

# Increasing salt tolerance in the tomato

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Received 17 August 2005; Accepted 21 December 2005

## Abstract

In this paper, a number of strategies to overcome the deleterious effects of salinity on plants will be reviewed; these strategies include using molecular markers and genetic transformation as tools to develop salinity-tolerant genotypes, and some cultural techniques. For more than 12 years, QTL analysis has been attempted in order to understand the genetics of salt tolerance and to deal with component traits in breeding programmes. Despite innovations like better marker systems and improved genetic mapping strategies, the success of marker-assisted selection has been very limited because, in part, of inadequate experimental design. Since salinity is variable in time and space, experimental design must allow the study of genotype×environment interaction. Genetic transformation could become a powerful tool in plant breeding, but the growing knowledge from plant physiology must be integrated with molecular breeding techniques. It has been shown that the expression of several transgenes promotes a higher level of salt tolerance in some species. Despite this promising result, the development of a salt-tolerant cultivar by way of transgenesis has still not been achieved. Future directions in order to overcome the present limitations are proposed. Three cultural techniques have proved useful in tomato to overcome, in part, the effects of salinity: treatment of seedlings with drought or NaCl ameliorates the adaptation of adult plants to salinity; mist applied to tomato plants grown in Mediterranean conditions improves vegetative growth and yield in saline conditions; and grafting tomato cultivars onto appropriate rootstocks could reduce the effects of salinity.

Key words: Conditioning, grafting, misting, pretreatments of salt and drought, quantitative trait loci, transgenic plants.

## Introduction

Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces yield. As saline soils and saline waters are common around the world, great effort has been devoted to understanding physiological aspects of tolerance to salinity in plants, as a basis for plant breeders to develop salinity-tolerant genotypes. In spite of this great effort, only a small number of cultivars, partially tolerant to salinity, have been developed. Further effort is necessary if the exploitation of saline soils and saline waters that are not currently usable is to be achieved. Although there are comparatively salt-tolerant relatives of the cultivated tomato, it has proved difficult to enrich elite lines with genes from wild species that confer tolerance because of the large number of genes involved, most of them with small effect in comparison to the environment, and the high costs of recovering the genetic background of the receptor cultivar. Conventional breeding programmes to improve the salt tolerance of elite genotypes using wild species as donors are inefficient at the selection stages. If it were possible to reveal molecular markers tightly linked to the genes governing salt tolerance their favourable alleles could be selected in segregant populations by those markers and eventually incorporated into salt-tolerant cultivars: molecular markers are unaffected by the environment. The possibilities of marker-assisted selection for breeding complex characters such as tolerance to salinity are explored here.

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The paucity of success achieved by past attempts to generate salt-tolerant genotypes, both through conventional breeding programmes and through some biotechnological approaches (such as *in vitro* selection; Flowers, 2004), have fuelled hopes that the problems might be solved via transgenesis. Over the years, plant breeders have developed very productive cultivars of high quality, resistant to diseases and adapted to the demands of the market and of industry (Grandillo *et al.*, 1999). New cultivars bred for salt tolerance have not only to be salt tolerant, but also achieve the same attributes of productivity and quality seen in modern cultivars. The introduction of genes conveying salt tolerance to elite cultivars or elite parents of current hybrids, by transformation, is a very attractive idea because, hypothetically, susceptible but productive cultivars should be converted to tolerant cultivars, while maintaining all the very valuable characters current cultivars possess. The main problems of transgenesis will be reviewed and possible solutions suggested.

However, solving a problem as complex as the profitable use of saline water in irrigated agriculture requires more than one strategy. As well as tolerant cultivars, a battery of cultural techniques, each contributing to a small extent to allow plants to withstand better the deleterious effects of salt, needs to be applied (Cuartero and Fernandez-Muñoz, 1999). Some of those strategies, like the application of chemical fertilizers at levels somewhat above the optimum for freshwater irrigation, the application of chemical amendments or leaching salts to deeper soil layers, have doubtful compatibility with preservation of the environment. Others practices, like seed priming, seedling conditioning, increasing relative humidity, and grafting, are perfectly compatible with sustainable environmental management and will also be reviewed here. It is important to point out that growers would exploit immediately advantageous growing techniques that allow them to utilize saline conditions, while retaining the yield and quality of varieties for which they already have established markets.

Salinity affects all dicotyledonous crops, but basic research cannot address all these species. The tomato (*Solanum lycopersicum* L.) is advocated as a model crop to test the possibilities of marker-assisted selection and the introduction of foreign genes by transformation, because its genetics are better known than any other dicotyledonous crop and it can be transformed by different methods. At the same time, the physiology of the tomato in salty and non-salty conditions has been extensively studied providing an invaluable base to understand the responses of the plants to cultural practices.

### Breeding for increasing salt tolerance

Most of the processes found empirically to be important for salt tolerance in plants, exhibit quantitative inheritance and

a high degree of environmental sensitivity. Although many components of salinity tolerance have now been extensively described (e.g. compartmentation in halophytes, minimizing sodium and/or chloride uptake, maximizing selectivity of potassium over sodium, controlling salt flux to the shoot through the entry of ions into the xylem stream, reducing transpiration, synthesizing compatible solutes) the application of this knowledge to plant improvement remains hampered because of the large number of genes involved, most of them with a small effect in comparison to the environment. Moreover, these gene effects are dependent on other genes (genetic background, epistatic interaction), on the salinity level and composition and on the environmental conditions (genotype $\times$ environment, G $\times$ E, interaction). Due to this complexity, genetic variation in the wild germplasm for quantitative-agronomic traits such as tolerance to environmental stresses remains largely untapped. Besides, conventional breeding programmes to improve elite genotypes for adaptation to abiotic stresses using wild species as donors are inefficient at the selection stages, when trying to enrich the elite lines with tolerance genes while recovering most of their genetic background.

The joint analysis of segregation of markers and of phenotypes of individuals or lines makes possible detection and location of quantitative trait loci (Quantitative Trait Loci, QTLs). Molecular markers tightly linked to QTLs can unveil masked alleles and make for an easier introduction of genetic material from donors (wild species and old cultivars), without the drawbacks that are associated with the introduction of 'wild genes' through conventional methods. Despite innovations like better marker systems and improved genetic mapping strategies, the success of marker-assisted selection (MAS) has been very limited, mainly due to both the definition of salt tolerance itself as the target trait and to the experimental design for its QTL analysis.

#### *The first problem: how to define salt tolerance*

Salinity affects yield quality and quantity, so that yield characters must be taken into account when breeding for salinity tolerance. But not only yield-related characters are important. As salinity affects almost every aspect of the physiology and biochemistry of the plant, the enhancement of crop salt tolerance will require the combination of several to many physiological traits (Flowers and Yeo, 1995; Cuartero and Fernández-Muñoz, 1999), not simply those directly influencing yield.

The first step to using physiological characters in breeding programmes is to prove those characters show genetic variability in the available germplasm. Cuartero *et al.* (2002) demonstrated the existence of variability for four physiological characters in tomato: Na<sup>+</sup> transport to the shoot from the external root nutrient solution; the relation between leaf [Na<sup>+</sup>] and leaf area reduction; the capacity to accumulate Na<sup>+</sup> in old leaves while young leaves maintain

a low  $[\text{Na}^+]$  and leaf  $[\text{K}^+]/[\text{Na}^+]$  ratio. By contrast with salt tolerance studies in cereals, where  $\text{Na}^+$  uptake,  $\text{K}^+$  uptake,  $\text{Na}^+/\text{K}^+$  discrimination, and other physiological characters have been subject to QTL analysis (Koyoma *et al.*, 2001; Cattivelli *et al.*, 2002; Lin *et al.*, 2004; Lindsay *et al.*, 2004), this kind of phenotyping for QTL analysis is almost absent in dicotyledonous crop species. Exceptionally,  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  in different tissues and their accumulation in the whole plant have been subjected to QTL analysis in *Citrus* spp. (Tozlu *et al.*, 1999). However, the vigour of the plants was not taken into account in this case, so that the QTLs reported might be related to vegetative growth rather than to ion uptake. The genetics of these physiological characters, together with other tolerance components related to metabolic defences against salinity (compatible solutes such as polyols and antioxidant molecules), have to be studied in order to advance the breeding of genotypes tolerant to salinity. However, the large number of characters contributing to salt tolerance generally precludes their study in any one experiment.

The value of QTLs in a plant breeding programme depends not only on the physiological or metabolic importance of the traits being evaluated, but also their heritabilities: those showing high heritability in the population should provide the most useful QTL. The heritabilities of salt-tolerance traits subjected to QTL analysis in plant populations are usually ignored and sometimes (i.e. entry mean heritability in Lee *et al.*, 2004) their estimates are no longer related to selection. The existence of variability has been demonstrated for some physiological traits (Cuartero *et al.*, 2002) and their heritabilities estimated in 135 recombinant inbred lines (RILs) from a cross between *S. lycopersicum* × *S. pimpinellifolium* on six plants per genotype, grown in hydroponics, after 5 weeks of salt treatment (plants 9-weeks-old). Heritabilities were medium or low, as expected for polygenic characters highly influenced by the environment. Interestingly, shoot dry weight, a character often measured as an indicator of growth and yield, always showed lower heritability than measures of ion contents and water relations (Table 1), indicating the

importance of using those traits rather than dry weight alone. Leaf area showed the highest heritability and should be used in breeding programmes developing salt-tolerant tomato genotypes, although more work is needed in order to find quick and reliable measures of the character.

Heritabilities for water use efficiency (WUE, estimated as plant dry weight in g/water transpired in litres), leaf tissue tolerance (estimated as  $[\text{Na}^+]$  in leaf/leaf area reduction), and  $\text{Na}^+$  transport to shoot (estimated as total  $\text{Na}^+$  content in the plant/total  $\text{Na}^+$  content in the volume of water transpired) were medium (Table 1), and the estimates suggest that leaf-tissue tolerance should be employed to breed genotypes tolerant to high (200 mM) levels of salinity, while  $\text{Na}^+$  transport to shoot should be employed in breeding for tolerance at medium salinity levels (100 mM); heritabilities of WUE were less dependent on salinity level than the two characters employed for its calculation (Table 1). Heritabilities of WUE were particularly high under both control and saline conditions, which means that important additive genetic variation was in the set of 135 RILs studied. Genotypes with high WUE should save substantial amounts of irrigation water. In addition, in the case of saline water, less irrigation water means less salt incorporated in the soil and better preservation of the soil.

Another concern of QTL analysis is that the phenotyping of the plants has to be not only consistent but also simple, since screening large numbers of individuals is required for genetic analysis. Screening of genotypes on naturally saline soils is not usually feasible because of the extreme variability in soil salinity both spatially and temporally (Richards, 1983). To avoid this variability, plants are often grown in nutrient solutions to which NaCl has been added. Nevertheless, a constant level of salinity is not normal in nature, so several levels have to be considered. In one of the few studies where evaluation was carried out in both a saline field and a greenhouse, genotypic correlation between field and greenhouse ratings for salt tolerance was only 0.55, although significant at  $P < 0.01$  (Lee *et al.*, 2004). Furthermore, salt tolerance appears to depend upon growth stage; tolerance at one stage of plant development is often not correlated with tolerance at other stages, for example, tolerance at germination does not correlate with tolerance of seedlings or adult plants (Foolad and Lin, 1997; Cattivelli *et al.*, 2002; Foolad, 2004). Therefore, the most sensitive plant stage(s) should be recognized and breeders should focus firstly on these.

**Table 1.** Heritabilities in broad sense of nine characters related to salt tolerance, estimated in 135 RILs from *Solanum lycopersicum* × *S. pimpinellifolium*, grown at 0, 100 and 200 mM NaCl

Character	0 mM	100 mM	200 mM
Shoot dry weight	0.11	0.15	0.21
Total leaf area	0.72	0.71	0.78
Water transpired	0.61	0.33	0.29
Water use efficiency	0.53	0.48	0.59
$[\text{Na}^+]$ in leaf	0.33	0.22	0.26
$[\text{K}^+]$ in leaf	0.22	0.33	0.47
$[\text{K}^+]/[\text{Na}^+]$ in leaf	0.13	0.33	0.49
Leaf $[\text{Na}^+]$ /leaf area reduction		0.23	0.51
$\text{Na}^+$ transport to shoot		0.40	0.26

#### *The second problem: the experimental design must allow the integration of knowledge*

A great advantage of QTL analysis in the post-genomic era is that it allows an integrative approach to complex traits such as salt tolerance. This integrative approach concerns the analysis of multiple traits in the same segregating population; the possibility of studying important features

of quantitative genetics in classical plant breeding (like  $G \times E$ , epistatic interactions, heterosis, and pleiotropy) and, if certain genes are suspected to control the trait (candidate genes), to test their putative contributions through the QTL analysis itself (candidate gene analysis).

Except for candidate genes, all those features of salt tolerance (defined as fruit yield under salinity) have been evaluated using three  $F_2$  populations derived from crosses between *S. lycopersicum* and two wild relatives (two accessions of *S. pimpinellifolium* and one accession of *S. galapagense* S. Darwin & Peralta). Only 41% of the loci assayed were polymorphic (Bretó *et al.*, 1994) but, fortunately, 43% of marker loci were linked to QTLs for the trait under study. Genetic variability at these QTLs for additive and heterotic effects among the three families was found (Monforte *et al.*, 1997a). Most drastic differences concerned the epistatic interactions in which all genomic regions containing QTLs were involved. These interactions between unlinked genes increased the range of variation of means, mainly upwards, as compared with genotypes at individual QTLs. Epistatic interactions were also found to be influenced by the salt treatment (Monforte *et al.*, 1997b). These authors compared the behaviour of QTLs in two of the  $F_2$  populations (those sharing the *S. lycopersicum* female parent and differing for the wild species) grown under optimum and high salinity conditions. They found that only a few QTLs were 'constitutive' (i.e. were detected under both conditions); some were only detected under optimal conditions ('response sensitive'), and the significant effects of the majority of QTLs depended on the presence of salinity ('response tolerant'). Given that salinity is variable in time and space, MAS should take into account not only the constitutive and response-tolerant, but also the response-sensitive QTLs. A major problem arises when a valuable allele at the QTL is different depending on the salinity level. This is an inconvenience for MAS but logical since as genes and QTL effects may be environmentally sensitive (Gupta and Lewontin, 1982; Gurganus *et al.*, 1998). This sensitivity results in phenotypic plasticity: the ability of the organisms to take on alternative developmental fates, depending on environment cues. The conclusion is that the presence of those epistatic and  $G \times E$  interactions greatly complicates MAS to improve salt tolerance in tomato.

It is important to point out that the above-mentioned QTL studies simply show QTLs at different map positions, or with different effects in different environments, which might result from statistical uncertainty. Jansen *et al.* (1995), Tinker *et al.* (1996), Sari-Gorla *et al.* (1997), and Melchinger *et al.* (1998) explicitly included a QTL-environment interaction component in the interval-mapping model. These studies show that the expression of QTLs can vary among environments and they suggest that roughly 30% of identified QTLs show significant  $G \times E$  interaction. This percentage is expected to be larger as the difference among the target environments becomes larger, as in

the case of control versus stress experiments (Monforte *et al.*, 1997b; Hemamalini *et al.*, 2000).

There are few studies that specify the contribution of QTL  $\times$  E and QTL  $\times$  QTL interactions to the total phenotypic variance. Juenger *et al.* (2005) report contributions of interactions larger than most individual QTLs, 19% and 12%, respectively. These contributions have not been estimated yet for salt tolerance but they should be taken into account if this trait is to be improved through MAS. Moreover, selection of favourable QTL alleles from the donor wild species is not always enough, because pleiotropic or regulatory effects of genes controlling quantitative traits like earliness, have been shown in *S. galapagense* (Monforte *et al.*, 1999). These pleiotropic effects of earliness genes on yield and vegetative traits have important consequences for the management of genetic resources of *S. galapagense*, explaining why some authors have proposed *S. galapagense* as a salt-tolerance donor while others consider it as having low salt tolerance. To breed efficiently for salt tolerance, a better understanding of the whole plant behaviour under changing salinity levels is needed. Besides, this knowledge should involve not only the trait defined as salt tolerance but also all its correlated traits because correlated responses may constrain the success of breeding programmes. Correlated traits usually have QTLs in common genomic regions (Tanksley and Nelson, 1996; Monforte *et al.*, 1997a; Fulton *et al.*, 2000; García *et al.*, 2000) and, surprisingly, QTLs of non-correlated traits might be tightly linked too. QTL analysis of multiple traits unveils these masked associations, ensuring the success of MAS. Multi-trait analysis, and the study of epistatic interactions, under different salinity levels only can be properly and efficiently carried out using populations of doubled haploids (DHs) or RILs in the experimental design.

QTL analysis of salt tolerance using this kind of experimental design in dicotyledonous species is still required. The major technical advantage for such an approach lies in the fact that the lines can be reproduced independently and continuously evaluated with respect to additional quantitative traits, markers, and candidate genes with all the information being cumulative. In addition, more accurate estimates of the location are obtained, with less variance, compared with the backcross design (Carbonell *et al.*, 1993). Replicated progenies can bring about a major reduction in the number of lines that need to be scored, which is very convenient for a proper phenotyping of traits that are difficult to evaluate, such as most physiological components of salt tolerance. Besides, having replicates of each individual, the environmental variation can be minimized, which is a way to increase the heritability of individual QTLs and to reduce the size of their confidence intervals. There are two situations in plant genomics where the wideness of confidence intervals is important: distinguishing linked QTLs governing different traits from

a QTL with pleiotropic effects over the traits, and candidate gene analysis. QTLs with pleiotropic effects seem to be crucial in co-ordinating (or regulating) the connected physiological pathways of traits and to control undesired correlated responses. Preliminary results on QTL analysis of salt tolerance using two populations of *Solanum* RILs show clustering of QTLs governing several trait sets such as fruit weight and root weight; fruit set and  $[\text{Na}^+]$  in the leaf; dried weight of stem (DWS), fruit weight (FW), fruit set, fruit number (FN), and water use efficiency (WUE). It is noteworthy that only three pairs of these traits present significant correlations: FW and FN ( $P < 0.02$ ), FW and DWS ( $P < 0.016$ ), and FW and WUE ( $P < 0.0065$ ).

The utilization of DHs and RILs for QTL analysis of salt-tolerance-related traits (physiological and agronomic) will be a way to identify key candidate genes within the genetic resources that might be used to obtain the desired adaptation of crops to salinity.

### Genetic transformation as a tool for increasing tolerance to salinity

In numerous papers published from 1993 to date, various authors have claimed enhancement of salt tolerance through either overexpression of endogenous genes or, more frequently, expression of genes that supposedly act on mechanisms involved in the process of tolerance (Borsani *et al.*, 2003; Flowers, 2004). Overall, the results obtained suggest that the expression of individual genes in transgenic plants can increase salinity tolerance, at least to some extent. However, 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. As Flowers (2004) rightly indicates, it would be best to avoid excessive optimism when drawing conclusions on the current state of this topic. Furthermore, it would be advisable to take into consideration some questions, such as the species used in the transformation, the procedure for evaluating the tolerance to salinity, and the complexity of character.

#### *Crop species transformed*

There are numerous articles on transformation directed at increasing the salt tolerance of rice (Datta, 2002; Borsani *et al.*, 2003; Flowers, 2004), but fewer for tomato (Arrillaga *et al.*, 1998; Gisbert *et al.*, 2000; Moghaieb *et al.*, 2000; Rus *et al.*, 2001; Zhang and Blumwald, 2001; Pineda, 2005) and other species of agronomic interest (Borsani *et al.*, 2003; Flowers, 2004). Indeed, the majority of transformation experiments have been carried out with the model species *Arabidopsis* and tobacco. It is not, however, certain what will occur when the genes transferred to the models are expressed in a cultivated species, and the suitability of tobacco as a model has been seriously

questioned (Murthy and Tester, 1996). Furthermore, results from the evaluation of salt tolerance in transgenic *Arabidopsis* plants cannot easily be extrapolated to 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, as occurs in the case of tomato and citrus. If true advances are sought, the best approach is to focus efforts on a particular crop, where the transformation technology is available. Without doubt, the difficulties will be greater and the advances slower in the crop than in a model species, but the results will indicate the true importance of transgenes in the genetic context in which the tolerant phenotype will supposedly occur: any results should be of practical interest.

#### *Evaluation of transgenic plants*

When the published results are scrutinized, 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 in the very short-term, by using shock treatments and, furthermore, the data collected for very specific growth periods (e.g. germination), in spite of the fact that in most crop species salt sensitivity depends on the growth stage (Khatun and Flowers, 1995; Pérez-Alfocea *et al.*, 1993). Moreover, tolerance estimated on the basis of seed germination is not correlated with the 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, has also been questioned. 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 (Pérez-Alfocea *et al.*, 1994; Cano *et al.*, 1996), and for cultivated species of the Cucurbitaceae (melon, cucumber, and watermelon) and wild species of *Cucumis* and *Citrullus* (Barag , 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 been previously 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 provide quantitative data on yield (Flowers, 2004). Perhaps one of the most widely publicized transformations of any crop, has been of tomato, where Zhang and Blumwald (2001) indicated that transgenic tomato plants overexpressing *AtNHX1* (coding for a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter) produced similar fruit yield when grown at 200 mM NaCl to the control plants grown in normal conditions (5 mM NaCl); it was claimed that most plants

of wild type (cv. MoneyMaker) died under saline conditions. These results were questioned by Flowers (2004) since, according to this author, plants of cv. MoneyMaker do not die at 200 mM NaCl. A similar response has been observed here: plants of cv. MoneyMaker were highly affected by this salt level and the production was very much reduced, but the plants did not die. Moreover, 108 independent transgenic plants have been obtained of the tomato cv. p73 with *AtNHX1*. The long-term evaluations (on the basis of fruit yield) carried out in two different localities (in Valencia, V Moreno, and in Murcia, MC Bolarin) and using two different hydroponic systems, indicate that the tolerance level of four homozygous lines (TG3) assayed to date is similar to those of the azygous lines and the wild type. This does not seem to be due to the different cultivar used for the transformation (MoneyMaker in one case and p73 in the other one). It may be that the four selected homozygous lines were not the most tolerant, but the tolerance of different progenies (TG2) was also evaluated after about 60 d of salt treatment and, until now, no transgenic line has been found to be more salt tolerant than the controls, although more lines will need to be evaluated before definitive conclusions can be made.

Another important aspect of evaluating tolerance 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. The relative tolerance of these lines can be estimated in the short- and mid-term, although, finally, the long-term response (estimating yield with quantitative data) must also be reported.

#### *Complexity of the tolerance to salinity character*

Given the very complex nature of tolerance to salinity, 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, elimination of oxygen scavengers, stress response, restoration of enzymatic activity, photorespiration; Bohnert *et al.*, 1996). Of course a particular gene (e.g. one that codes a transcription factor) can have a cascade effect, modifying the expression of many genes. Alternatively, the expression of a gene involved in the compartmentalization 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. However, if the transfer of many genes were necessary, this would invalidate a transformation-based

approach. 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.

In the *Solanum* genus, the existence of halotolerant accessions of wild species (e.g. *S. pennellii* Correll, *S. galapagense*, *S. pimpinellifolium*), and their sexual compatibility with the cultivated species (*S. lycopersicum*) should have permitted the genetic dissection of the tolerance character by traditional methods. The studies carried out to date have provided valuable information (reviewed by Flowers, 2004; Foolad, 2004). However, the distortion of the segregation (Taylor and Ingvarsson, 2003), frequent in interspecific crosses, as well as the difficulties inherent to the evaluation of plants under saline conditions, have made the analysis difficult. As a consequence, it is still not known which are the main genes determining salt tolerance in wild species.

The use of functional genomic approaches may serve to overcome these and other problems. Transcriptomic approaches provide 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 (Zhang *et al.*, 2000; Bohnert *et al.*, 2001; Kawasaki *et al.*, 2001; Seki *et al.*, 2001, 2002; Chen *et al.*, 2002; Kreps *et al.*, 2002; Ozturk *et al.*, 2002; Maathuis *et al.*, 2003; Rabbani *et al.*, 2003; Atienza *et al.*, 2004; Gu *et al.*, 2004; Taji *et al.*, 2004; Takahashi *et al.*, 2004; Ueda *et al.*, 2004). Some of those studies have revealed the induction of genes putatively involved in the process of salt tolerance; however, most results of gene expression profiles show non-specific effects of saline treatments (Munns, 2005). Although these methods might lead to an overestimation of the number of genes supposedly involved, which would make more difficult the identification of relevant genes among an enormous number of other genes with purely secondary or irrelevant functions, it is foreseeable that transcriptomic approaches 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 state of development at which the saline treatment is applied, to perform tissue-specific studies and, as pointed out by Munns (2005), to avoid traumatic or unnatural treatments. Furthermore, rather than apply these approaches to model species, it would be better to apply them in crop species or related wild species and thus, by comparison, try to identify the genes responsible for tolerance.

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 (Baulcombe, 2004; Herr *et al.*, 2005), and

high throughput systems are available to infer gene function (Smith *et al.*, 2000; Wesley *et al.*, 2001). Candidate gene analysis by post-transcriptional gene silencing in halotolerant wild-type accessions may be a very valuable tool in this area.

Functional analysis of candidate genes using either over-expression of endogenous or foreign genes can help to identify functionally relevant genes that determine salt tolerance. A functional analysis programme is being carried out using this experimental strategy in tomato. To date several transgenes have been introduced into the same cultivar (p73) to compare their effects on tolerance levels: 35s::ScHAL1, 35s::ScHAL2, 35s::ScHAL3, 35s::AtHAL3, 35s::ScHAL5, 35s::ScIMP1, tas::ScIMP1, 35s::LeImp3, tas::LeImp3, tas::TPS1, 35s::CAT (cloned by the team of Dr Serrano) mas::AtNHX1 (kindly supplied by Dr Blumvald), PCpea::asnA (supplied by Van der Have BV NL), and 35s::tas14 and 35s::tsw12 (cloned by the team of Dr Pintor). Some of the transgenes have also been introduced in the tomato cultivar UC82B and in melon and watermelon. Evaluation of response to salt in normal and high salt conditions has been carried out in two different environments (Valencia and Murcia, Spain) using two hydroponic culture systems. In both cases, short- and long-term assays evaluated crop yield in TG2 plants (for pre-selection of material), but mainly in homozygous and azygous (TG3) lines for each transgene. Results have shown that: (i) some of those genes promote a higher level of salt (and/or drought) tolerance in tomato cv. p73 (Pineda, 2005); (ii) their effect is not restricted to a specific cultivar as some genes also promote a higher level of tolerance in another tomato cultivar (UC82B) as well as in transgenic plants of melon and watermelon; (iii) there are great differences in the expression of a given gene in independent tomato transgenic lines, indicating the necessity of selecting those with the best expression for the trait; and (iv) instead of using constitutive promoters (e.g. 35S) it would be better to use inducible regulator elements.

The study of regulatory elements is a key point because, traditionally, transgene research has mainly been based on the use of strong and constitutive promoters (e.g. p35S). The underlying idea is that by over-expressing a certain gene or by expressing it in a constitutive way it would always have an effect on the phenotype. But increasing evidence supports the idea that constitutive promoters involve a high energetic cost and yield penalty in transgenic plants (Rus *et al.*, 2001; Pineda, 2005). In some cases, beneficial effects of the transgene are masked by pleiotropic effects derived from the use of strong promoters. In fact, evidence from research into salt tolerance supports the advantages of using inducible promoters (Kasuga *et al.*, 1999; Garg *et al.*, 2002; Rohilaa *et al.*, 2002; Pineda, 2005). It seems that a proper modulation of gene expression in time and space is more important than mere over-expression of the transgene. Moreover, the use of inducible or

specific promoters is essential for co-transference and co-expression of several genes. For example, in this programme of functional analysis in tomato, the first step is the identification of genes involved in the main effects induced by salinity, but the final objective is to introduce selected genes using a multiple construction or, alternatively, by sexual crossing between homozygous lines. However, transgenes with the same regulatory elements should not be combined, to avoid problems of gene silencing; nor should constitutive promoters be used, as previously indicated. Currently, the lack of knowledge of regulatory elements limits the two possible alternatives.

Fortunately, there are several genomic approaches allowing identification and tagging of regulatory elements; for example, those based on the use of enhancer, promoter or gene traps (Springer, 2000). A tomato gene trap collection has been generated using the vector pD991 (kindly supplied by Dr T Jack) and the identification in tomato of regulatory elements involved in saline response is now being undertaken. One advantage of trap lines is their dual purpose, since they also work as T-DNA insertion lines for the identification and tagging of genes involved in other processes.

Most of the genes used so far in plant transformation to improve salt tolerance have been cloned from species phylogenetically distant from the target species used in breeding programmes. It is suggested that the genes be cloned from the crop of interest or from related wild species. Within the genus *Solanum* there is a wide diversity of accessions from wild species showing high tolerance levels. *S. pennellii* accession '20164' is being used in an additional gene-trapping programme addressed at identifying tolerance genes in wild tomato species. Lines showing differential expression in different salt conditions are currently being identified, but a deeper study in time and space of expression patterns in the selected lines is required. In any case, identification of new halotolerance genes is expected in the mid- to long-term.

## Cultural techniques contributing to withstand better the deleterious effects of salt

### *Increasing salt tolerance by pretreatments*

The successful adaptation of cell lines to salinity (Binzel *et al.*, 1985) suggests that a genetic potential for salt tolerance is present in cells of plants from which the lines were derived and that exposure of the cells to salt triggers the expression of this information. Salt-adaptation has also been observed in *Sorghum bicolor* L. Moench. (Amzallag, 1999), although the induction of adaptation was only possible during specific times, called developmental windows (Amzallag *et al.*, 1993). Similar adaptive responses and developmental windows probably exist in other species, such as tomato. It is shown here that the pretreatment at

particular growth stages increases the capacity of plants to adapt to salinity, and, consequently, these adapted plants tolerate salinity better than non-adapted plants.

*Seed priming:* Pretreatment of seed has been used not only to increase salt tolerance during germination and early growth stages (Sivritepe *et al.*, 2003), but also can have effects during fruiting. Plants from tomato seeds primed in 1 M NaCl for 36 h produced a greater fruit yield at low (35 mM NaCl) and moderate (70 mM NaCl) salt levels in the irrigation water than non-primed seed (Cano *et al.*, 1991). The positive effect of seed priming on yield was not clear at 140 mM since the negative effect of high salinity during the growing period dominates the positive effect of seed-priming (Cano *et al.*, 1991). This adaptation could be due to the sum of the adaptation induced by priming the seed with salt, plus adaptation induced by salinity during radicle emergence, as fruit yield also increased in plants primed at germination (Bolarín *et al.*, 1993). The faster growth of tomato plants from primed seeds seems to be the result of a higher capacity for osmotic adjustment because plants from primed seeds have more Na<sup>+</sup> and Cl<sup>-</sup> in roots and more organic acids and sugars in leaves than plants from non-primed seeds (Cayuela *et al.*, 1996). Another mechanism can also help to explain the faster growth of plants from primed seeds. Kaur *et al.* (2005) proved higher activity of sucrose-cleaving enzymes in sinks of *Cicer arietinum* L. plants from primed seeds: acid invertase was enhanced in the apex of the main stem and acid invertase and sucrose synthase in pod walls.

Priming induces increased solubilization of seed storage proteins like the B-subunit of the 11-S globulin in *Beta vulgaris* L. (Bourgne *et al.*, 2000) and it reduced lipid peroxidation and enhanced antioxidative activity in seeds of *Momordia charantia* L. (Yeh *et al.*, 2005) and *Zea mays* L. (Randhir and Shetty, 2005).

*Salt pretreatment applied at the seedling stage:* Adaptation can occur not only during germination and early growth stages, but also during late vegetative development. Tomato seedlings (5-leaf stage) of the salt-sensitive genotype Durinta grown for 15 d in 35 mM NaCl, half the concentration used during later plant growth, showed up to 29% more fruit yield than non-adapted plants (Cayuela *et al.*, 2001). However, no positive effect was observed in the long-term in the adapted-plants of the more salt-tolerant genotype GC-72, perhaps because salt treatment (70 mM) was too low (salinity reduced yield only by 20%) (Cayuela *et al.*, 2001). Seedling pretreatment at the 2-leaf stage did not trigger any long-term adaptive response. It seems that there is a developmental window for adaptation at the 5-leaf stage, and that the stress level necessary to trigger the adaptive response is related to the tolerance degree of the genotype.

*Drought pretreatment applied at the seedling stage:* Plants exposed to a stress often show tolerance to other stresses

(cross-tolerance). For example, salt stress stimulates cold hardiness in potato (*Solanum tuberosum* L.) and spinach (*Spinacia oleracea* L.) seedlings (Ryu *et al.*, 1995); water stress induces chilling resistance in rice (*Oryza sativa* L.) (Takahashi *et al.*, 1994); heat stress increases tolerance to several abiotic stresses (Sabehat *et al.*, 1998). This strategy has not been used practically to improve salt tolerance of horticultural crops, although drought pretreatment could increase the tolerance to the osmotic effect, the main effect induced by salinity when moderate salt levels are used. Gonzalez-Fernandez (1996) observed that tomato plants, which had previously been subjected to a drought stress pretreatment, were able to grow better than non-pretreated plants after 21 d of salt treatment, and Balibrea *et al.* (1999) found a positive effect on the salt tolerance of tomato plants proceeding from pretreated seedlings with PEG. The pretreatments were applied at different growth stages (when seedlings had 4–5 true leaves and just after germination, respectively) and the procedures were very different (withholding watering until seedlings wilt, keeping them flaccid for 20–24 h, and seedlings immersion in a PEG solution), which suggest that water stress, in general, induces salt tolerance in tomato, at least at the plant growth level. Recently, it has been observed that adaptation of pretreated plants was maintained throughout the growth cycle, as fruit yield of drought pretreated plants was 40% greater than that of the non-pretreated plants at the end of the harvest period (150 d of irrigation with 70 mM NaCl in the water). Moreover, the most productive plants maintained low Na<sup>+</sup> and Cl<sup>-</sup> accumulation in their leaves until the end of growth cycle, which shows that adaptation is a long-term response during which the plants adjust their physiology to the environmental conditions (MT Estañ, E Cayuela, A Muñoz-Mayor, MC Bolarín, personal communication).

Considerable effort has been directed at understanding the mechanisms underlying adaptation. Adaptation is a complex process and involves numerous physiological and biochemical changes (Guy, 2003). To date, many varied mechanisms explaining the phenomenon of adaptation in general and cross-tolerance in particular have been proposed; in the case of cross-tolerance, it has been suggested that specific proteins are induced by one kind of stress and are involved in the protection against other kinds of stress (Pareek *et al.*, 1995; Sabehat *et al.*, 1998), although a common mechanism has not been found. It is possible that conventional transcriptome and proteome analyses do not provide direct information about how a change in mRNA or protein is coupled to a change in biological function, as a change at one level in multiple complex regulatory networks does not necessarily lead to a particular change in function. Metabolomic studies could be useful to understand the plant response to adaptation (Fiehn, 2002).

### Increasing salt tolerance by relative humidity

High temperature and low humidity, typical of spring and summer in a Mediterranean climate, impose water stress in most crop plants (Stanghellini, 1994; Li *et al.*, 2001). Salinity in soils or in the irrigation water also restrict water availability to plants in a similar manner to water stress, which causes reductions in growth rate and even in production (Munns, 2002) and alters plant water relations (Romero-Aranda *et al.*, 2000). One strategy to alleviate water deficit imposed by salinity could be to modify relative humidity around the plants (Li *et al.*, 2004). This hypothesis was tested with the tomato cultivar Daniela grown in a greenhouse with non-saline (NS) and saline (S, 50 mM NaCl) irrigation water, non-misted (NM) and misted (M, 10 s every 8 min) that, when applied together, generated the four treatments: NS-NM (control), S-NM, NS-M, and S-M. Salinity reduced the dry weight of leaves by 30%, of the stem by 50%, and plant height by 10% in plants grown without mist. However, no reduction was observed in misted plants (Table 2). An *et al.* (2002, 2005) also found increased plant growth with increasing relative humidity in melons (*Cucumis melo* L.) and tomatoes. Mist increased total plant leaf area of NS and S plants by 39% and 100%, respectively, because of larger leaves. The total yield of NS increased 18% with mist while yield of S plants increased more than twice with mist. Mist increased fruit weight by 15% in NS plants and doubled fruit weight of S plants. The number of fruit was significantly reduced by salinity in NM plants, but was unaffected in M plants. Li *et al.* (2001, 2004) also demonstrated increased tomato fruit production when lower vapour pressure deficit was applied to salinized plants. Mist reduced plant water uptake significantly in NS and S plants. Less Na concentration in leaves (low toxicity), higher plant leaf area, leaf gas exchange, and leaf water status in misted plants were the physiological causes of the higher growth and fruit yield (Romero-Aranda *et al.*, 2002).

### Increasing salt tolerance by grafting

One possibility of avoiding or reducing yield losses caused by salinity would be to graft cultivars on to rootstocks able to reduce the effect of external salt on the shoot. This strategy could also provide the opportunity to growers of combining good shoot characters with good root characters

and to researchers of studying the contribution of genes expressed in the roots or in the shoot and their interaction (Zijlstra *et al.*, 1994; Pardo *et al.*, 1998). Grafting was widely used to limit the effects of soil and vascular diseases, like fusarium wilt, in tomato (Scheffer, 1957; Lee, 1994), but the reasons for grafting, as well as the number of species grafted, have increased dramatically over the years. Grafts have been used to increase yield (Edelstein *et al.*, 2004) and fruit quality (Lopez-Galarza *et al.*, 2004); to induce resistance against low (Bulder *et al.*, 1990) and high (Rivero *et al.*, 2003) temperatures; to alleviate iron chlorosis in calcareous soils (Romera *et al.*, 1991); to enhance nutrient uptake (Ruiz *et al.*, 1997); to increase the synthesis of endogenous hormones (Proebsting *et al.*, 1992) and to improve water use (Cohen and Naor, 2002).

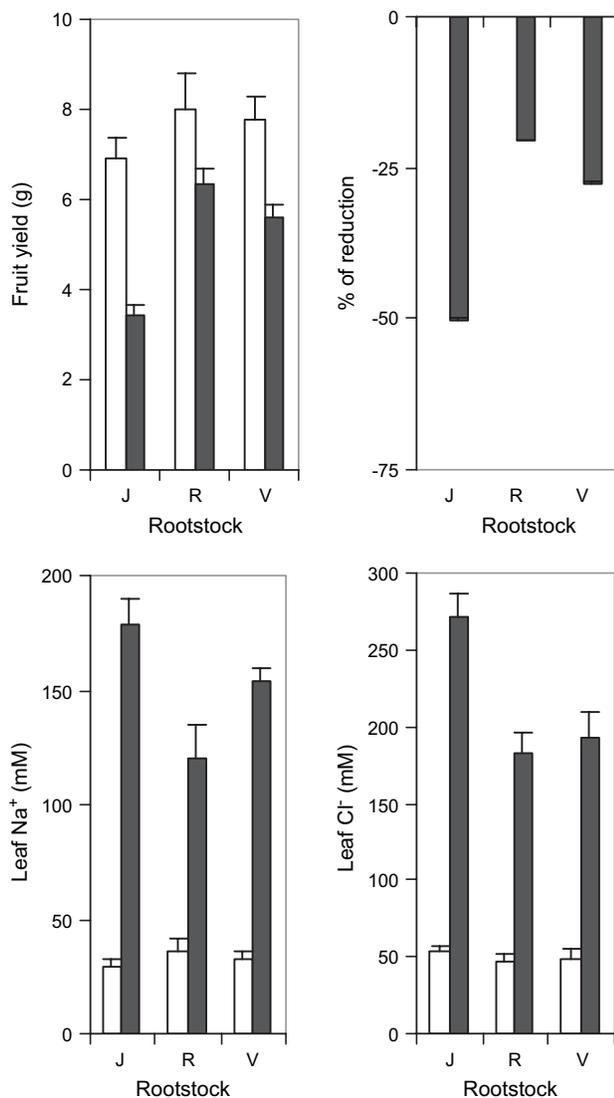
Many studies have been carried out to determine the response of grafted trees and grafted grapevines (*Vitis vinifera* L.) to saline conditions. The positive effect of rootstock in *Citrus* spp. is related to the ability of the rootstock to exclude chloride (Bañuls and Primo-Millo, 1995; Fernandez-Ballester *et al.*, 2003; Moya *et al.*, 2003; Ferguson and Grattan, 2005). In grapevines, where the damage induced by salinity is also primarily caused by Cl<sup>-</sup> ions, *Vitis* species rootstocks differ widely in their ability to exclude Cl<sup>-</sup>, and thus in their salinity tolerance when used as rootstocks (Antcliff *et al.*, 1983; Stevens *et al.*, 1996; Fisarakis *et al.*, 2001; Agaoglu *et al.*, 2004; Walker *et al.*, 2004); for example, the 'Ramsey' rootstock has been used the world over to restrict Cl<sup>-</sup> transport to the shoot of grapevines (Gibberd *et al.*, 2003). However, grafting has rarely been used to increase the productivity of vegetables growing under adverse conditions (Ruiz *et al.*, 1997). In tomato, the results obtained until now suggest that grafting does improve plant adaptation to salt stress in cultivars of determined and undetermined growth (Santa-Cruz *et al.*, 2002; Estañ *et al.*, 2005).

The commercial tomato cultivar Jaguar as scion has been grafted onto roots derived from the same genotype (J/J) or onto the roots of cvs Radja (J/R) and Volgogradskij (J/V) and the grafted plants have been grown at 0 and 50 mM NaCl. The rootstock did not have a significant effect on fruit yield in the absence of salt, while in saline conditions, fruit yield increased more than 60% in both graft combinations, with respect to J/J (Fig. 1). The increased tolerance of grafted plants seems to be related

**Table 2.** Dry weight of leaves and stems, plant height, total plant leaf area, yield, individual fruit weight, number of fruits/plant and plant water uptake of tomato cv. Daniela grown with (S) and without (NS) 50 mM NaCl and with (M) and without (NM) mist

Figures followed with the same letter in each column do not differ at  $P > 0.95$ .

Treatment	Leaves (g)	Stems (g)	Height (cm)	Leaf area (cm <sup>2</sup> )	Yield (kg/plant)	Fruit weight (g)	Fruit number	Water uptake (L/plant)
NS-NM	96 b	122 a	259 b	12088 c	5.8 b	125 b	47 a	190 a
S-NM	65 c	63 b	240 c	6796 d	1.7 d	53 d	32 b	92 c
NS-M	117 a	121 a	259 b	16620 a	7.1 a	139 a	51 a	121 b
S-M	123 a	122 a	265 a	13967 b	3.7 c	85 c	44 a	79 d



**Fig. 1.** Fruit yield, reduction percentage of fruit yield in saline medium respect to the non-saline medium, and Na<sup>+</sup> and Cl<sup>-</sup> concentrations in leaves of self-grafted Jaguar (J) plants and grafted onto Radja (R) and Volgogradskij (V) grown without salt (white bars) and at 50 mM NaCl (black bars). Error bars represent SE of the mean.

to the ability of rootstocks to regulate the transport of saline ions throughout the plant growth cycle, as the most productive grafted plants maintained lower leaf Na<sup>+</sup> and Cl<sup>-</sup> accumulation even after 90 d of salt treatment. With the aim of corroborating these data, the salinity response of the grafted plants is currently being studied by using the same cultivar (Jaguar) as scion and, as rootstocks, recombinant inbred lines (RILs) from two crosses (*S. lycopersicum* × *S. pimpinellifolium* and *S. lycopersicum* × *S. galapagense*), which show very different abilities to transport saline ions to the leaves. The selection of adequate rootstocks could reduce the toxic effect of saline ions, which is the main deleterious effect in the long-term.

## Conclusions

The genetics of physiological characters together with other tolerance components related to metabolic defences against salinity have to be studied in order to advance the breeding of genotypes tolerant to salinity. Heritabilities of the characters should be used to select the most relevant characters to evaluate the salt tolerance of segregating populations in QTL studies. As salinity in soils is variable and plant tolerance depends on the stage of plant development, plants should be phenotyped at several salinity concentrations and at the most sensitive plant stage(s).

QTL analysis allows an integrative approach to complex traits, such as salt tolerance, in three main ways: the analysis of multiple traits in the same segregating population; the contribution of QTL × E and QTL × QTL interactions to the total phenotypic variance and pleiotropic or regulatory effects of genes controlling quantitative traits; and candidate gene analysis. Such analyses under different salinity levels and plant stage(s) only can be properly and efficiently carried out using populations of DHs or RILs.

Genetic transformation will be a powerful tool in the breeding of complex characters, such as tolerance to salinity, if progress is made on the identification of genes involved in the process of salt tolerance, the isolation of genes from other sources of variation (i.e. crop species and wild related species), the design of vectors which allow the transfer and co-ordinate expression of several genes (as salt tolerance is a multigenic trait), and the identification and use of regulatory elements, which spatially and temporarily modulate the level of expression of the transgenes. Despite the present limitations, it is foreseeable that our ability to design the future breeding programmes based on genetic transformation will be strengthened with the data obtained from ongoing functional genomics projects.

Seedling pretreatment with drought or NaCl are interesting strategies to be applied when tomato plants have to be grown in saline soils or soils irrigated with saline water: the stress level necessary to trigger any adaptive response seems to be related to the degree of tolerance of the genotype. Increasing humidity around tomato plants effectively alleviates the deleterious effects of salt on tomato-plant growth and on fruit yield. Grafting tomato plants onto appropriate rootstocks also increased salt tolerance. Additional research is necessary to demonstrate if these three practices can be joined: priming seeds of rootstocks and scions or of rootstocks alone, grafting scions onto rootstocks, and growing the grafted plants under high relative humidity.

## Acknowledgements

This work has been partially supported by grants AGL2003-07427 (CICYT) RTA2001-113 and RTA2004-00075 (INIA). We thank Dr MJ Díez (COMAV, Universidad Politécnica de Valencia) for

supplying seeds of *S. pennellii* '20164', and Professor TJ Flowers for very valuable commentaries while editing the manuscript. We dedicate this paper to the memory of Professor Manuel Caro who initiated physiological salinity research in CEBAS and La Mayora (Spain).

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