity

Since NaCl is the major component of most saline soils, our usage of the terms salinity and salt stress here refers to stress caused by NaCl. The ionic stress starts generally later than osmotic stress, when the toxic saline ions are transported to the shoot and build up to toxic levels within the leaves. Thus, roots must exclude most of the Na^+ and Cl^- dissolved in the growing medium, or the salt in the shoot will gradually build up with time to toxic levels. The cause of injury is probably salt load exceeding the ability of the cells to compartmentalise salts in the vacuole. Salts would then build up rapidly in the cytoplasm and inhibit enzyme activity (Yokoi et al. 2002). Alternatively, they might build up in the cell walls and dehydrate the cell (Flowers et al. 1991). The rate of cell death is crucial for the survival of the plant. If new leaves are continually produced at a rate greater than that at which old leaves die, there will be enough photosynthesizing leaves on the plant to produce flowers and seeds, although reduced in number. However, if old leaves die more quickly than new ones develop, the plant may not survive.

In most studies on salinity it has not been possible to determine whether the toxic effects observed are due to Na^+, Cl^- or to a contribution of the two. In only a few species such as citrus (Moya et al. 2003) has there been conclusive evidence of greater sensitivity to Cl^- than to Na^+. Consequently, Na^+ is considered the primary cause of ion-specific damage for many plants (Tester and Davenport 2003). However, similar relationships between fruit yield and leaf ionic concentrations for Na^+ and Cl^- were observed in tomato, which suggests that the toxic effects are due, at least in the long term, to the contribution of both ions (Estañ et al. 2005).

Salt tolerance of cultivated species is generally correlated to an efficient Na^+ and Cl^- exclusion mechanism and better maintenance of leaf K^+ concentration at

high external NaCl (Gorham et al. 1990; Matsushita and Matoh 1991). However, the higher salt tolerance of wild tomato species over cultivated forms has generally been associated with the halophytic character of Na^+ accumulation in the wild relatives (Cuartero and Fernandez-Muñoz 1999). Tester and Davenport (2003) suggested that although halophytes accumulate Na^+ in the shoot, it is unlikely that halophytic species have higher rates of Na^+ transport at high salinities or over the long term than salt-sensitive species. Studies of halophytes at low salinities tend to obscure the true situation, because many halophytes show growth stimulation upon addition of NaCl to a growth medium when NaCl is rapidly accumulated and employed preferentially as an osmoticum (Alarcon et al. 1993; Glenn et al. 1999). Several recent reviews of this area of study have been carried out (Tester and Davenport 2003; Apse and Blumwald 2007; Munns and Tester 2008), and these describe in detail the transport of Na^+ from the growing medium into the roots, the Na^+ loading into and unloading from the xylem, and its redistribution within the plant.

16.2.3 Plant Response to Oxidative Stress Induced by Salinity

In addition to its known components of osmotic stress and ion toxicity, salt stress is also manifested as an oxidative stress, all of which contribute to its deleterious effects (Hernandez et al. 2000; Mittova et al. 2002). Oxidative stress is characterised by the overproduction of reactive oxygen species (ROS) represented predominantly by superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen. Plants have defensive mechanisms and utilise several biochemical strategies to avoid damage caused by ROS. Plant enzymatic defences include antioxidant enzymes such as the phenol peroxidase, ascorbate peroxidase, glutathione peroxidase, superoxide dismutase, and catalase which, together with other enzymes of the ascorbate-glutathione cycle, promote the scavenging of ROS (Hernandez et al. 2001; Hong et al. 2007). The biochemical defence system also includes carotenoids, ascorbate, glutathione and tocopherols. Several authors have suggested that the function of sugars, poliols, glycine-betaine and proline could be to protect cells against the hydroxyl radical (Sickler et al. 2007).

The sources of ROS under stress, mechanisms of ROS detoxification and the role of ROS in stress signalling are all active areas of current research and have been extensively studied and reviewed (Ben Amor et al. 2007). However, more studies are necessary before a definitive conclusion can be reached about the role of the ROS production under stress. Thus, increased ROS production has long been known under the heading of ‘oxidative stress’, which in itself is a negative term implying a harmful process. In contrast to this negative term, implying a state to be avoided, Foyer and Noctor (2005) proposed that the syndrome would be more usefully described as ‘oxidative signalling’, that is, an important and critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology.

16.2.4 Homeostasis and Protection or Damage Repair Induced by Salinity

Responses to salt stress are often discussed in terms of homeostasis and protection or damage repair (Zhu 2001). Mechanisms of ion homeostasis and osmotic homeostasis attempt to restore the cellular ion or water content to levels similar to those present under unstressed conditions. Protection and damage repair mechanisms attempt to prevent or repair cellular damage caused by altered ion or water content under stress. To understand the molecular mechanisms that plants have developed to cope with salinity, key genes for salt stress should be identified. Present engineering strategies for enhanced salt tolerance rely on the transfer of one or several genes either involved in signalling and regulatory pathways or encoding enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, as is indicated in the next sections. Osmotic homeostasis probably depends on the action of genes for solute synthesis, and a number of channels and carriers for uptake and compartmentalisation of inorganic solutes, especially K^+ (Rodriguez-Navarro 2000). Aquaporins may also have a role in osmotic homeostasis by facilitating water movement, but despite some progress achieved recently, not much is currently known about the levels of physiological relevant aquaporin function and regulation, and its effects on plant water balance (Kaldenhoff et al. 2008). Because of the most important effect induced by salinity to long-term is the toxic effect induced by the transport of saline ions to the shoot, most approaches have been directed to studying cation transporters and their regulation, especially the Na^+ transporters genes, like *SOS1* (Shi et al. 2002), *AtHKT1* (Rus et al. 2004) and *AtNHX1* (Apse and Blumwald 2002), whereas the Cl^- transport mechanisms in plants have been rarely studied (Colmenero-Flores et al. 2007).

In spite of the important work carried out on the Na^+ transport mechanism in plants in recent years, more advances are necessary to understand the role and regulation of genes involved in the re-establishment of Na^+ homeostasis under salt stress (Maathuis 2006; Pardo et al. 2006; Apse and Blumwald 2007; Munns and Tester 2008). Moreover, these genes should be identified not only in model species like *Arabidopsis* but also in crop species, since their role may be different depending on the species, as seems to occur in the *HKT1* genes. Recent studies on the role of *AtHKT1* in Na^+ transport have shown that this gene appears to control retrieval of Na^+ from the xylem before it reaches the shoot (Davenport et al. 2007). However, the *HKT* gene family is quite diverse, and this diversity led to early reports of apparently contradictory properties (see '*AtHKT1;1*, A case study of confusion', in Munns and Tester 2008). Increased clarity has been provided by dividing the *HKT* gene family into two distinct subfamilies (Platten et al. 2006). Recently, Nagata et al. (2008) did a comparative molecular-biological analysis of membrane transport genes in different organisms, ranging from bacteria to animals and plants. They compared the numbers of membrane transporter genes in *Arabidopsis* and rice. Although many transporter genes are similar in these plants, *Arabidopsis* has a more diverse array of genes for multi-efflux transport and for

response to stress signals, and rice has more secondary transporter genes for carbohydrate and nutrient transport.

Another of the genes controlling long-distance transport in *Arabidopsis thaliana* is the *SOS1* gene, which encodes a plasma membrane Na^+/H^+ antiporter that is essential for salt tolerance (Shi et al. 2003). Key intermediaries in the regulation of SOS1 are the protein kinase SOS2 and its associated Ca^{2+}-sensor protein SOS3 (Pardo et al. 2006). Co-expression of the three proteins in a yeast strain lacking endogenous Na^+ transporters restored salt tolerance to a much greater extent than SOS1 alone, whereas SOS2 or SOS3 individually failed to stimulate SOS1 activity (Quintero et al. 2002). Despite these advances, the function of *SOS1* in plants needs to be clarified, as this transporter seems to function in retrieving Na^+ from the xylem to prevent excess Na^+ accumulation in the shoot under severe salt stress (Shi et al. 2003). On the contrary, *SOS1* functions to load Na^+ into the xylem for controlled delivery to the shoot when salinity is moderate (Shi et al. 2002).

Na^+ has to be compartmentalised in the vacuole to prevent excess Na^+ accumulation in the cytoplasm. *Arabidopsis* AtNHX1 protein was the first Na^+/H^+ exchanger identified in plants (Gaxiola et al. 1999). The presence of Na^+/H^+ antiporter activities has been physiologically characterised in tonoplast vesicles and it is molecularly represented by six *Arabidopsis* genes, *AtNHX1–6* (Yokoi et al. 2002). *AtNHX1* steady-state transcript levels were increased in response to NaCl, KCl, sorbitol, and ABA, suggesting that *AtNHX1* transcript upregulation is not specific to ionic stress but is common to osmotic stress (Gaxiola et al. 1999; Shi and Zhu 2002). Overexpression of *NHX* antiporters has been used to improve salt tolerance in several plant species (Zhang and Blumwald 2001; Wu et al. 2004). Pardo et al. (2006) summarised the important work carried out in transformed plants with *NHX* antiporters and concluded that relevant information is still missing about the way *NHX* expression affects ion compartmentalisation within the cell, overall ion homeostasis, and osmotic adjustment, in order to fulfil the physiological premises basis that sustain the increase in salt tolerance.

With respect to the Na^+ pumps, there are no classical pumps in higher plants. In fungi and mosses there is a Na^+ pump, *ENA1*, which hydrolyses ATP to pump Na^+ out of the cell (Benito and Rodriguez-Navarro 2003). Also in yeast are the *HAL* genes (Serrano et al. 1999). Overexpression of *HAL1* conferred salt tolerance in yeast by facilitating intracellular K^+ accumulation and decreasing intracellular Na^+ (Rios et al. 1997). According to Munns (2005), the expression of these genes, which do not have orthologues in higher plants, may introduce new mechanisms for Na^+ and/or K^+ homeostasis. *HAL1* was introduced into tomato and its overexpression led to higher salt tolerance in the progeny of different transgenic plants, and furthermore, a similar mechanism to that in yeast was observed, namely by facilitating K^+/Na^+ selectivity under salt stress (Gisbert et al. 2000; Rus et al. 2001). Afterwards, a transgenic line with a very high gene expression level was selected in order to corroborate the tolerance induced by *HAL1* in tomato. However, the fruit yield of the homozygous plants was lower than that of the azygous plants, in spite of the lower Na^+ uptake and Na^+ translocation to the shoot that persisted over time in the homozygous line (Muñoz-Mayor et al. 2008).

Moreover, the ability of *HAL1* to reduce Na^+ uptake in the long-term was shown by the low accumulation of Na^+ in fruits. In-depth physiological characterisation of these plants showed that the greater ability for Na^+ exclusion in the homozygous line caused another type of osmotic problem, as leaves required increased synthesis of organic solutes to maintain osmotic balance, which has a high energy cost (Balibrea et al. 2003), and hence a growth penalty that negatively impacted on fruit yield. These results demonstrate the importance of considering the osmotic component of salt stress, especially when overexpression is used as a tool to identify genes involved in salt tolerance. It may be concluded that the constitutive expression of a gene may induce improvements in a trait, e.g. Na^+ exclusion, but this positive effect may provoke other physiological problems in the plant. In this sense, studies with *AtNHX1*-overexpressing tomato showed greater K^+ uptake than control lines but nevertheless were prone to K^+ deficiency symptoms at leaf K^+ concentrations greater than the control line (Leidi et al. 2005). According to Pardo et al. (2006), this paradoxical phenotype is likely to be due to exacerbated activity of the *AtNHX1* antiporter in the transgenic lines which could increase the vacuolar pool at the expense of the cytoplasmic K^+ pool, thereby inducing a K^+ starvation signal and eliciting greater K^+ uptake by roots. Thus, proper modulation of gene expression in time and space may be more important than mere overexpression of the transgene (Tonsor et al. 2005), as is pointed out below.

Taken together, tolerance to salinity stress is a complex phenomenon at both the cellular and the whole plant level, and a considerable gap still exists between the knowledge gained by physiological and molecular studies in response to salt stress and the knowledge required to develop crop plants with enhanced tolerance to field saline conditions. A focus on comprehensive physiological, molecular and metabolic aspects of salt stress in crop plants is needed to advance in the knowledge of the salt tolerance basis and to facilitate the development of crop plants with enhanced stress tolerance. New tools for functional genomics emerged in recent years may enhance significantly the descriptive power of physiological analysis (Sanchez et al. 2008; Weckwertha 2008), although it is absolutely necessary to elucidate not only processes involved in ionic stress tolerance and compatible solute synthesis, but also other processes as mechanisms of osmotic tolerance, which remain unknown (Munns and Tester 2008). Furthermore, to benefit more from the new genomic approaches, molecular studies with plants grown in physiologically realistic conditions are needed. Finally, it should be mentioned that differences in salt tolerance mechanisms between salt-sensitive glycophytes and salt-tolerant halophytes may result from changes in regulation of the same basic set of genes involved in salt tolerance. Many genes encoding potential salt tolerance determinants have been identified in model plant species, but a comparative study of the expression of those genes in both halophytes and glycophytes has been hampered by the lack of genetic and molecular tools available for the halophytic species. An important step forward has been provided by the use of *Arabidopsis* molecular and genetic tools to characterise the halophyte *T. halophila*, which is allowing have shown that the two species exhibit both shared and

divergent responses to salt stress (Gong et al. 2005a; Kant et al. 2006). Currently, we are trying to identify genes involved in salt tolerance in the wild salt-tolerant tomato species *S. pennellii*, using insertional mutagenesis as a genetic tool, as discussed in next sections.

16.3 Breeding for Salinity Tolerance

The first question that arises when breeding for salinity tolerance is the possibility to develop genotypes tolerant to salinity. Natural evolution has shaped flowering plant species and ecotypes to live under saline conditions, so that there is some compatibility between plant life and saline conditions and, therefore, it should be possible to artificially reproduce this process. However, the term 'tolerant genotype' depends upon the context. In natural environments a tolerant genotype is one that competes in the ecosystem and produce offspring. In an agricultural setting a tolerant genotype has to produce an economic yield (Yeo 2007). Breeders try to obtain genotypes not only able to live under saline conditions (biological tolerance) but also to grow and maintain agricultural productivity (agricultural tolerance) with the aid of appropriate cultivation methods. The success achieved in producing salt-tolerant varieties of crops has, however, been very limited. About 29 cultivars in only 12 species had been released for their salt tolerance until year 2000 (Flowers et al. 2000) and there has been little advance since then.

Obtaining salt-tolerant cultivars comprises three steps: (1) the presence of genetic variability for salinity tolerance in the species to be bred, in species that can be crossed with the target species, or even in organisms genetically far from the target species (2) to find out the gene or genes underlying the tolerance, and (3) the transmission of the gene(s) responsible of the tolerance to the cultivars.

16.3.1 Variability in Salinity Tolerance

All the crops where genetic variability for salt tolerance has been investigated have shown some degree of tolerance, either in the cultivated species or in closely related species that can be crossed with the cultivated one. Examples of species showing intra-specific variability are sorghum (Igartua and Garcia 1999), strawberry clover (Rumbaugh et al. 1993), rice (Alia et al. 2006), cotton (Ashraf 2002), lentil (Ashraf and Waheed 1990), etc. Inter-specific variability among genetically related species has been demonstrated in crops as different as tomato (Bolarin et al. 1991), durum wheat (Munns et al. 2000), eucalyptus (Niknam and McComb 2000) and many others.

Tolerance to salinity in higher plants affects numerous plant processes at all levels of organization (ion transport, osmotic adjustment, ion selectivity, nutrition, compartmentation, growth, water use, water use efficiency, etc.). Variability for osmotic adjustment has been demonstrated in wheat (Morgan 1992) barley (Blum 1989), rice

(Lilley and Ludlow 1996), sorghum (Basnayake et al. 1993), maize (Bolaños et al. 1993), tomato (Borsani et al. 2002) etc.; for Na^+ transport, in rice (Yeo 1992) and tomato (Cuartero et al. 2002). Cuartero et al. (2002) also demonstrated phenotypic variability in tomato for the relation [Na^+]/leaf area reduction, for the relation [K^+]/[Na^+] in leaf, and for selective Na^+ accumulation in old leaves.

It seems that the necessary variability for starting a breeding program is not hindering the development of salt tolerant cultivars in most crops. However "tolerance to salinity" is a very inaccurate term because it depends on the stage of plant development and where the tolerance has been measured. The easiest stage of development for determining tolerance to salinity is germination because experiments can be performed in the lab under controlled environmental conditions and with a high number of genotypes at the same time. But tolerance to salinity at germination stage has proved without relation to tolerance at adult stage in sorghum (Krishnamurthy et al. 2007), lentil (Ashraf and Waheed 1990), tomato (Foolad and Lin 1997), and rice (Moradi et al. 2003). Lack of relation between tolerance in vegetative stages and harvest has also been demonstrated (Greenway and Munns 1980; Shannon et al. 1987; Caro et al. 1991). Tolerance to salinity in one stage of development seems quite independent from tolerance to salinity in other stages what complicates indirect selection and comparison of results coming from different experiments and researchers.

Quantification of salt-tolerance is also difficult in the field, because (1) the stress may be experimentally uncontrollable because of variable rainfall, (2) the notoriously high field heterogeneity for salinity is liable to confound any planting plan designed for field experiments, and (3) salt uptake and sensitivity are modulated by environmental conditions that may affect each variety differently: any parameter which affects the transpiration rate (such as light intensity, temperature and humidity), can change dramatically a plant's susceptibility to salinity (Yeo et al. 1990). To avoid field problems measuring tolerance, plants are usually grown on inert substrates supplemented with a nutrient solution salinized with NaCl or a known mixture of salts enriched in NaCl.

Salt concentrations at which plants are grown to measure tolerance to salinity deserve also attention. The degree of salt sensitivity of genotypes at the germination stage is generally maintained when different salt concentrations are tested (Cuartero and Fernandez-Muñoz 1999; Foolad 2004), but this behaviour is not general and Cruz (1990) points out that the most tolerant tomato cultivars at 5–7 dS m^{-1} do not correspond with the most tolerant ones at 13 dS m^{-1}. It is necessary to define salinity conditions on the field and selection and experiments should be performed at those established salinity conditions (Cuartero et al. 2008).

16.3.2 Determinants Underlying Salinity Tolerance

It is known that most of the processes empirically determined to be important in plant tolerance to salinity exhibit quantitative inheritance (they show continuous variation) and a high degree of environmental influence (Cuartero and Fernandez-Muñoz 1999; Zhang et al. 1999; Mikiko et al. 2001).

Direct selection under field conditions for quantitative traits is difficult because fluctuating environmental factors adversely affect the accuracy and repeatability of such traits. Indirect selection for markers linked to quantitative traits has been suggested and put into practice for some time (Sax 1923; Falconer 1981). For an indirect selection marker to be useful in a breeding program, it has to exhibit both a significant genetic correlation with the trait and higher heritability than the trait itself (Falconer 1981). Molecular (DNA) and biochemical markers closely linked to quantitative trait loci (QTLs) affecting salt tolerance are good candidates because their expression are almost independent from the environment, and powerful biometric methods have been developed and applied to QTL mapping.

The main goal of QTL detection is marker-assisted selection (MAS) and QTL cloning (Asins 2002). The success in both of them depends on our ability to locate the QTL in chromosome regions as narrow as possible.

Salinity affects most of the biological processes in plants; hence tolerance to salinity is determined by many components. Some of them have been reviewed in the first part of this chapter, that is: accumulation of solutes (inorganic and organic) to maintain cell volume and turgor to counteract osmotic stress; ion exclusion from the root and ion compartmentalization to fight against ionic stress; preferential transport of water, K^+ and Ca^{2+} to restore nutritional status; and over-production of antioxidant enzymes and antioxidant compounds to avoid oxidative stress. All of those characters are quantitatively inherited and will require the detection of the QTL governing their expression.

The basis of QTL detection is the identification of significant statistical association between phenotypes and specific genetic markers, established by the joint analysis of segregation of markers and phenotypes in individuals or lines. The most extensively segregating generation used has been the F_2 because it can be rapidly obtained. Additive and dominant effects can be properly detected in F2 generations, but F_2 have two important drawbacks: the genotypes cannot be replicated and evaluated several times and in several environments, and epistatic interactions are frequently undetected. According to Asins (2002) recombinant inbred lines (RIL) or double haploids (DH) can be reproduced independently and continuously evaluated with respect to additional quantitative traits and markers, with all the information being cumulative. Additive and epistatic interaction can be determined with RIL and DH but not dominant or over-dominance effects.

When dealing with a complex character as tolerance to salinity that depends on a number of quantitative traits, QTL analysis allows an integrative approach. Total phenotypic variation can be explained in three main ways: the identification of QTLs partially controlling the tolerance by analysing multiple traits in the same segregating population; the contribution of QTL × Environment (E) interaction; and the contribution of QTL × QTL interactions or epistastatic effects. Such analyses under different salinity levels and plant stage(s) can only be properly and efficiently carried out using populations of DHs or RILs (Cuartero et al. 2006).

16.3.2.1 QTL × E Interaction

Since salt concentration in the soil is highly variable, it is frequent to test plant tolerance to salinity in several salt concentrations applied to the root system. Genotype × salt treatment interaction has been found in several occasions and species (Bolarin et al. 1991; Asins et al. 1993; Igartua 1995; Lee et al. 2004). It is then not surprising that QTL also show interaction with the environment, which means that expression of particular chromosome regions differs across environments and markers identified would be significant in only one or some of the environments. However, genotype × salt interactions are detected with ANOVA in the numerous experiments including several genotypes and two or more environmental conditions, but to detect QTL × E interaction it is necessary to study segregant populations with several replicates of each genotype in two or more environments, and unfortunately this kind of studies are rare. In addition F_2 population, the most frequent segregant population analyzed, is a poor plant material to detect QTL × E interactions and studies with RIL or DH populations in two or more environments are uncommon.

When appropriate segregant populations (RIL or DH) are grown in at least two salinity conditions (control and saline), more QTL have been identified in saline than in control conditions, and significant QTL × E interaction has been found in all the experiments designed to detect the interaction. Monforte et al. (1997) found only 6 QTL with expression in saline and control conditions, while 9 where expressed in the control and 18 specific for saline conditions. Takehisa et al. (2004) described 37 and 20 QTL in the fields irrigated with saline and fresh water respectively. Manneh et al. (2007) reported 8 markers expressed in control and saline conditions while 28 where expressed in only one of the conditions tested. Villalta et al. (2008) detected 4 QTL in control and 20 in saline conditions in one population, and 11 in saline, 2 in control and 2 in saline and control conditions in another population. Those examples have used only two experimental environments: control and saline conditions. It is necessary to asses that QTL detected under saline condition are expressed in different salt concentrations, otherwise QTL should be found for each specific salt concentration on which tolerant genotypes were to be grown.

QTL × E interaction, as genotype × environment interaction, has an evolutionary base because, according to Asins (2002), this sensitivity to the environment results in phenotypic plasticity (the possibility to take alternative development fates depending on environment) which is likely to be of particular importance in plants because they cannot move from one environment to another. But, from the viewpoint of the breeder, QTL × E interaction complicates the use of QTL in MAS for tolerance to salinity because salinity in the soils is not constant but variable, spatially in the field and also temporarily due to rain and irrigation variation from one year to another. To deal with this unpredictable situation breeders have to manage as many QTL related to tolerance to salinity as possible in order to breed salt tolerant genotypes. Some authors see the QTL × E interaction as lack of consistency of

QTL effects in different environments and suggest using only QTL expressed in most of the environments in MAS programmes. However, as mentioned before, there are many more QTL related to tolerance to salinity expressed only under saline conditions and, according to their importance, they are key QTL to breed salt tolerant genotypes.

The number of QTL related to some characters important in salt tolerance is high: 33 in the case of Monforte et al. (1997), 57 detected by Takehisa et al. (2004), 38 by Manneh et al. (2007), and 17 or 21, depending on the population considered, by Villalta et al. (2008). This number will probably increase when more characters and environmental conditions (salt concentrations) are considered, making very difficult to handle all of them in a MAS programme. Fortunately some QTL associations have been found when they have been mapped (Villalta et al. 2008) which will assist in the introduction of those QTL clusters in elite lines.

16.3.2.2 QTL × QTL Interaction

QTL detection experiments are notoriously poor at detecting interactions between loci (Frankel and Schork 1996). For this reason, we do not have a good idea as to how common such epistatic effects are, but when experiments have been designed to test specifically for their presence, these interactions have been found (Flint and Mott 2001). The molecular dissection of variation in bristle number in *Drosophila* indicated that the combination of two QTL had much larger effect than predicted from their individual effects (Gurganus et al. 1999). Epistasis has also been documented in plants. Lark et al. (1995) demonstrated epistasis in soybean, Eshed and Zamir (1996) in tomato, Lukens and Doebley (1999) in maize, Maheswaran et al. (2000) in rice, etc. The detection of epistasis seems to be independent of the species and the lack of information about QTL × QTL interaction could be explained by the plant material employed in the experiments. According to Asins (2002), epistasis between QTL can hardly be detected in advanced backcrosses or F_2 populations. The reason is that every backcross generation reduces the number of gene combinations while increasing genes from the recurrent genotype and in F_2 populations, even if large, there are few individuals with two-locus double homozygotes. RIL or DH populations are, definitely, the appropriate plant material to detect epistasis.

MAS will lose efficiency if QTL × QTL interactions do exist and are ignored in the selection process because they have not been detected and quantified.

16.3.3 Transmission of Determinants Responsible of Salt Tolerance

The traits governing tolerance to salinity are quantitatively inherited with low heritability (Cuartero et al. 2006). In addition, these traits are difficult to measure in segregating populations, requiring meticulous control of environmental variables

and replication of progenies over locations and seasons. MAS is an attractive approach when a few QTL control a significant portion of the variability for the traits under selection (Zhang et al. 1999).

Translating results from gene or QTL discovery to farm applications requires an agronomic approach rather than a purely academic perspective. We need not only an understanding of the genetic mechanisms underlying a particular trait but also a keen sense of the target environments and the appropriate germplasm to use as vehicles to deliver the traits (Leung 2008). It is important to note that cultivars bred for salt tolerance have to be not only salt tolerant, but also achieve the same desirable traits of productivity, quality, resistance to diseases, and adaptation to cultural techniques the current cultivars have. Elite lines, cultivars or parents of today's hybrids would be the ideal candidates to be improved for tolerance to salinity by introducing the tolerant QTL from the donors.

Today cultivars are the result of many years of selection and incorporation of traits demanded by consumers and growers. The delicate equilibrium among productivity, quality, resistance to diseases, and other agronomic traits showed by those cultivars could be probably altered with the introduction of a substantial number of QTL as those required for tolerance to salinity. Even with the help of codominant molecular markers tightly linked to the QTL to be transmitted, MAS programmes will need the phenotypic selection of the breeder generation after generation. Phenotypic selection can only be properly practised in generations approaching homocigosity slowly. Accordingly, MAS programmes should be designed to slowly approach the recurrent parent genotype. Cuartero and Fernández-Muñoz (1999) described an example of such a programme. A partial solution would be to breed rootstocks tolerant to salinity, grafting onto them the shoot of today cultivars. Martinez-Rodriguez et al. (2008) have recently demonstrated the beneficial effects of some rootstocks chosen because of their tolerance to salinity on tomato yield irrigated with saline water.

Despite innovations like better marker systems and improved genetic mapping strategies, the success of MAS has been very limited even taking into account that tolerance of the breeding lines is not expected to be as high as that of the tolerant donors. Manneh et al. (2007) pointed out the reliability of MAS to identify superior yielding rice genotypes under stress in the field. Several rice lines have been bred and released in the Philippines, Bangladesh, and India demonstrating significant yield advantages over salt-sensitive varieties (Ismail et al. 2007). Those two examples of success in breeding for salinity tolerance have been performed with rice. Rice has two important advantages over other species, especially dicotyledonous species: (1) tolerance to salinity is controlled by a few QTL with large effects, and (2) it is especially sensitive to salinity only during early seedling and reproduction stages (Leung 2008). The partial success on breeding salt tolerant rice varieties, a feasible plant model, should encourage the work with other species.

Salinity increases steadily in agricultural soils because of the irrigation water employed and because inadequate irrigation practices. If a soil salinity target has been fixed at the beginning of a breeding programme, it can substantially increase in the about 10 year's period needed to obtain a new cultivar with tolerance to

salinity. So, breeding salinity tolerance cultivars cannot be alone a long term solution to grow in saline soils but it must be complemented by other cultural techniques to stabilize the soil salinity level (Cuartero et al. 2008).

16.4 Improving Salt Tolerance Trough Gene Transformation

Despite great efforts to increase the level of salinity tolerance in species of agronomic interest, the results obtained through conventional breeding methods, as well as by some biotechnological approaches (e.g. *in vitro* selection) have been rather scarce (Flowers 2004; Yamaguchi and Blumwald 2005; Ashraf et al. 2008). The difficulty in obtaining practical results through these and other approaches explains the expectations generated to obtain salt tolerant cultivars via genetic transformation.

In numerous papers published from early 1990s until the present time, several authors have claimed enhancement of salt tolerance through either overexpression of endogenous genes or, more frequently, heterologous expression of genes that supposedly act on different mechanisms involved in the process (Cuartero et al. 2006).

Genes that have proven somewhat effective in providing stress tolerance using a transgenic approach belong to different categories. Preliminary research in this field was mainly focused on the overproduction of metabolically compatible (organic) solutes in transgenic plants (Chen and Murata 2002; Penna 2003; Kavi Kishor et al. 2005). More recently, attention has been paid to the modification of the glycine betaine biosynthesis pathway by using genes isolated from different sources (Su et al. 2006; Shirasawa et al. 2006; Zhou et al. 2007; Waditee et al. 2007; Park et al. 2007), most probably because the quaternary ammonium compound glycine betaine is accumulated in numerous halophytes from several families (Rhodes and Hanson 1993; Flowers and Colmer 2008). In this respect, it has been shown that the accumulation of glycine betaine in genetically modified plants of tomato is more effective in the chloroplasts than in the cytosol (Park et al. 2007), in a similar way to that previously observed in rice (Sakamoto et al. 1998). Notably, the accumulation of glycine betaine in transplastomic plants of carrot led to high levels of salt tolerance (up to 400 mM NaCl; Kumar et al. 2004).

Another strategy to increase the level of salt tolerance has been the transfer of genes codifying different kinds of proteins functionally related to macromolecules protection (LEA proteins, osmotin, chaperons, mRNA binding proteins; Wang et al. 2003; Zhang et al. 2007) or the protection of key metabolic enzymes (Arrillaga et al. 1998).

The scavenging of reactive oxygen intermediates through the transfer and expression of genes encoding detoxification enzymes (e.g. glutathione S-transferase, superoxide dismutase, ascorbate peroxidase, catalase) is an alternative way to protect cells against oxidative stress, thus limiting the damage produced by salt treatments (Apel and Hirt 2004). Interestingly the co-expression of more than one gene involved in oxidative stress protection in both the chloroplasts and cytosol gave rise to plants with increased tolerance to different types of abiotic stress (Zhao and Zang 2006; Tseng et al. 2007; Lee et al. 2007).

Genetic manipulation with genes encoding membrane proteins involved in the uptake and transport of water and ions, such as water channel proteins and ion transporters, is an alternative approach (Yamaguchi and Blumwald 2005; Chinnusamy et al. 2005; Forrest and Bhave 2007). As ion transport across the tonoplast into vacuoles is energised by a proton moving force (Gaxiola et al. 2007), the strategy based on the use of antiporters has generated high expectations in recent years. By overexpressing the vacuolar Na^+/H^+ antiport from *Arabidopsis thaliana* (*AtNHX1*), a high level of salt tolerance was reported in genetically modified plants of arabidopsis (Apse et al. 1999), tomato (Zhang and Blumwald 2001) and canola (Zhang et al. 2001). Thereafter, the *AtNHX1* gene was overexpressed in genetically modified plants of wheat (Xue et al. 2004), corn (Yin et al. 2004), beet (Yang et al. 2005), cotton (He et al. 2005) and tall fescue (Tian et al. 2006; Zhao et al. 2007), and in all the cases the authors indicated enhancement of salt tolerance. The greater tolerance conferred by the *AtNHX1* gene has been attributed to a process of Na^+ compartmentalisation into the vacuoles (Yamaguchi and Blumwald 2005). Despite being an attractive hypothesis, additional compelling evidence is needed before drawing a definitive conclusion. Since monovalent ions are judged toxic at the concentrations required for osmotic adjustment, it is generally assumed that Na^+ and Cl^- are compartmentalised in halophytes, predominantly in vacuoles, so that concentrations in the cytoplasm are maintained within tolerable limits. However, as stated by Flowers and Colmer (2008), experimental evidence for compartmentalisation of Na^+ into vacuoles is still limited, even in halophytes.

New *AtNHX* genes have been cloned and characterised (Yokoi et al. 2002; Aharon et al. 2003; Wang et al. 2007; Liu et al. 2008) and much effort has been made to identify orthologous genes in different species and perform the functional analyses, usually by overexpression in genetically modified plants. Examples are *AgNHX1* from *Atriplex gmelini* (Ohta et al. 2002); *BnNHX1* from *Brassica napus* (Wang et al. 2004); *GhNHX1* from *Gossypium hirsutum* (Wu et al. 2004); *OsNHX1* from *Oryza sativa* (Fukuda et al. 2004; Wu et al. 2005; Chen et al. 2007); *HbNHX1* from *Hordeum brevisubulatum* (Lu et al. 2005); *GmNHX1* from *Glycine max* (Sun et al. 2006; Li et al. 2006); *SsNHX1* from *Suaeda salsa* (Zhao et al. 2006b, c; Li et al. 2007); *PgNHX1* from *Pennisetum glaucum* (Verma et al. 2007; Rajagopal et al. 2007); *TNHX1* from *Triticum aestivum* (Brini et al. 2007); and *AeNHX1* from *Agropirum elongatum* (Qiao et al. 2007).

Overexpression of a vacuolar H^+-pyrophosphatase (*AVP1*) from *Arabidopsis thaliana* in transgenic plants of the same species increases the level of salt tolerance (Gaxiola et al. 2001). Similar results have been achieved by overexpressing the homologues from *Thellungiella halophila* (*TsVP*) in tobacco (Gao et al. 2006) and cotton (Lv et al. 2008), *Suaeda salsa* (*SsVP*) in arabidopsis (Guo et al. 2006), and *Triticum aestivum* (*TVP1*) also in arabidopsis (Brini et al. 2007). Interestingly, *TsVP* and *SsVP* genes have been cloned from halophytes (*Thellungiella halophila* and *Suaeda salsa*, respectively).

Likewise, a higher level of salt tolerance has been described through the overexpression of genes that codify plasma membrane Na^+/H^+ antiports cloned from different sources. For example, *AtSOS1* from *Arabidopsis thaliana* (Shi et al. 2003);

SOD2 from *Schizosaccharomyces pombe* (Gao et al. 2003; Zhao et al. 2006a); *nhaA* from *Escherichia coli* (Wu et al. 2005); and *OsSOS1* from *Oryza sativa* (Martínez-Atienza et al. 2007).

Regulatory genes such as transcription factors and those codifying signal transduction components or receptor-related proteins are another target (Kaur and Gupta 2005; Agarwal et al. 2006). Cloning of genes codifying transcription factors is a promising field as they lie upstream of many other genes. Recent research has allowed the identification of several transcription factors that are important in regulating plant stress responses, including not only different kinds of abiotic stress but also pathogen-induced defence responses, various physiological processes, hormonal signalling pathways and several developmental processes (Agarwal et al. 2006; Ham et al. 2006; Sohn et al. 2006; Seong et al. 2007; Ogawa et al. 2007; Liu et al. 2007; Dai et al. 2007; Nakashima et al. 2007).

It has also been suggested that genes codifying calcium sensors (Cheong et al. 2003) or even DNA helicases (pea DNA helicase 45, PDH45, Sanan-Mishra et al. 2005) and RNA helicases (DEAD-box helicase, Gong et al. 2005b; Owttrim 2006) could be involved in the process of salt tolerance. The role of siRNAs in stress conditions is also under study (Sunkar and Zhu 2004; Borsani et al. 2005; Jung and Kang 2007). Finally, knowledge of the processes related to DNA/RNA metabolism and G-protein signalling pathways could be useful in elucidating the less known stress signalling networks and thereby be helpful for engineering salinity-tolerance in crop plants (Tuteja 2007).

16.5 Functional Analysis of Salt Tolerance-Related Genes

Overall, the results obtained in this field show that the expression of different kinds of genes in transgenic plants can increase salinity tolerance, at least to some extent. Unfortunately, it is not possible to conclude for the moment that true halotolerant cultivars (i.e. with a sufficient tolerance level from an agronomic point of view) have been obtained via transformation. In fact, it would be best to avoid excessive optimism when drawing conclusions on the current state of this topic (Flowers 2004). Furthermore, when performing the functional analysis of a salt tolerance-related gene it would be advisable to take into consideration aspects such as the species used in the transformation, the procedure for evaluating the tolerance to salinity, and the complexity of the trait.

With respect to the first issue, the majority of transformation experiments have been carried out with the model species arabidopsis and tobacco (Vij and Tyagi 2007) which means we should be cautious when drawing conclusions. It would be best to perform such experiments in crops (Grover et al. 2003; Yamaguchi and Blumwald 2005; Cuartero et al. 2006; Bhatnagar-Mathur et al. 2008), as it is not certain what will occur when the genes that have given a positive result in models are expressed in cultivated species. The suitability of tobacco as a model in this field has been seriously questioned (Murthy and Tester 1996) and results from the evaluation of salt tolerance in transgenic arabidopsis plants cannot easily be

extrapolated to a crop species, as the important trait in the latter is the maintenance of production under stress. Extrapolations between different crop species cannot even be made, since the effects of salinity can be very different between species. If true advances are sought, the best approach is to focus efforts on cultivated species where the transformation technology is already available. Without doubt, the difficulties will be greater and the advances slower in crops than in model species, but the results will indicate the true importance of a certain transgene in the genetic context in which the tolerant phenotype will supposedly occur. Last but not least, any results could be of practical interest (Cuartero et al. 2006).

Regarding the procedure for evaluating the tolerance to salinity, if the published results are scrutinised, some of the methods of evaluation of transgenic materials appear of doubtful value. Results of a descriptive type or those based upon photographic evidence of the performance of plants may lead to confusing or erroneous conclusions (Flowers 2004). Responses to salt are frequently studied using small samples, in the very short-term, by using shock treatments and, furthermore, the data are collected during very specific growth periods (Yamaguchi and Blumwald 2005), in spite of the fact that in most crop species salt sensitivity depends on the growth stage (Perez-Alfocea et al. 1993; Khatum and Flowers 1995). Moreover, tolerance estimated on the basis of seed germination is not correlated with tolerance at later growth stages (Foolad and Lin 1997; Cuartero and Fernandez-Muñoz 1999). The usefulness of *in vitro* tests, frequently used for the evaluation of salt tolerance, could also be questioned because transpiring conditions have a major influence on Na^+ transport and tolerance (Moller and Tester 2007). However, a clear relationship between tolerance to salinity *in vitro* (callus) and *in vivo* (plants grown in greenhouse) has been observed for cultivated and wild tomato species (Perez-Alfocea et al. 1994; Cano et al. 1996) and similar results were obtained for cultivated species of *Cucumis* and *Citrullus* (melon, cucumber, and watermelon) and related wild species (Barage 2002). *In vitro* tests can provide complementary information on the effect of some transgenes (e.g. genes involved in ionic homeostasis) and can be useful for the pre-selection of transgenic lines (if an *in vitro* and *in vivo* correlation has previously been shown), but they should not be used as the only criterion to determine the degree of salt tolerance. In evaluating the tolerance of transgenic crops, it is important to perform long-term experiments, focus on growth and yield, and provide quantitative data (Flowers 2004; Munns and Tester 2008).

Bressan et al. (2008) have discussed the convenience of defining a minimum set of criteria for establishing unambiguously that transgenic plants do indeed show tolerance that is attributable to the transgene. In this respect, an important aspect in the functional analysis of a salt tolerance-related gene is the plant material to be used. The use of TG1 plants (primary transformants) is questionable because epigenetic effects (which are very important in some cases) may lead to erroneous conclusions. The evaluation in TG2 avoids the above problem, but it is necessary to take into account that this is a segregant progeny. In the authors' opinion, the best materials are the homozygous and azygous lines obtained in TG3. Thus, each homozygous line should be compared with two controls: the wild type and the corresponding azygous line without the transgene. Positional effects can generate

great differences in the expression of a given transgene in independent transgenic lines, indicating the necessity for selecting those with the best expression for the trait (Pineda 2005). Dose effects of the transgene can be estimated by comparing the behaviour of homozygous *versus* hemizygous lines (i.e. those derived from the sexual crossing between the homozygous and azygous lines). The relative tolerance of these lines can be estimated in the short- and mid-term, although, ultimately, the long-term response (estimating yield with quantitative data) must also be reported.

16.5.1 Complexity of the Trait and Sources of Genetic Variation

Salt tolerance is a complex trait (Bohnert et al. 1996; Tuteja 2007; Munns and Tester 2008). If one takes into account the diversity of mechanisms involved, the question that immediately arises is whether the introduction of a single gene can produce a sufficient level of tolerance or whether it is necessary to introduce several genes involved in different processes (e.g. osmotic adjustment, osmoprotection, ionic homeostasis, oxygen free radical scavengeing, stress response, restoration of enzymatic activity, photorespiration; Bohnert et al. 1996). Of course a particular gene (e.g. one that codes for a transcription factor) can have a cascade effect, modifying the expression of many genes. Alternatively, the expression of a gene involved in the compartmentalisation of ions in the vacuoles may alleviate toxic effects. Even so, it seems unlikely that a single gene could affect all the processes influenced by salinity. What is most likely is that the transference and expression, in a co-ordinated way, of a series of genes, each of which would affect one of the principal mechanisms of the process, would produce tolerant plants. The problem is that there is still not a clear idea of which genes have to be transferred.

When considering the future targets in this field, one can argue that rather than looking for salt tolerant-related genes in salt sensitive species, like arabidopsis, it would be better to focus on halotolerant plants. Flowers and Colmer (2008) have recently reviewed the mechanisms of tolerance in halophytes, plants that are able to survive and reproduce in environments where the salt concentration is around 200 mM NaCl or more. In this respect, the authors have proposed that research should be concentrated on a number of 'model' (halotolerant) species that are representative of the various mechanisms that might be involved in tolerance. As these halophytes are evolutionarily distant from the main crop species, from a breeding point of view it would perhaps be better to take advantage of the existence of halotolerant accesions of wild species related to a given crop, as occurs in the genus *Solanum* (Cuartero et al. 2006), *Citrullus* (Barage 2002), *Cucumis* (Barage et al. 2002) and many others. Unfortunately, despite the wealth of sources of variation, it is still not known which are the key genes determining the high level of salt tolerance in those plants.

16.6 Genomic Approaches for Dissection of Salinity Tolerance

The use of functional genomic approaches may serve to overcome the above mentioned problems. Transcriptomic analysis provides the expression profiles of hundreds or thousands of genes. At present, this kind of approach is being used to identify those genes that are expressed or deactivated in response to saline or other types of abiotic stress (Cuartero et al. 2006, and references therein). Although these methods might lead to an overestimation of the number of genes supposedly involved, which would make the identification of relevant genes among an enormous number of other genes with purely secondary or irrelevant functions more difficult, it is foreseeable that transcriptomic analysis will become a valuable tool in the near future. However, in order to fulfil the expectations created in this field, it would be sensible to take into account the stage of development at which the saline treatment is applied, to perform tissue-specific studies and to avoid traumatic or unnatural treatments (Munns 2005). In fact, transcriptomics studies can produce different answers depending on the tissue examined and whether the plant is growing or dying (Munns and Tester 2008). The relationship between the intensity of saline treatment and the degree of salt tolerance is another point to be considered. A high-salt treatment for a sensitive plant like arabidopsis will induce changes predominantly associated with senescence; however, a low-salt treatment may not result in discernable changes in gene expression (Munns and Tester 2008). The opposite situation should also be taken into account, as a high-salt treatment for a salt-tolerant plant (e.g. halophyte) may not produce any remarkable change in gene expression (as those plants grow normally in that situation and tolerate salt concentrations that kill or inhibit the growth of the majority of glycophytes) and a low-salt treatment may just reflect an abnormal situation for that species. Rather than apply these approaches either to model (salt-sensitive) species or halophytes, it would be better to apply them in both crop species and halotolerant accessions of related wild species and thus, by comparison, try to identify the genes responsible for tolerance (Bohnert et al. 2006; Cuartero et al. 2006).

Other genomic approaches should provide very useful information. For example, major advances have been achieved in the study of mechanisms of post-transcriptional gene silencing and high throughput systems are available to infer gene function (Baulcombe 2004; Herr et al. 2005; Cherian et al. 2006). We foresee that, if systematically applied in a large-scale program using halotolerant plants, this approach would be particularly valuable for the identification of genes involved in different mechanisms related to salt tolerance.

Overexpression has hitherto been the most widely used strategy for both the functional analysis of candidate genes as well as for the increase in salt tolerance in transgenic plants. The underlying idea is that by overexpressing a certain gene or by expressing it in a constitutive way it would always have a positive effect on the phenotype. But increasing evidence supports the idea that sometimes strong and constitutive promoters (e.g. CaMV-35S, mostly for dicots, or actin and ubiquitin1, for monocot species) involve a high energetic cost and yield a penalty in transgenic plants (Rus et al. 2001; Grover et al. 2003; Pineda 2005; Muñoz-Mayor et al. 2008) and, in other cases, the beneficial effects of the transgene are masked by pleiotropic

effects derived from the use of strong promoters (Romero et al. 1997; Capell et al. 1998; Kasuga et al. 1999). Yield penalty and/or pleiotropy not only make the interpretation of the results on the functional analysis of a given transgene difficult but also hamper any practical application. In fact, evidence from research in this field supports the advantages of using inducible promoters (Kasuga et al. 1999; Garg et al. 2002; Rohila et al. 2002; Lee et al. 2003; Su and Wu 2004; Nakashima et al. 2007). Moreover, the use of inducible or specific promoters will be essential when tackling the co-transference and co-expression of several genes to avoid homology-based gene silencing (Grover et al. 2003; Cuartero et al. 2006). It is expected that the identification of new *cis* regulatory elements, which allow proper expression in time and space, will be a major target in the near future (Yamaguchi and Blumwald 2005; Cherian et al. 2006; Cuartero et al. 2006; Bhatnagar-Mathur et al. 2008).

The use of mutants as genomic tools should also be one of the main research areas in the coming years. One of the key factors explaining our present knowledge in several areas of plant development lies in the detection and characterisation of mutants that have altered developmental traits, for example those affected in tomato fruit development and maturation (Emmanuel and Levy 2002; Tanksley 2004; Giovannoni 2007; Lozano et al. 2009). By comparison, the number of mutants with the level of salt tolerance affected in other species than arabidopsis which are already available for the scientific community is rather scarce, perhaps due to the difficulty in performing proper evaluation of the trait. Occasional spontaneous mutants or, alternatively, those generated by chemical (e.g. EMS) or physical (e.g. fast neutrons) methods could provide the basis for advancing the knowledge of physiological processes related to salt tolerance. However, in the absence of obvious candidate genes, the isolation of a gene altered in the mutant through a positional cloning strategy requires huge effort. Fortunately, at present we can overcome these problems by using alternative approaches.

Insertional mutagenesis with T-DNA or transposable elements constitutes a basic tool for the identification of genes and the analysis of their function. With respect to insertional mutagenesis with T-DNA, we can approach the tagging of genes by using a simple construction with a marker gene. In this way, the integration of T-DNA within the structural sequence or the controlling elements of a given gene will lead to its disruption and the consequent loss-of-function or, depending of the characteristics of the T-DNA-insert, gain-of-function or change in its level of expression (Krysan et al. 1999). Upon detecting the mutant phenotype in TG1 (in the case of dominant, semidominant or additive effects) or TG2 (recessive), its cloning can be easier as the gene is tagged by the T-DNA.

By comparison with classical insertional mutagenesis, trapping systems (Springer 2000) can be particularly useful for the identification of genes related to salt tolerance. The advantage of using enhancer, promoter or gene traps resides in its self dual nature. Like any other T-DNA, those traps act as insertion mutagens, but also, when T-DNA is integrated inside an endogenous gene in the appropriate orientation, the reporter gene lies under the control of the regulatory elements of the tagged gene. By analysing the reporter gene expression one can obtain a precise picture of the spatial and temporal expression pattern of the endogenous gene tagged by the trap. In this respect, trapping strategies bring great advantages over insertional mutagenesis

by allowing identification of functionally redundant genes, those expressed at multiple developmental stages (generating confusion during phenotyping), genes whose disruption causes early lethality, and genes whose disruption causes a soft phenotype that may not be detected (in this case, the reporter expression gives a clue to identify the phenotype during evaluation). In addition, gene identification is independent of the expression level of the gene, avoiding the risk of rejecting genes that are expressed at low levels, even though they have major effects on the phenotype. Finally, this is the best way to identify genes that are activated or repressed in response either to an external stimulus or biotic and abiotic stress situations.

Using an enhancer trap (kindly provided by Dr. Thomas Jack, Department of Biological Sciences, Dartmouth College, USA) and a promoter trap (developed in the laboratory of Drs. Rafael Lozano and Trinidad Angosto, Universidad de Almería, Spain), a collection of more than 2,000 T-DNA lines of halotolerant accessions of the wild tomato-related species *Solanum pennellii* has been generated (Anton et al 2009). Scrutiny of this collection to identify insertion mutants with altered levels of saline stress (mainly hypersensitive) is under way. Given that the collection of T-DNA lines is going to enlarge progressively, identification of new salt tolerance related-genes is expected in the near future. It is foreseeable that the use of these genomic approaches will allow the genetic dissection of the trait and, thereafter, the proper design of a breeding program.

16.7 Conclusions

A significant gap still exists between the knowledge gained in physiological and molecular studies of responses to salt stress, and the knowledge required to develop crop plants with enhanced tolerance to saline conditions. A focus on physiological, molecular and metabolic aspects of salt stress in crop plants would help to bridge this gap. It is still necessary to elucidate some processes involved in ionic stress tolerance, synthesis of compatible solute and also other processes as the mechanisms of osmotic tolerance. New tools arising from functional genomics may significantly enhance the descriptive power of physiological analysis and molecular studies with plants grown under realistic saline conditions.

It seems then that the necessary variability for starting breeding programs is not hindering the development of salt tolerant cultivars in most crops, although it is necessary to look for variability to some key physiological traits related to osmotic and ionic stresses, in specifically salt concentrations and plant development stages. QTL detection and mapping requires the development of large RIL or DH generations from parents with a wide variation in a number of traits related to tolerance to salinity. These populations will help to: (1) locate the QTL in chromosome regions as narrow as possible, (2) accumulate QTL information gained in different experiments and growing seasons, and (3) quantify QTL × E and QTL × QTL interactions. Elite cultivars or elite lines, parents of today's hybrids, are the ideal candidates to receive the salt tolerance QTL from donor genotypes. Introduction of QTL should be made by combining

marker assisted and phenotypic selection in breeding programs approaching slowly the recurrent elite lines. This will allow breeders to maintain the delicate equilibrium among productivity, quality, resistance to diseases, and other agronomic traits showed by elite cultivars and lines, that could be otherwise be altered with the introduction of a substantial number of QTL as those required to obtain tolerance to salinity.

Expression of some transgenes promotes a high level of salt tolerance in different species. Despite these interesting results, it is not possible to conclude yet that cultivars tolerant enough from an agronomic point of view have been obtained via transformation. To fulfil the expectations created by the plant transformation technique, it is necessary to advance on different aspects: (1) identification of genes actually involved in the process of salt tolerance, (2) isolation of genes from adequate sources of variation (wild species related to a given crop), (3) design vectors that allow the transfer and coordinate expression of several genes (since salt tolerance is a complex trait), and (4) identification of regulatory elements modulating spatially and temporarily the expression level of the transgenes. Despite present limitations, it is foreseeable that breeding programmes will benefit from ongoing functional genomics projects that could allow the use of genetic transformation as a regular breeding tool.

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