COMPARATIVE ANALYSES OF PLANT RESPONSES TO SALINITY IN RELATED TAXA: A USEFUL APPROACH TO STUDY SALT STRESS TOLERANCE MECHANISMS

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Abstract

The progressive salinisation of irrigated cropland is causing substantial losses in agricultural production, a problem that will worsen due to climate change effects. Enhancing crop salt tolerance is a sensible strategy to achieve significant increases in crop yields, but requires a deep understanding of the underlying mechanisms. When challenged by salinity, all plants, regardless of their degree of tolerance, activate a series of basic responses, including the control of ion transport, the synthesis of compatible solutes for osmotic adjustment, or the activation of antioxidant systems. Yet, for a given species, the biological relevance and the relative contribution of different responses to the mechanisms of salt tolerance remain largely unknown. Over the last years, we have performed comparative analyses on the responses to salinity in different taxa, genetically related but with varying levels of tolerance. Correlating salt-induced changes in the concentrations of suitable biochemical stress markers with the relative tolerance of the investigated species, we are obtaining novel and interesting information on those mechanisms. Some examples with taxa of several genera are discussed, to show the usefulness of our approach.

Key words: climate change, ion transport, osmolytes, salt stress, salt tolerance.

INTRODUCTION

Modern agriculture is largely dependent on a limited number of cultivars of a few major crops, derived, directly or indirectly, from the 'Green Revolution' of the 1960s and 1970s (Borlaug & Dowswell, 2005). These cultivars were developed for a high-input, industrialised agriculture and can provide high yields under optimal – artificial – growing conditions, in either greenhouses or open fields, although generally require large amounts of agrochemicals (chemical fertilisers, herbicides and pesticides) and irrigation water; they are, however, relatively sensitive to stressful conditions, such as cold, high temperatures, waterlogging, drought or salinity. In fact, for all major crops there is a large difference between average yields and the record yields obtained under the most favourable growing conditions; these losses, which can vary from 50% to more than 80%, depending on the species, are mostly due to abiotic stress conditions affecting the plants in the fields; among them, drought and soil salinity are the environmental factors most relevant for this reduction of crop productivity (Buchanan et al., 2000).

In the current climate change scenario – with increasing average temperatures, reduced rainfall and alteration of the normal seasonal weather patterns – crops in arid and semi-arid regions are being affected by drought periods which are longer, more frequent and more intense than in the near past.

The progressive 'secondary' salinisation of irrigated land – by the accumulation of toxic ions dissolved in irrigation water – is also contributing to the extension of desertification of former fertile cropland.

Therefore, increasing, or even maintaining the present production levels is becoming a severe challenge for agriculture.

Additional factors, not directly dependent of climate change effects – such as the massive movement of rural population to the cities, change of land use (for urban development, tourism or industrialisation) leading to a further reduction of the area of available farmland, or

the growing demand of cereals and oilseed crops for biofuel production, competing with food – are also limiting the potential productivity of agriculture at the global level.

Crop production is still growing, both in absolute terms and *per capita*; however, the rate of growth has been decreasing since the mid-1980s. Nowadays, there is enough food to feed everybody on earth – although that food is not evenly distributed – but if this trend continues, soon this will not be true anymore.

STRATEGIES FOR INCREASING AGRICULTURAL PRODUCTION

In the present circumstances, it is clear that an increase in crop production cannot be based on a significant extension of the total agricultural land area, which is actually decreasing. As water for irrigation is becoming an increasingly scarce resource, it is also not possible to enlarge the area of land cultivated under irrigation, which is much more productive than rain-fed farmland. Also, there is an urgent need to switch the present agricultural practices to a more sustainable agriculture, stopping or reducing depletion of natural resources and the destruction of areas of high ecological value. Therefore, we should also exclude growing our present crops in low-fertility, marginal land, which would require the use of large amounts of chemical fertilisers and would not be sustainable.

An extension of the global area of biotech (transgenic) crops, including the development of varieties with new traits, will also contribute to increasing food production, as they provide higher yields than the corresponding conventional crops (ISAAA, 2017). Nevertheless, these transgenic plants are derived from 'Green Revolution' varieties and pose the same problems than non-transgenic cultivars regarding high inputs requirements and sustainability issues.

Many other strategies are being tried to increase, or at least maintain crop yields, but in the frame of a more sustainable agriculture. They include, for example, organic agriculture, which is obtaining good results in terms of productivity as well as a business, due to the interest of consumers on food products obtained without the use of agrochemicals.

There is also a 'new generation' of fertilisers, namely, slow-release and controlled-released fertilisers; they can (modestly) increase crop yields when used at the same doses than traditional fertilisers – or maintain the same production at lower doses – but their main advantage is that they could help protect the environment, being less contaminating for soil and water and having a smaller 'carbon footprint'. The application to crops of 'biostimulants', a disparate group of different substances (humic substances, protein hydrolysates, seaweed extracts, chitin-derived biopolymers, or some chemical elements) or microorganisms (beneficial fungi and bacteria) should also be mentioned as a means to enhance the efficiency of plant nutrition, facilitate growth under stress conditions and/or improve the quality of the harvested product (Boscaiu et al., 2018; Xu & Geelen, 2018).

Developing drought and salt-tolerant crops

All approaches mentioned above will no doubt contribute to improving crop yields, but the expected increase in food production will not be sufficient to cope with population growth. Since the most substantial reduction in productivity is due to environmental abiotic stress conditions, especially drought and salinity, the most effective alternative would be to develop crop varieties more resistant to salt and water stress. For this, all available strategies should be used including, obviously, traditional breeding techniques. This approach has not been very successful in the past, due to the complexity of the stress-tolerance traits, but now the breeder can use an array of modern molecular tools – marker-assisted selection (MAS), next-generation sequencing (NGS) technologies, high-throughput genotyping platforms, among others – which significantly increase the efficiency and reduce the time required to carry out breeding programmes; in fact some successful examples of crops with enhanced salt or water stress tolerance obtained by 'classical' breeding, have been reported in recent years, for instance several specific cultivars mentioned by Fita et al. (2015). Genetic engineering can also be used to express 'stress-tolerance' genes in transgenic crops; here again, many laboratory experiments point to the feasibility of this strategy. However, up

to now no biotech crop with enhanced abiotic stress tolerance is commercially cultivated in our fields, except for a drought-resistant maize variety, developed by Monsanto and BASF, expressing a bacterial RNA chaperonin gene (Castiglioni et al., 2008). Progress is also being made in the recovery and improvement of local or neglected crop varieties, and in the domestication of wild plants with relatively higher stress tolerance than our standard crops. Some of those wild species are extremely resistant in nature to salinity (halophytes) or drought (xerophytes), and could be the basis of a sustainable, 'saline' or 'arid' agriculture (Fita et al., 2015; Boscaiu et al., 2018).

We can conclude that the biotechnological improvement of crop abiotic stress tolerance, especially to drought and soil salinity, is the most promising strategy to quickly increase crop yields and food production, needed to feed a growing human population in the next few decades. To reach this goal, applying the different strategies mentioned in the previous paragraph, a deep understanding of the mechanisms underlying abiotic stress tolerance in plants is required.

ELUCIDATION OF THE MECHANISMS OF STRESS TOLERANCE IN PLANTS

Paradoxically, most studies on drought and salt tolerance in plants have been carried out using species that are not tolerant, mostly the model *Arabidopsis thaliana* or some crops, such as tobacco or rice. It is doubtful that the results obtained can be generalised to all species, but this is generally assumed, partly due to the confusion in the literature between two related, but distinct concepts: *responses* to stress and stress *tolerance*.

Stress responses *vs.* tolerance to stress
All plants, independently of their degree of tolerance, use the same general responses to water and salt stress (and also to other abiotic stresses), based on the activation of a series of conserved mechanisms, including: **i)** control of ion transport and ion homeostasis, at the cell and whole plant levels; **ii)** biosynthesis and accumulation of compatible solutes or osmolytes for osmotic adjustment; **iii)** activation of antioxidant enzymes and synthesis

of antioxidant compounds – since drought and high soil salinity generate oxidative stress as a secondary effect; **iv)** changes in gene expression leading to the synthesis of 'protective' proteins, such as heat-shock proteins, LEA proteins, osmotin, and many others.

Stress tolerance is obviously based on the activation of these conserved responses, justifying to some extent the use of non-tolerant models for its study. However, it is clear that the *efficiency* of the response must largely vary in different species, as plants show a very wide range of tolerance. Furthermore, the relative contribution of specific responses to the mechanisms of tolerance may also vary and, for a given species, is generally unknown.

Our experimental approach for the study of abiotic stress tolerance in plants
We are investigating the mechanisms of salt and drought tolerance in different plants, including some wild species, vegetable and ornamental crops and forest trees, such as taxa of the genera *Plantago*, *Juncus*, *Limonium*, *Phaseolus*, *Tagetes*, *Portulaca* or *Picea*, among others. Our work is based on the hypothesis that performing comparative analyses of the responses to stress of taxonomically – and, therefore, genetically – closely related taxa that show different degrees of stress resistance should help to distinguish those responses that are relevant for tolerance from those which are not, and thus contribute to elucidate general mechanisms of tolerance in plants. In our studies, as 'closely related taxa' we have included: **i)** wild species of the same genus, adapted to natural habitats affected by varying types and levels of environmental stress; **ii)** different ecotypes/varieties/cultivars of the same species, which often also show differences in stress resistance; **iii)** a crop and some of its wild relatives; or **iv)** different populations/provenances of the same species.

Our experimental strategy consists on applying salt stress (different concentrations of NaCl) and water deficit (withholding irrigation) treatments to the plants under controlled greenhouse conditions and determining the stress-induced changes in the levels of different biochemical stress markers associated to specific response pathways – ions, osmolytes,

antioxidant compounds, antioxidant enzyme activities. Correlation of the contents of these markers with the relative tolerance of the investigated taxa – estimated from their distribution in nature and/or the relative growth inhibition caused by the stress treatment – allows establishing which specific responses are involved in each case in tolerance mechanisms.

In the following sections, we describe a brief selection of published results to show the usefulness of the strategy outlined above. These examples are limited to salt tolerance mechanisms based on the control of ion transport and osmotic adjustments, in taxa of three genera: *Phaseolus*, *Plantago* and *Juncus*. Salt tolerance based on other plant responses, such as the activation of antioxidant systems, or the mechanisms of tolerance to drought, will not be mentioned here.

SALT TOLERANCE IN *Phaseolus*

Experimental material and salt treatments

Plants of three cultivars of common beans (*Phaseolus vulgaris*) – 'The Prince', 'Judía de Franco' and 'Maxidor' – and one cultivar of the runner bean (*P. coccineus*) – 'Moonlight' – were grown for three weeks in the presence of 0 (control), 50, 100 or 150 mM NaCl. After the treatments, the plants were harvested, and several growth parameters were determined: stem length, number of leaves, leaf fresh and dry weight, and leaf water content.

Based on the salt-induced inhibition of growth, the following ranking of salt tolerance was established: 'Maxidor' > *P. coccineus* ('Moonlight') > 'Judía de Franco' > 'The Prince'

Control of ion transport

Generally, Na^+ and Cl^- ions accumulate in plant leaves, to a greater or lesser extent, in response to NaCl treatments. In *Phaseolus*, Na^+ leaf levels increased with increasing external salinity, in a concentration-dependent manner, only in the most salt-sensitive cultivar, 'The Prince'. In 'Maxidor', the most tolerant, Na^+ content was maintained at the same level than in the non-stressed control at all external salt concentrations tested. In the second-most tolerant cultivar, 'Moonlight' (of *P. coccineus*)

and in 'Judía de Franco', Na^+ increased significantly (but only slightly in 'Moonlight') in the presence of the highest NaCl concentration tested (150 mM), but not at lower salinities. Cl^- ions accumulated in leaves in parallel with increasing external salinities, in all tested cultivars, and reaching in all cases absolute levels much higher than those of sodium. However, the qualitative patterns of accumulation were similar to those of Na^+; that is, the highest contents were measured in the least salt tolerant cultivar ('The Prince'), and the lowest in the most tolerant *cv*. 'Maxidor'. There is, therefore, a *negative* correlation between tolerance and the efficiency of ion transport to the aerial part of the plants, indicating that, in *Phaseolus*, salt tolerance is based, at least partly, on the inhibition of Na^+ (and, to a lesser extent, Cl^-) transport to the leaves.

Osmotic adjustment

According to the 'ion compartmentalisation hypothesis' (Wyn Jones et al., 1977), Na^+ and Cl^- ions must be sequestered in the cell vacuoles to avoid reaching toxic levels in the cytoplasm; this requires the accumulation of compatible solutes in the cytosol, to maintain cellular osmotic balance. Proline (Pro) is one of the commonest plant osmolytes. Leaf Pro contents increased in response to the salt treatment in the four *Phaseolus* cultivars, but its accumulation showed a negative correlation with their relative salt tolerance: Pro reached the highest levels in *cv*. 'The Prince' and the lowest in 'Maxidor'. This means that Pro cannot be directly involved in the mechanisms of salinity tolerance in *Phaseolus*, although it appears to be a reliable biochemical salt stress marker, accumulating in those cultivars that are relatively more sensitive and therefore more stressed in the presence of salt.

Regarding other putative osmolytes, glycine betaine did not accumulate in response to the salt stress treatment in any of the four *Phaseolus* cultivars. Sucrose and fructose contents clearly increased in *cv*. 'The Prince' in parallel with increasing external salinity, but not in the other three cultivars. Of those tested, only *myo*-inositol appears to be a functional osmolyte in this genus as its accumulation in

leaves correlates positively with the relative salt tolerance of the bean cultivars.

SALT TOLERANCE IN *Plantago*

Experimental material and salt treatments

Three species of the genus *Plantago*, *P. crassifolia*, *P. coronopus* and *P. major*, were selected for this study. The two first species are halophytes, growing in natural saline ecosystems, whereas *P. major* is considered a glycophyte and is only found in the field in habitats of low salinity. According to their distribution in nature, the relative salt tolerance of these species is: *P. crassifolia* ≥ *P. coronopus* > *P. major*. Plants of the three species were grown in the greenhouse for four weeks, watered with NaCl solutions of increasing concentration, from 0 (control) to 800 mM. Quantification of the degree of salt-induced growth inhibition confirmed the relative tolerance to salinity indicated above, for the three analysed *Plantago* species.

Control of ion transport

Leaf ion (Na^+, Cl^-) contents increased in the three *Plantago* species in parallel with increasing external salinity, both ions reaching similar absolute levels. Contrary to *Phaseolus*, in this case, the highest ion contents were measured in the two halophytes, slightly higher in the most tolerant *P. crassifolia*; the most sensitive *P. major* showed substantially lower concentrations of Na^+ and Cl^- in the leaves. This accumulation pattern indicates that, in *Plantago*, salt tolerance is based on the active transport of ions to the leaves. Interestingly, both halophytes showed relatively high leaf concentrations of Na^+ (in the most tolerant *P. crassifolia*, also of Cl^-) in control, non-stressed plants, whereas the two ions were present at much lower levels in the glycophyte *P. major*. This observation suggests that salt-tolerant plants of this genus can use inorganic ions as osmotica, even under conditions of low soil salinity, which supports the existence of active mechanisms of ion transport from roots to the aerial part of the plants.

Osmotic adjustment

It is well established that sorbitol is the functional osmolyte in the genus *Plantago*.

Accordingly, we measured relatively high leaf concentrations of this polyalcohol in the three selected *Plantago* species. The highest value, ~ 2 mmol g^{-1} DW, was observed in the presence of 800 mM NaCl in *P. crassifolia*, the most tolerant species. However, both the absolute levels and the patterns of accumulation of sorbitol in response to salt stress were similar in the three species. Therefore, even though sorbitol could be necessary for cellular osmotic balance, it cannot be responsible for the differences in salt tolerance, since there is no differential accumulation of the osmolyte in the three analysed taxa. Pro contents, on the other hand, were very low in the controls and largely increased in response to the salt treatment, but only at high external salt concentrations (600-800 mM NaCl) and, most important, only in the halophytes, not in the salt-sensitive *P. major*. Therefore, salt tolerance in *Plantago* seems to be partly dependent on the activation in tolerant species (but not in salt-sensitive ones) of the synthesis of a secondary osmolyte, Pro, in response to high salinity stress.

SALT TOLERANCE IN *Juncus*

Experimental material and salt treatments

In this case, we also selected three species, two halophytes (*Juncus maritimus* and *J. acutus*) and one glycophyte (*J. articulatus*). According to the salinity of the natural habitats of these species, and the degree of salt-induced growth inhibition in controlled salt treatments, the relative tolerance to salinity of these species is *J. maritimus* > *J. acutus* >> *J. articulatus*. Salt treatments were carried out by watering the plants with increasing NaCl concentrations, from 0 (controls) to 400 mM, during eight weeks.

Control of ion transport

Plants of the three selected *Juncus* species showed a progressive increase in the *root* levels of Na^+ and Cl^-, correlated to the increase of NaCl concentration in the watering solution. The absolute ion concentrations reached and the patterns of accumulation were similar for the two ions and the three species, regardless of their relative tolerance to salt. We also observed a concentration-dependent increase of Na^+ and Cl^- contents in the *shoots*, in response

to increasing salinity; although the concentration of the two ions was similar, as in roots, there were significant differences between species. We observed a clear *negative* correlation between ion concentrations and salt tolerance: the highest levels were reached in the less tolerant *J. articulatus*, followed by *J. acutus*, whereas *J. maritimus*, the most tolerant, showed the lowest accumulation of Na^+ and Cl^-. Therefore, salt tolerance in *Juncus* is associated with the *inhibition* of ion transport from the roots to the aerial part of the plants.

Osmotic adjustment

Glycine betaine (GB) and total soluble sugars (TSS) both increase with increasing salinity in the shoots of salt-treated *Juncus* plants. Although these osmolytes may contribute to osmotic balance in this genus, their patterns of accumulation and the concentrations reached were similar in the three selected species, so that salt-induced GB and TSS biosynthesis cannot be responsible for the observed differences in tolerance. Pro contents, on the other hand, were very low in the controls and increased > 20-fold in the presence of 400 mM NaCl, but only in the salt-tolerant *J. maritimus* and *J. acutus*; in the glycophyte *J. articulatus*, the increase of Pro concentration with respect to the control, although statistically significant, was less than twofold. Therefore, Pro accumulation is most likely involved in the mechanisms of salt tolerance in *Juncus*, as it correlates *positively* with the relative degree of tolerance of the investigated species.

SALT TOLERANCE AND K^+ HOMEOSTASIS

An increase in cellular Na^+ concentration is generally accompanied by a decrease in K^+ levels, as the two cations compete for the same transport proteins (Rodríguez-Navarro, 2000). Mechanisms leading to the maintenance of relatively low Na^+/K^+ ratios under high salinity conditions are considered relevant for tolerance. A reduction in leaf K^+ contents in response to salt treatments has been observed in different species, in agreement with the general behaviour mentioned above, whereas in other species K^+ is maintained at the same level than in the non-stressed controls. Interestingly,

some tolerant plants show a peculiar pattern of salt-induced changes in leaf K^+ contents, first decreasing at low or moderate salinities, and then increasing again in the presence of higher external salt concentrations. This indicates the specific activation of K^+ transport from roots to leaves in response to strong salt stress conditions. We have observed this phenomenon, for example, in the tested halophytes of the genera *Plantago* (*P. crassifolia* and *P. coronopus*) and *Juncus* (*J. maritimus* and *J. acutus*), but not in the more salt-sensitive species *P. major* and *J. articulatus*, respectively. These data support the relevance of the activation of K^+ transport to the aerial part of the plants in different tolerant taxa.

SUMMARY OF SALT STRESS TOLERANCE MECHANISMS IN THE SELECTED GENERA

In *Phaseolus*, salt tolerance is dependent, at least in part, on specific mechanisms blocking transport of Na^+ cations (and, to a lesser extent, of Cl^- anions) from roots to leaves, and on the accumulation of *myo*-inositol for osmotic balance and as an osmoprotectant. Pro is a reliable marker of stress, accumulating to higher levels on those taxa which are more sensitive to stress (and, therefore, more stressed at the same salinity levels), as compared to related more tolerant taxa. However, Pro does not seem to be directly involved in salt tolerance mechanisms (Al Hassan et al., 2016c).

In *Plantago*, salt tolerance depends on the efficient transport of toxic ions (Na^+, Cl^-) to the leaves – where they are predominantly stored in vacuoles – and on the activation of K^+ transport under high external salinity. Plants of this genus use sorbitol as the main osmolyte for osmotic adjustment, but tolerant species also accumulate a secondary osmolyte (Pro) at high external salinity. Salt-tolerant *Plantago* taxa can accumulate Na^+ (or both, Na^+ and Cl^-) in the leaves, to be used as osmotica, also under low soil salinity conditions (Al Hassan et al., 2016d).

Finally, in *Juncus*, salt tolerance is associated with the inhibition of Na^+ and Cl^- transport from roots to shoots, the activation of K^+

transport to the leaves in the presence of high external salt concentrations, and the salt-induced accumulation of Pro for osmotic adjustment and osmoprotection (Al Hassan et al., 2016a).

A GLIMPSE TO OTHER SPECIES

Additional studies have revealed that some of the responses to salt stress mentioned above for *Phaseolus*, *Plantago* or *Juncus* are relevant for tolerance also in other plants species, unrelated taxonomically. For example, in oleander (*Nerium oleander*) salt tolerance is dependent on mechanisms blocking transport of toxic ions to the leaves (as in *Juncus*), and on the accumulation of GB and TSS as functional osmolytes (Kumar et al., 2017). In Norway spruce (*Picea abies*), salt stress activates transport of Na^+ and K^+ from the roots to the needles and accumulation of Pro, whereas soluble sugars do not seem to be involved in tolerance mechanisms (Schiop et al., 2015). In *Inula crithmoides*, an extremely tolerant succulent halophyte, salt tolerance is based on the efficient transport of toxic ions to the aerial part of the plants (as in *Plantago*), the activation of K^+ transport from the roots at high external salinity, and the use of GB as the main physiological osmolyte, with contribution of some sugars (arabinose, fructose and glucose) to osmotic adjustment under stress (Al Hassan et al., 2016b); similar mechanisms seem to operate in the genus *Limonium* – which includes a large number of halophytes – except that Pro, not GB, is the major functional osmolyte (Al Hassan et al., 2017). Inhibition of Na^+ transport to the leaves and maintenance of high K^+ concentrations even at high salinity levels are also important for salt tolerance in the genus *Silene* (Kozminska et al., 2018).

CONCLUSIONS

The general conclusion of the work carried out in our laboratory over the last years, and partly summarised here, is that the mechanisms of salt stress tolerance – as well as tolerance to drought and other abiotic stresses – vary widely in different plant species, even though they are based on the same conserved responses. Our strategy of performing comparative studies of the responses to stress in closely related taxa, but showing varying degrees of tolerance, has been proved to be very useful for the elucidation of those mechanisms. However, the studies must be carried out in different genera, species, cultivars or populations, as no single model, not even *Arabidopsis thaliana*, can provide a general and accurate view of the subject.

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