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Evans review

Tissue tolerance: an essential but elusive trait for salt-tolerant crops

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Abstract. For a plant to persist in saline soil, osmotic adjustment of all plant cells is essential. The more salt-tolerant species accumulate Na^+ and Cl^- to concentrations in leaves and roots that are similar to the external solution, thus allowing energy-efficient osmotic adjustment. Adverse effects of Na^+ and Cl^- on metabolism must be avoided, resulting in a situation known as 'tissue tolerance'. The strategy of sequestering Na^+ and Cl^- in vacuoles and keeping concentrations low in the cytoplasm is an important contributor to tissue tolerance. Although there are clear differences between species in the ability to accommodate these ions in their leaves, it remains unknown whether there is genetic variation in this ability within a species. This viewpoint considers the concept of tissue tolerance, and how to measure it. Four conclusions are drawn: (1) osmotic adjustment is inseparable from the trait of tissue tolerance; (2) energy-efficient osmotic adjustment should involve ions and only minimal organic solutes; (3) screening methods should focus on measuring tolerance, not injury; and (4) high-throughput protocols that avoid the need for control plants and multiple Na^+ or Cl^- measurements should be developed. We present guidelines to identify useful genetic variation in tissue tolerance that can be harnessed for plant breeding of salt tolerance.

Additional keywords: barley, chickpea, chloride, osmoregulation, rice, sodium, wheat.

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Introduction

Osmotic adjustment is an essential plant response to a saline soil. The osmotic pressure in every cell must increase in order to maintain turgor, and in every organelle in order to maintain volume. This is achieved by increased accumulation of Na^+, Cl^- or organic solutes. Halophytic plants can grow in soils with over 500 mM NaCl (in excess of seawater), and tolerate over 500 mM Na^+ and Cl^- in their tissues (Flowers and Colmer 2008). This must be, at least in part, due to their ability to sequester much of the salt in the cell vacuoles, as there is a limit to which Na^+ and Cl^- can accumulate in the cytoplasm without an adverse effect on metabolic processes. It is not known what this limit is, because the cytoplasm is made up of many compartments that are difficult to isolate and measure, but for chloroplasts the upper limit is possibly 100–200 mM Na^+ (Flowers *et al*. 2015). The concentration in the cytosol is likely to be considerably less, possibly 10–30 mM (Munns and Tester 2008; Kronzucker and Britto 2011).

Salt-tolerant crop species like barley are able to grow productively in soils with up to 200 mM NaCl in the soil solution (Ayers *et al*. 1952) and tolerate similar concentrations of Na^+ and Cl^- in their leaves (e.g. Fricke *et al*. 1994; Boyer *et al*. 2008). The ions therefore provide osmotic adjustment with little need for organic solutes except in the cytoplasm.

In contrast, in some of the more salt-sensitive species, Na^+ is at much lower concentration than Cl^- in the leaves and Cl^- contributes more to osmotic adjustment. Examples are grapevine (Walker *et al*. 2004), bread wheat (Gorham *et al*. 1990; Husain *et al*. 2004) and soybean (Ledesma *et al*. 2016). In chickpea, a particularly salt-sensitive species, Na^+ rather than Cl^- is the major cause of damage to leaves (Khan *et al*. 2016). Such findings are consistent with the conclusion that Na^+ is potentially a more toxic ion than Cl^-. A large portion of osmotic adjustment in some salt-sensitive species occurs with organic solutes that are expensive to synthesise and divert assimilate from growth processes such as cell wall and protein

synthesis (Munns and Gilliham 2015). The 'cost' of this osmotic adjustment can be seen in the yield reductions in saline soil despite lowered leaf Na^+ concentration. Although introduction of *Nax2* (Na^+-excluding gene, *TmHKT1*;*5-A*) into durum wheat increased yield by 25% in saline soil, the yield was still less than in non-saline soil (Munns *et al*. 2012).

The capacity of tissues to function while containing a high internal Na^+ and Cl^- concentration is known as 'tissue tolerance'. A key mechanism is intracellular compartmentation of Na^+ and Cl^- so that most of the ions are contained in vacuoles and the concentrations in the cytoplasm remains relatively low (Flowers *et al*. 1977). This mechanism can also be called 'cellular tolerance' and defined as the ability of a cell to compartmentalise Na^+ and Cl^- in vacuoles at concentrations that would be toxic in the cytoplasm. However, as we explain below, other factors are also involved in tissue tolerance.

This viewpoint paper explores the concept of tissue tolerance of Na^+ and Cl^- in crop plants – the ability of organs and their component cells to maintain function in the presence of elevated tissue Na^+ and Cl^- concentrations. The aim is to clarify the present understanding of tissue tolerance and to identify methods that allow selection of genotypes with greater ability to tolerate high concentrations of Na^+ and Cl^- in their leaves, to enable tissue tolerance to be used as a trait in plant breeding for saline soils.

Three inter-related concepts: osmotic adjustment, intracellular compartmentation, and tissue tolerance

Osmotic adjustment

For a plant in saline soil, osmotic adjustment is essential. The osmotic pressure of each cell should increase to match the increase in the osmotic pressure of the soil solution, and be due to an increase of solute content, not loss of water, so turgor and volume are maintained. The increased osmotic pressure could be generated by the synthesis of organic solutes such as sugars that reach concentrations great enough to contribute a significant osmotic pressure, but this would be at the expense of growth as those solutes are no longer available for cell wall and protein synthesis (Yeo 1983; Munns 1988). If Na^+ and Cl^- were completely excluded, osmotic adjustment in plants growing in a soil solution of 200 mM NaCl would need increases in organic solutes of 400 mM in order to balance the osmotic pressure of the external salinity, and with a simple sugar like glucose (molecular weight 180), the amount needed would make up 36% of the plant dry mass (Munns and Gilliham 2015). The percentage would be almost double this if sucrose (molecular weight 342) were used. It would be impossible for more than half of the plant's incoming assimilate to be diverted to pools of solutes for osmotic adjustment as normally 60–90% of the photosynthate is respired to produce energy for general maintenance of metabolic functions (Amthor 2000; Jacoby *et al*. 2011).

Alternatively, the required increase in osmotic pressure could come from Na^+ and Cl^- taken up from the soil, which demands much less ATP than the synthesis of organic solutes (Greenway and Munns 1983; Yeo 1983; Raven 1985). This energy-efficient strategy is used by halophytes (Flowers and Colmer 2008) and the more salt-tolerant non-halophytes such as barley, where the increase in both Na^+ and Cl^- concentration in the shoot tissues is similar to the increase in the external medium (Boyer *et al*. 2008). As explained below, the increases in Na^+ and Cl^- in the tissue are largely confined to the vacuole, so organic solutes are still needed – but only for osmotic adjustment in the cytoplasm. This is not such a big energy drain on the plant as the volume of the cytoplasm of fully expanded cells is relatively small, in the order of one tenth of the total cell volume. In leaves it is ~2% for epidermal cells (Storey *et al*. 1983) and parenchyma cells (Hajibagheri *et al*. 1984), but for cells containing chloroplasts is up to 33% for mesophyll cells of C_3 plants (James *et al*. 2006*b*) and 56% for bundle sheath cells of C_4 plants (Storey *et al*. 1983).

Intracellular compartmentation

The concept of 'intracellular compartmentation' of Na^+ and Cl^- was established in the 1970s after the finding that enzymes extracted from halophytes were significantly inhibited *in vitro* when NaCl concentrations increased above 80 mM, yet halophyte leaves had concentrations approximately five times this (reviewed by Flowers *et al*. 1977). Enzymes extracted from halophytes had a similar response to NaCl *in vitro* to those extracted from non-halophytes (Greenway and Osmond 1972; Flowers 1972). This led to the consensus that the tolerance to high concentrations of these ions in the tissues was determined by the ability of cells to compartmentalise most of the Na^+ and Cl^- within the vacuoles, rather than any special tolerance of enzymes in the cytoplasm to high Na^+ or Cl^-. The osmotic pressure in the cytoplasm would be balanced with organic solutes and K^+ (Wyn Jones and Gorham 2002). It was argued that salt-tolerant plants would have low Na^+ and Cl^- in the cytoplasm and its various organelles and compartments, and so avoid toxicity, and also have low ion concentrations in the cell walls and avoid dehydration. In addition, coping with the formation of reactive oxygen species (ROS) and their removal to minimise oxidative stress (Bose *et al*. 2014) can also contribute to maintenance of organelle and cell functioning in salinised plants, although ROS are also involved in cellular signalling (Miller *et al*. 2010).

Tissue tolerance

This concerns the capacity of organs to function while their tissues or cells contain high concentrations of Na^+ or Cl^-. Observations by Yeo and Flowers (1983) that rice genotypes differed in the severity of chlorosis that was not simply related to differences in tissue concentrations of Na^+ resulted in the concept of 'tissue tolerance'. Intracellular compartmentation is the main component of tissue tolerance, but other mechanisms contribute, as discussed below.

Tissue tolerance – definition and component parts

Our definition of tissue tolerance is the ability of an organ to maintain function in the presence of elevated tissue Na^+ and Cl^- concentrations. The concentration should be as high as necessary for the purpose of osmotic adjustment, but toxicity in the cytoplasm must be avoided.

Another definition of tissue tolerance could be a relative concept that has a practical application for breeding within a given species: the ability of one genotype to tolerate leaf Na^+

and Cl^- concentrations above that in another; 'tolerance' being defined as functional normality, that is no loss of chlorophyll, no dehydration, and sufficient photosynthetic activity to sustain growth (the growth rate being set by the osmotic stress of the soil solution).

It would be satisfying to have a more precise definition, such as 'the ability of a cell or tissue to tolerate internal Na^+ or Cl^- above *x* mM', that is, a concentration that is toxic to the cytoplasm, or a specific component of it, and therefore indicates that these ions are preferentially compartmentalised in the vacuole. However, we do not know exactly what this potentially toxic concentration of Na^+ or Cl^- is (Cheeseman 2013). Increases in tissue Na^+ are usually accompanied by decreases in tissue K^+, and it is possible that regulation of cytosolic K^+ concentrations at an optimum level or 'homeostasis' might be critical for tolerance (Shabala and Pottosin 2014). It is also possible that the toxic effect of Na^+ might be related to its competition with K^+ for K^+-requiring enzymes so that the cytoplasmic Na^+/K^+ ratio might be more significant than the Na^+ concentration itself (Shabala and Cuin 2008). We note that some *in vitro* studies comparing the effects of NaCl and KCl on enzymes involved in cellular metabolism or photosynthesis have found the inhibitory effects of higher concentrations to be identical (e.g. Greenway and Osmond 1972; Osmond and Greenway 1972; Besford and Maw 1976). This means that further work is needed to understand exactly where and how Na^+ and Cl^- are damaging or toxic.

Component parts are defined as a range of factors acting together that are likely to confer tissue tolerance. These factors are shown in Fig. 1 and can be broadly separated into two main categories; (1) those involved in the transport of Na^+ or Cl^-; and (2) those involved in maintaining the functional water status of the leaf.

Fig. 1 indicates the component parts of tissue tolerance to Na^+ and Cl^-. These ions can be partitioned between different cell types, and Cl^- is often reported to be higher in epidermal cells than mesophyll cells (Conn and Gilliham 2010). In wheat and barley, Na^+ was similar in the mesophyll and epidermis; however, K^+ accumulated at much higher concentrations in the mesophyll compared with the epidermis so that the K^+/Na^+ ratio was more favourable in these key cells (James *et al*. 2006*b*). Ideally, Na^+ and Cl^- concentrations should be high in the vacuole as these ions are a 'cheap' source of osmotic adjustment, but at the same time be below potentially toxic concentrations in the cytoplasm. Vacuolar compartmentation is required to maintain low cytoplasmic concentrations of Na^+ and Cl. Once the vacuolar storage capacity of cells is reached, and these ions are still entering the leaf via the xylem, concentrations in cytoplasmic compartments will increase substantially and approach concentrations that induce cell damage. Alternatively, Na^+ and Cl^- could build up in the apoplast, leading to cellular dehydration (Oertli 1968; Flowers and Yeo 1986). Preventing oxidative stress from ROS can be a factor when plants are exposed to salinity (Miller *et al*. 2010).

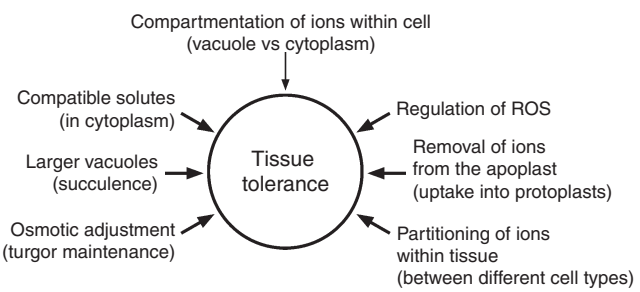

Fig. 1. Tissue tolerance of potentially toxic ions such as Na^+ and Cl^- is a complex trait where a number of factors contribute to the maintenance of low Na^+ and Cl^- concentrations in the cytoplasm of key cell types (e.g. mesophyll cells in leaves) and the maintenance of cell volume and turgor. The factors include vacuolar compartmentation which removes Na^+ and Cl^- from the cytoplasm and provides osmotica in vacuoles, adequate K^+ and compatible solutes to provide osmotic balance in the cytoplasm, and regulation of ROS. These together enable the tissue to function in the presence of elevated Na^+ and Cl^- concentrations. See text for full description.

An increase in succulence (measured as water per unit leaf area) is a common response of dicotyledonous halophytes to external salinity (Polle and Chen 2015). Succulence leads to larger cells with a resultant dilution of salt without an increase in leaf area. Increased succulence also occurs in many dicot non-halophytes. In four species of *Brassica*, the water per unit leaf area increased by 50–70% when plants were grown in 200 mM NaCl; for example the succulence of canola (*Brassica napus* L.) increased from 176 to 306 g m^{-2} (Ashraf and McNeilly 1990). Development of succulence is due to changes in orientation of cellulose fibrils in enlarging cell walls. The cell wall enzymes xyloglucan endotransglucosylase/hydrolases (XTHs) are likely involved in salt-elicited leaf succulence in higher plants (Polle and Chen 2015).

Tissue tolerance is therefore physiologically and genetically complex, a result of the coordinated contribution of a range of components. We now discuss ways to screen for tissue tolerance and how these various approaches might be used in a breeding program.

Genetic variation in tissue tolerance for the purpose of plant breeding

Measurement of genetic variation of salt tolerance in rice (*Oryza sativa* L.) by Flowers and Yeo (1981) showed that genotypes differed in survival of a period in NaCl that was not simply related to differences in leaf tissue concentrations of Na^+. Examination of the possible reasons underlying the variation in tolerance showed that several traits could be involved (Yeo *et al*. 1990). Apart from a low shoot Na^+ accumulation, the other traits were partitioning of salt into older rather than younger leaves, plant vigour and the tolerance to salt within the leaves. This latter trait could be quantified by the rate at which chlorophyll was lost from the leaves with increase in leaf Na^+ concentration following salinisation of seedlings and resulted in the concept of 'tissue tolerance' (Yeo and Flowers 1983; Fig. 2).

Experiments on durum wheat (Munns and James 2003) resulted in similar findings to rice. Fig. 3 compares the effect of salinity on the growth of nine durum genotypes with different rates of Na^+ transport to leaves. The figure is drawn with leaf 3 concentration Na^+ on the *x*-axis, and with biomass as the dependent variable, as leaf 3 was the major photosynthesising leaf at that stage of plant development and would be supplying

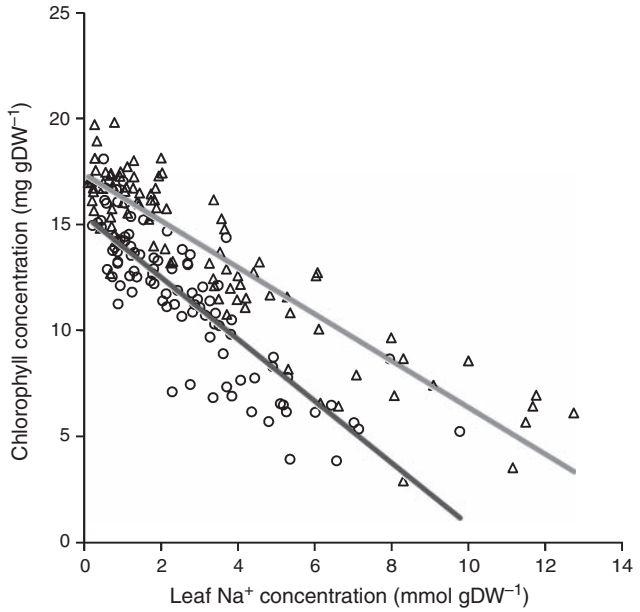

Fig. 2. Relationship between chlorophyll and Na^+ concentrations in leaf 3 of ~100 individual plants of two genotypes of rice (*Oryza sativa*) after exposure to 50 mM NaCl (for details of the method see Yeo and Flowers 1983). The two genotypes illustrated lost 50% of their chlorophyll when the leaf Na^+ reached 5.25 mmol g^{-1} DW (○) or 7.83 mmol g^{-1} DW (△).

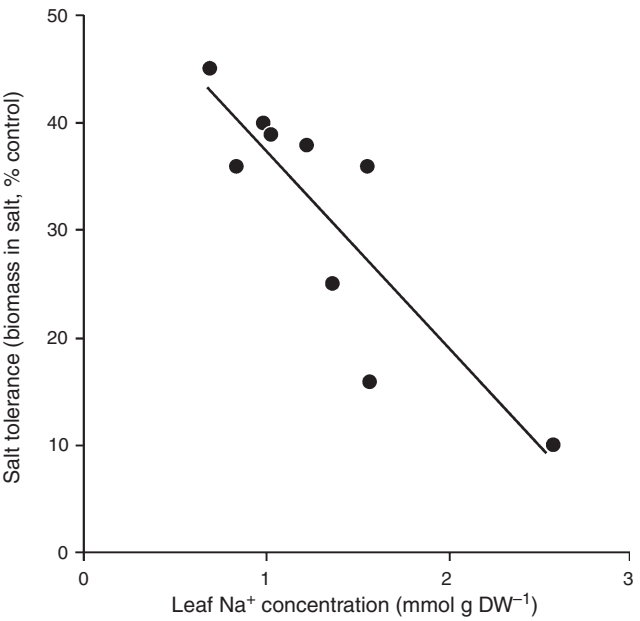

Fig. 3. Relationship between salinity tolerance (shoot dry mass in salt treatment as % of control) and leaf Na^+ concentration in nine durum wheat genotypes (*Triticum turgidum* ssp. *durum*). NaCl (150 mM) was added gradually before leaf 3 appeared. Na^+ concentrations were measured on leaf 3 after 10 days, and shoot dry mass after 24 days. Values are means ($n = 5$). Data presented in Munns and James (2003). The leaf water content was ~5 : 1 (g water g^{-1} DW) so the concentration of Na^+ in tissue water for 1 mmol g^{-1} DW was ~200 mM.

the bulk of assimilate to the growing leaves. In Fig. 3 it can be seen that two durum wheat genotypes had a leaf Na^+ concentration of 1.5 mmol g^{-1} DW (equivalent to 300 mM in tissue water), but one had twice the biomass production of the other (as a % of control) indicating a higher tolerance of Na^+ in the leaf tissue. If these data can be replicated in different experiments and in different environments, then there is good grounds for assuming that these two genotypes differ in tissue tolerance.

Barley also appears to vary in tissue tolerance. A study of 187 Tibetan wild barley (*Hordeum vulgare* L. ssp. *spontaneum*) accessions by Qiu *et al*. (2011) showed a significant correlation ($r = 0.455$) between Na^+ accumulation and salt tolerance (% biomass after 27 days at 300 mM NaCl) but there were many accessions with the same Na^+ concentration and with different salt tolerance. A similar finding can be seen in the study by Chen *et al*. (2005) for seven barley cultivars grown at 320 mM NaCl. This type of analysis indicates that variation in tissue tolerance exists, but does not allow it to be quantified.

Similar to toxicological approaches, and presuming that the chlorosis in rice was a response to (and therefore a useful indicator of) the toxic effect of the Na^+ in the tissues, Yeo and Flowers (1983) suggested a tissue tolerance index using the concentration of Na^+ in the leaf at which the chlorophyll concentration was reduced by 50%. These authors sought to establish a quantitative screening method to enable quantitative trait loci (QTL) analysis and marker-assisted selection of this trait for breeding salt tolerant rice. Further work showed a five-fold difference in tissue tolerance trait between genotypes. However, it was not correlated positively with survival (Yeo *et al*. 1990), rather it was a contributory trait, along with other traits such as low leaf Na^+ concentration, sequestering Na^+ in older rather than younger leaves, and plant vigour, which all contributed to salt tolerance. Only plant vigour was strongly correlated with survival (Yeo *et al*. 1990).

The concept of tissue tolerance first proposed as a potential trait for rice breeding by Yeo and Flowers (1986) has regrettably not yet progressed to utilisation in plant breeding.

Efforts at quantifying genetic variation in tissue tolerance

A study on genetic variation in salt tolerance of tetraploid wheat (i.e. durum wheat and its relatives) had identified an outstanding landrace, ssp. *polonicum* (Line 455), with high Na^+ concentrations in leaves coupled with better maintenance of green leaf area and biomass production (described in Munns and James 2003). To quantify its tissue tolerance, a method similar to that proposed in rice by Yeo and Flowers (1983) was used to compare Line 455 with the salt-sensitive Australian durum cultivar Wollaroi (James *et al*. 2002). The results, shown in Fig. 4, indicated that cv Wollaroi had a greater degree of chlorosis for a given Na^+ concentration above 1 mmol g DW^{-1} (above 200 mM on a leaf tissue water basis) than Line 455. The Na^+ concentration at 50% loss of chlorophyll was higher in Line 455 than Wollaroi, indicating it had greater tissue tolerance; however, at this stage turgor was very low, and photosynthesis was undetectable. At this stage, increases in Na^+ concentration were rapid, having been steady for the previous 17 days (the circled data).

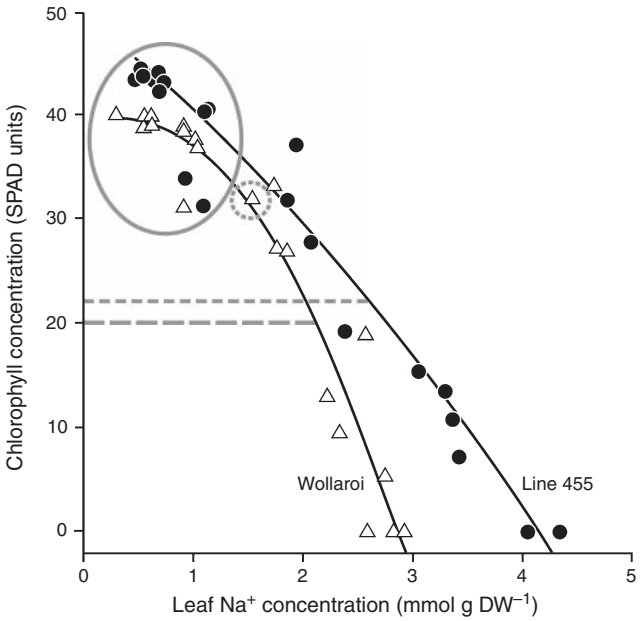

Fig. 4. Relationship between chlorophyll concentration and leaf Na^+ concentration (leaf 3) for durum wheat cv. Wollaroi and Line 455 grown in 150 mM NaCl over 3 weeks (adapted from James *et al*. 2002). The dashed lines indicate a 50% drop in chlorophyll concentration for Wollaroi (– –) and Line 455 (- - -). The large circle indicates leaves with the same turgor and photosynthesis rate (on an area basis) as controls. The small dashed circle indicates a sample with reduced turgor and photosynthesis. All samples with Na^+ concentrations higher than 1.6 mmol g^{-1} DW had very low or zero turgor and photosynthesis.

The differences between genotypes in leaf Na^+ concentration at 50% loss of chlorophyll were relatively small, so follow-up experiments were done to confirm these values. This proved difficult as the relationship between loss of chlorophyll versus tissue Na^+ concentration was different for leaves 1, 2 and 3, indicating there was no absolute relationship between the degree of leaf chlorosis and the Na^+ concentration in that leaf. Key lessons learnt from these experiments were that measurements should not be made on dying or dead tissues, and that the focus should be on still-healthy functional leaf tissue to determine the highest Na^+ concentration tolerated before injury occurs or decline in function such as photosynthetic capacity.

The study also indicated that measurements of injury such as membrane leakage or lipid peroxidation, even at the early onset of tissue damage, would be too late to provide a quantitative measure of tissue tolerance, as would be Na^+ measurements after visible injury. Na^+ concentrations, which increase rapidly after any loss of chlorophyll (e.g. James *et al*. 2002), can rise because of increased import (for instance, if the storage capacity of the lower part of the leaf or stem is exceeded), decreased export (if retranslocation processes are significant), or loss of water. Measurements that would predict the time at which injury occurred or when Na^+ concentrations might start to escalate were investigated. It was found that chlorophyll concentration (as measured with a SPAD meter) were as good as the chlorophyll fluorescence parameter F_v/F_m in detecting when irreversible damage occurs to photosystem II and that the only fluorescence parameter that predicted this injury was NPQ, a measurement not rapid or feasible for a large number of genotypes (James *et al*. 2002).

Salinity stress, like other abiotic stresses, is associated with changes in levels of ROS and the various antioxidants that detoxify them or regulate their concentration (Miller *et al*. 2010). However, such measurements have not yet proved to be of diagnostic use for screening for salt tolerance. For example, consistent correlations with antioxidant activity and salt tolerance across genotypes were not found with barley (Maksimovic *et al*. 2013) or halophytes (Bose *et al*. 2014).

Another approach is to look for genetic variation in K^+ concentration in leaves of plants in saline conditions, as maintaining the optimal K^+ concentration in the cytosol is critical for cell viability. A lower K^+ efflux from leaves in the presence of NaCl treatment may indicate a greater ability to maintain a higher K/Na^+ ratio in the cytoplasm. A positive correlation was found between the ability of salt-treated leaves to retain K^+ and the salt tolerance of whole plants in wheat and barley (Wu *et al*. 2013).

'Tissue tolerance' therefore should be broadly defined as the capacity of the cells or tissues to continue to function without injury despite high internal Na^+ or Cl^- concentrations, and in the face of a significant osmotic stress caused by the external NaCl concentration in the root zone. Thus, quantification of 'tissue tolerance' requires a measure of metabolic or physiological functioning in the face of increasing internal Na^+ or Cl^- concentrations (high concentrations experienced over a prolonged period of time). As one example, the relationship between photosynthesis and leaf Na^+ concentration differed between two chickpea genotypes of known differences in tolerance to saline soil. The salt-sensitive one had a greater reduction in photosynthesis per increase in tissue Na^+ (Khan *et al*. 2016). The same salt-sensitive genotype also suffered a greater reduction in photosynthesis via non-stomatal limitations and greater assessed damage to photosystem II at similar leaf ion concentrations as the more tolerant genotype (Khan *et al*. 2015), indicating that these two chickpea genotypes differ in leaf tissue tolerance of Na^+.

Genes involved in tissue tolerance – can these provide a molecular approach to screening?

At the cellular level tissue tolerance is achieved by compartmentation of Na^+ and Cl^- in the vacuole, together with the synthesis of compatible solutes and their location within the cytoplasm to balance the osmotic pressure of the ions in the vacuole. Previous reviews have covered the tonoplast-located Na^+ and Cl^- transporters and the H^+-pumps likely to be involved in compartmentation (Hedrich 2012; Shabala 2013). Compatible solutes have been reviewed for halophytes (Slama *et al*. 2015) and glycophytes (Deinlein *et al*. 2014; Roy *et al*. 2014).

Briefly, transporters traditionally thought to be of primary importance in vacuolar Na^+ sequestration are tonoplast-localised H^+-ATPases and H^+-PP_iases, which generate the membrane potential and proton motive force needed to drive Na^+ uptake into the vacuole via tonoplast-localised Na^+/H^+ exchangers such as NHX1 and NHX2. The greater ability of halophytes

than non-halophytes to sequester Na^+ in their vacuoles may be related to the constitutive expression of tonoplast Na^+/H^+ antiporters and the further stimulation of their activity under saline conditions (Barkla *et al*. 1995; Glenn *et al*. 1999), whereas in non-halophytes tonoplast Na^+/H^+ antiporters are salt-inducible (summarised by Shabala 2013). The activity of Na^+/H^+ antiporters is energised by the vacuolar H^+ pumps, so increased activity of Na^+/H^+ antiporters should be accompanied by increased activity of tonoplast H^+-ATPases or H^+-PP_iases. This activity, however, would place an additional demand on the ATP (or PP_i in the case of the H^+-PP_iase) pool and on respiratory activity. Knockouts of both H^+ pumps and NHX proteins have salt-sensitive phenotypes (Krebs *et al*. 2010). However, the exact role of NHX proteins in salt tolerance and plant development is unclear. The NHX1 proteins may operate principally as K^+ rather than Na^+ exchangers (Bassil *et al*. 2011; Barragan *et al*. 2012), with their major role being the regulation of cytosolic K^+ or pH. Furthermore, transcriptional changes in *NHX1* and *NHX2* expression were not correlated with salt tolerance in three barley cultivars (Adem *et al*. 2014), and overexpression of *AtNHX1* in barley did not improve its salt tolerance (Adem *et al*. 2015). This may be because these proteins are regulated post-translationally. It is also perplexing that a quadruple knockout of vacuolar and Golgi NHX proteins in *Arabidopsis thaliana* (L. Heynh.) had a reduced salt sensitivity, although root growth was slower under non-saline conditions (McCubbin *et al*. 2014). However, the knockouts of Golgi-localised NHX proteins are salt sensitive and these proteins are suggested to be involved in pH homeostasis of vesicle trafficking compartments (Bassil *et al*. 2011), and possibly of secretion of Na^+ into the vacuole via these (Reguera *et al*. 2015).

A chloride-cation-cotransporter (CCC) that is present on the Golgi may also provide a mechanism of Na^+ and Cl^- loading of vesicles: examples have been reported in grapevine and *Arabidopsis* (Henderson *et al*. 2015). In rice, a similar transporter (OsCCC1) was found to transport Na^+, K^+ and Cl^- into roots and shoots. It was speculated to be on the plasma membrane, and is thought to have a role in maintaining the osmotic potential of plant cells required for elongation (Chen *et al*. 2016).

Successful Na^+ sequestration into vacuoles requires both efficient loading and retention, i.e. very low back-leakage of Na^+, which is of particular importance for halophytes with very high internal Na^+ concentrations (Leach *et al*. 1990). This may be due to slow vacuolar (SV) and fast vacuolar (FV) channels with particularly low rates of leakage (Shabala 2013). These channels were studied in the mesophyll cells of the salt tolerant *Chenopodium quinoa* Willd. and it was found that the numbers of SV and FV channels differed between different genotypes and correlated with differences in salt tolerance (Bonales-Alatorre *et al*. 2013). Polyamines and choline, a precursor of the compatible solute glycine betaine, may also play a role in prevention of Na^+ leakage out from the SV channel as these compounds in the cytosol inhibit SV channel activity (Pottosin and Shabala 2014; Pottosin *et al*. 2014). In addition to avoiding possible toxicity in the cytoplasm, prevention of Na^+ and Cl^- back-leakage would also diminish the need for excessive expenditure of energy to maintain ion compartmentation (Shabala and Mackay 2011).

A study with three barley cultivars with contrasting salt tolerance looked at expression of five candidate genes mentioned above that are considered to be involved in cellular tissue tolerance (Adem *et al*. 2014). The study confirmed that tissue tolerance was a dominating component of the overall plant responses to salinity. However, it was not possible to infer which cultivars were salinity tolerant based solely on expression profiling of candidate genes at one specific time point.

Many of the genes mentioned above are not transcriptionally regulated by salt, and approaches that have searched for differential transcriptional responses in crop cultivars that differ in salt tolerance have not revealed any clear patterns that could be used to reveal marker genes for tolerance (Roy *et al*. 2014). So, at present there does not seem to be a reliable or feasible molecular-based method for screening for tissue tolerance, even at the cellular level. The molecular basis of other components of tissue tolerance (Fig. 1) are even more complex.

We now comment on experimental approaches and protocols that are most appropriate for screening genotypes for tissue tolerance, and measurements that can be employed.

Future approaches to measure tissue tolerance

Tissue tolerance is the result of the coordinated contribution of several physiological components (summarised in Fig. 1). It would be beneficial to assess the relative contribution of each of these components to overall tissue tolerance, and their contribution to energy-efficient growth and key yield determinants. However, energy costs associated with complex physiological processes are difficult to measure. A solution would be to measure the overall impact of these processes on net carbon availability for growth and development. Tissue tolerance resulting from effective intracellular compartmentation and osmotic adjustment using Na^+ and Cl^- rather than organic solutes would increase carbon availability for growth in terms of overall biomass, and for development in terms of more lateral shoots (or tillers in the case of cereals), more fertile florets or flowers, and larger seeds.

Physiological indicators

Physiological indicators of tolerance of a given soil salinity that indicate sustained growth rate and functional integrity are most useful when non-destructive. Stomatal conductance is a non-destructive measurement of leaf health and functional integrity, and measurements of stomatal conductance with a porometer are rapid and reliable (James *et al*. 2008). Leaf temperature provides a high-throughput alternative to measuring stomatal conductance (Sirault *et al*. 2009). Modern phenotyping methods using image analysis coupled with physiological measurements such as photosynthesis (fluorescence) and conductance (infrared) were summarised by Walter *et al*. (2015). These methods are also non-destructive, and can reveal mechanisms when accompanied by specific physiological measurements.

Stomatal conductance is more sensitive to salinity than rates of photosynthesis per unit leaf area, as leaves that develop after the soil becomes saline are smaller in area but considerably thicker with a higher concentration of chlorophyll per unit area, so that photosynthesis per unit area may not decrease

(e.g. James *et al*. 2002). The downside of these measurements of stomatal conductance or CO_2 assimilation is that they vary with time of day and with leaf age, as well as ambient conditions, so measurements need to be confined to controlled environments. They must be done on a defined leaf with known-age, and Na^+ concentrations must be measured at the same time the functional index is measured.

Turgor maintenance using Na^+ and Cl^- rather than organic solutes for osmotic adjustment is the successful result of tissue tolerance, and perhaps its best indicator, but measurements of turgor require special expertise in the use of thermocouple psychrometers (Boyer *et al*. 2008). Osmotic pressure of the leaf sap is a simple measurement and an indicator of osmotic adjustment if normalised for changes in water content of the tissue (e.g. for wheat, Colmer *et al*. 1995). It should be noted that measurements of 'relative water content' for tissues that have undergone osmotic adjustment can lead to underestimates of turgor (Boyer *et al*. 2008), because of cellular swelling and leakage when leaves from salt-stressed plants are transferred to distilled water. Measurement of the water content of leaves without transfer to distilled water (water : dry weight ratio), avoids this artefact but is subject to diurnal changes in dry weight. Measurement of succulence (water : area ratio), an important adaptation in dicotyledonous species, avoids diurnal changes in DW. However, both water content per unit area and per unit dry weight decrease as a leaf ages under control conditions as well as accelerating under stress, so matching control tissue is needed.

Screening a large number of genetically diverse genotypes with different intrinsic growth rates or early vigour is very challenging and resource-intensive if the protocol relies on comparisons of plants grown under control conditions as well as under salinity stress. The glasshouse or growth cabinet space required to grow control plants under optimum conditions of light and pot size is prohibitive for a large number of genotypes; yet pot size matters and plants with restricted root volume will grow more slowly and have different biomass allocation to roots than those in large pots (Poorter *et al*. 2012). As much as possible, protocols should avoid the need to compare biomass production of plants in salt versus control conditions, as providing optimum control conditions for the latter is so difficult to achieve in experiments lasting more than several weeks.

Protocols should also avoid the need for repeated tissue Na^+ measurements, which again can be time consuming. Ideally they are done only at the point of loss-of-function or incipient damage. However this is hard to predict. A protocol that exposes all genotypes to the same apoplastic Na^+ or rate of Na^+ influx to leaves would aid assessments of leaf tissue tolerance.

Protocols that minimise the need for Na^+ measurements

A novel method involving leaves detached from salt-stressed plants of wheat and barley was proposed by Wu *et al*. (2015). The plants had been grown at 300 mM NaCl and 50 mM NaCl was fed in dilute nutrient solution to detached leaves over 48 h. The benefit of this method is that all leaves were receiving the same concentration of salt, whereas in intact plants there could be genetic differences in exclusion by the roots which complicates the interpretation of results. Internal Na^+ or Cl^- concentrations do not need to be measured if transpiration (i.e. salt uptake rates) are the same. However one drawback of this method is that the change in water relations: leaves are no longer under osmotic stress. The osmotic stress is now very low as the tissue is at a water potential the same as the fed solution instead of the saline soil. In barley, this protocol indicated tissue tolerance correlated with whole plant salt tolerance as assessed in saline soil. With wheat, however, the detached leaf injury did not correlate with whole plant injury, probably because the wheat was also grown at 300 mM NaCl which was a lethal treatment and the fed solution of 50 mM was far too high. The method of feeding detached leaves or any part of the shoot should be mindful that the concentration in the xylem to a transpiring leaf is normally less than 10% of the soil solution. In barley it was ~5% of the soil solution (Munns 1985) and less in wheat (James *et al*. 2006*a*). Feeding higher concentration may cause salt to build up in the apoplast outside the cells and result in dehydration. This may explain the lack of correlation with wheat.

A second novel method for assessing tissue tolerance as distinct from Na^+ exclusion in rice was suggested by the studies on submerged intact seedlings in aerated solution which measured the growth of very young plants at NaCl concentrations up to 200 mM (Kurniasih *et al*. 2013). Again this has the advantage of all plants being exposed to the same concentration of salt, and that initially at least, internal salt concentrations need not be measured (but to date only one genotype has been studied in this way). An advantage over the detached leaf method is that water relations are as normal, i.e. the osmotic stress has not been relieved. A drawback is that it might be applicable only to submergence-tolerant species such as rice seedlings.

Suggested treatments and measurements

For salt-tolerant species like barley, the treatment should be at least 200 mM NaCl in the root-zone. Osmotic adjustment demands a total of 400 mM solutes in the leaf tissue, and we know that 200 mM Na^+ and Cl^- in the cytoplasm is toxic, so if the concentration of Na^+ or Cl^- in the tissue as a whole is 200 mM, there must be intracellular compartmentation of these ions. It would be sufficient to measure some indicator of growth rate or health such as osmotic adjustment, photosynthesis, or chlorophyll (with a SPAD meter), and this could be done over time at one NaCl treatment or at increasing NaCl treatments up to 350 mM NaCl. Na^+ measurements could be restricted to selected, contrasting genotypes. For salt-sensitive species, the treatment should be lower than 200 mM, such as 50, 100 or 150 mM NaCl depending on species, e.g. chickpea, rice and wheat respectively.

A given fully-expanded leaf should be measured rather than the shoot as a whole for two reasons. One that Na^+ concentration is not uniform across the shoot (Wolf *et al*. 1991) and the second that other factors (such as the osmotic pressure of the soil solution) may have affected the shoot growth rate and hence the concentration of Na^+ in the growing regions, as concentration depends not only on the delivery rate to the shoot in the xylem but is inversely related to the relative

growth rate of the shoot. (Note that it is also influenced by the root : shoot ratio and the uptake rate of the root).

An alternative approach is to grow plants for a long period of time at a lower salinity, or for shorter periods in gradually increasing salinity, and measure genotype variation in final biomass, % survival, or degree of injury.

Screening in the field

For genetically complex salt tolerance traits such as tissue tolerance, would screening be carried out most effectively in the field in saline soil using grain yield as the fundamental indicator of salt tolerance? Yield encapsulates the complexity of all the physiological processes involved in tissue tolerance into a quantifiable product that is both recognisable and relevant to breeders. For example, efficient osmotic adjustment through effective cellular and sub-cellular compartmentation of salt ions would result in a greater allocation of carbon to key yield determinants such as number of productive tillers, and grain number and size. Screening in the field for tissue tolerance could be feasible if two key factors were either regulated or measured: phenology and the spatial variability in soil salinity.

Flowering time is a key factor that determines yield of wheat and barley in saline fields in a rain-fed environment where water limits yield (Setter *et al*. 2016). Genotypes that flower earlier have an advantage through avoiding an increase in soil salinity that typically increases later in the season as the soil dries out. To deal with this issue, breeding populations or germplasm collections for screening for salt tolerance *per se* would need to be truncated to limited range in flowering time to no more than 1 week, preferably even shorter.

Well characterised field trial sites with appropriate spatial designs and adequate replication are required to account for the spatial heterogeneity of salinity, and also other abiotic and biotic factors that may influence yield. Variation of salinity within the field can be exploited if soil conductivity readings with an electromagnetic induction meter such as EM38 (preferably calibrated against soil cores from the same site) are taken for each plot and used as a covariate in the statistical analysis (James *et al*. 2012; Setter *et al*. 2016). Only then can yield differences between genotypes be reliably detected.

Field trials are unpredictable. Unusual climatic patterns can be disastrous or serendipitous. Even with the best agronomic management, unseasonal rain, hail or frost can ruin a trial, as can a severe drought. These issues mean that field trials need replicating at different sites and in different years. However, these issues can throw up unexpected and useful results, as found by Setter *et al*. (2016). Barley showed the benefit of the tissue tolerance trait, as it yielded more than wheat, which could have been due to its early flowering, but it yielded more in a saline than a dry soil indicating that it could use Na^+ and Cl^- for osmotic adjustment.

Field validation of putatively tissue-tolerant germplasm selected from controlled environment screening protocols is essential for uptake by breeders and incorporation into breeding programs. Typically, breeders will not incorporate novel traits in supplied germplasm into breeding programs unless there is convincing proof of trait value (improved grain yield relative to current varieties under the appropriate saline field conditions), that the traits are present in well adapted germplasm, and additionally, that there is no yield penalty - that the germplasm yields as well as current cultivars under non-saline conditions.

Concluding remarks

In summary we have suggested that 'tissue tolerance' should be broadly defined as the capacity of the cells or tissues to continue to function without injury, with a high internal Na^+ and/or Cl^- concentration, in the face of a significant osmotic stress caused by the external NaCl concentration in the root zone. Tissue tolerance is physiologically and genetically complex, and so far there is no evidence that any single gene or molecular marker is a quantitative predictor of this complex trait.

What would be the exact benefit of a greater degree of tissue tolerance to a crop like barley? A higher turgor is not expected to make any single shoot grow faster as turgor itself is not limiting the rate of leaf growth (Termaat *et al*. 1985; Munns *et al*. 2000). However, a more energy-efficient osmotic adjustment using Na^+ and Cl^- rather than organic solutes is expected to stimulate an increase in lateral shoot development. It is well known in dry or saline soils that the number of tillers or lateral branches are reduced and recent research has shown that outgrowth of lateral buds is determined by sugar supply (Kebrom *et al*. 2012; Mason *et al*. 2014). Another expectation is that plants with greater tissue tolerance will be able to grow in, or at least survive, a higher salinity.

The challenge ahead lies in the development and validation of high throughput methods that can be used to screen germplasm, and can be validated in the field. Only then will useful genetic variation be harnessed for plant breeding.

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