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OPINION PAPER

# Salt stress or salt shock: which genes are we studying?

**Yuri Shavrukov**

Australian Centre for Plant Functional Genomics, University of Adelaide, Waite Campus, SA 5064, Australia

To whom correspondence should be addressed. E-mail: yuri.shavrukov@acpfg.com.au

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## Abstract

**Depending on the method of NaCl application, whether gradual or in a single step, plants may experience either salt stress or salt shock, respectively. The first phase of salt stress is osmotic stress. However, in the event of salt shock, plants suffer osmotic shock, leading to cell plasmolysis and leakage of osmolytes, phenomena that do not occur with osmotic stress. Patterns of gene expression are different in response to salt stress and salt shock. Salt stress initiates relatively smooth changes in gene expression in response to osmotic stress and a more pronounced change in expression of significant numbers of genes related to the ionic phase of salt stress. There is a considerable time delay between changes in expression of genes related to the osmotic and ionic phases of salt stress. In contrast, osmotic shock results in strong, rapid changes in the expression of genes with osmotic function, and fewer changes in ionic-responsive genes that occur earlier. There are very few studies in which the effects of salt stress and salt shock are described in parallel experiments. However, the patterns of changes in gene expression observed in these studies are consistently as described above, despite the use of diverse plant species. It is concluded that gene expression profiles are very different depending the method of salt application. Imposition of salt stress by gradual exposure to NaCl rather than salt shock with a single application of a high concentration of NaCl is recommended for genetic and molecular studies, because this more closely reflects natural incidences of salinity.**

**Key words:** gene expression, gradual salt application, osmotic shock, plasmolysis, salt shock, salt stress, sudden (single-step) salt application.

## Preface

Salt stress and salt shock are two distinct phenomena, both triggered by the application of salt. However, the terms 'salt stress' and 'salt shock' are used incorrectly or without consideration of their precise meaning in many studies. The aim of this paper is to clarify both phenomena and answer the question: Are salt-responsive genes that have been discovered and described in the literature controlling reactions of plants to salt stress, salt shock, or something else? This is not a comprehensive analysis of all published data but highlights some of the most pertinent studies showing differences between salt stress and salt shock.

## Scientific terms

It is first necessary to define several important scientific terms used here. Salt stress is the exposure of plants to salinity, the main component of which is NaCl. Salt stress can be in one of two forms -(1) gradual exposure to increasing levels of NaCl or (2) exposure of plants to low levels of salinity – or it may be a combination of both. Salt shock is an extreme form of salt stress, where plants are exposed suddenly to a high level of salinity. Salt shock rarely occurs in either agricultural practice or in natural ecosystems because increases in NaCl concentration in soils occur gradually, via rising water tables, deep penetration of roots, or slow, seasonal drying of the soil profile and removal of soil water via evaporation and transpiration. A graphical depiction of the common methods of salt application used in experiments, and their comparison to natural increases in soil salinity, is presented in Fig. 1.

There are two main components of salt stress/shock: osmotic and ionic components. Salt shock immediately induces osmotic

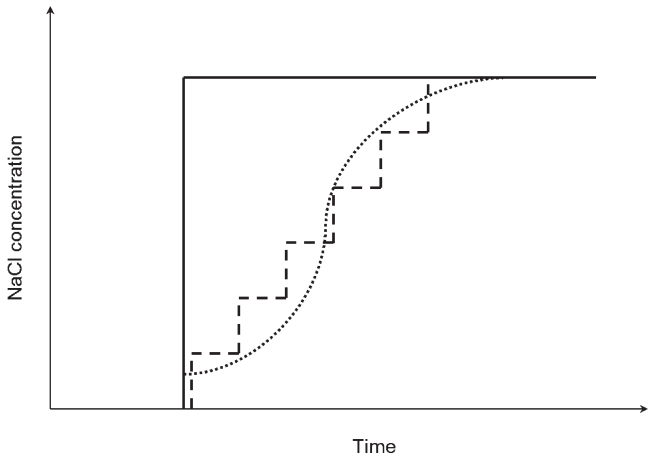


**Fig. 1.** Schematic representation of two different forms of salt application used in experiments with plants – sudden (in one step) NaCl application (solid line) and gradual salt application (dashed line) – in comparison to salinity changes in the top soil profile during soil drying (dotted line).

shock, when plants are suddenly exposed to large differences in osmolarity (osmotic pressure) between external solutes with a high concentration of NaCl and internal solutes in the cell cytoplasm. Osmotic stress as a first component of salt stress is characterized by relatively small differences in osmotic pressure inside and outside of the cells, and occurs several consequent times if plants are exposed to gradually increasing NaCl concentrations. Osmotic adjustment refers to the adjustment of osmotic pressure inside plant cells in response to osmotic stress, and needs to be much stronger if plants are to survive the effects of salt (osmotic) shock. The ionic component of salt stress/shock follows the osmotic response, with a time delay because the concentration of $Na^+$ ions has to reach a toxic level in the plant cell cytoplasm.

## Introduction

Interest for studying salt stress is growing rapidly because salinity is now a major environmental factors limiting crop production. In the context of global warming, the impact of salinity seems to be increasing, and it is expected that more attention will be given to research in this area in future. Physiological mechanisms of plant reaction to NaCl have been described in much detail (reviewed in Munns and Tester, 2008). It is well documented that the osmotic and ionic components of salt stress represent primary and secondary phases of the stress, respectively, where plants react to each component at different times. Osmotic stress (or the osmotic component of salt stress) occurs immediately when roots come into contact with solutions containing unfavourably high concentrations of salts in hydroponic systems or in soil. At this stage, plants need to adjust osmotically for water potential and turgor to achieve homeostasis. Tolerance to osmotic stress is an important component of the plant reaction to salt stress. But osmotic stress is also a component of the initial stages of drought stress, which similarly involves increasing cellular concentrations of osmolytes and regulation of stomatal conductance (James *et al*., 2008). After NaCl application, plants usually achieve osmotic homeostasis relatively quickly, either during several hours or at least within the first day following salt stress (Munns, 2002). The ionic stress component of salinity stress, as a second phase, becomes gradually more severe, typically beginning after 1–3 days of NaCl application despite rapid influx of $Na^+$ ions and transport to the shoots. This is because the concentration of $Na^+$ must reach a certain toxic level in cell protoplasts of shoots and this process requires some days (Munns, 2002, 2005; Roshandel and Flowers, 2009). After 24–72 hours of NaCl application, the concentration of $Na^+$ in cytoplasm may be close to toxic levels and, therefore, plants react to ionic stress progressively. $Na^+$ exclusion and tissue tolerance are two common mechanisms of tolerance to the ionic component of salt stress, where one or both of these mechanisms are employed by tolerant plant types (reviewed in Munns and Tester, 2008).

Plants growing in a salinized field similarly experience osmotic and ionic components of salt stress as described above. Rains flush salts through to the deeper subsoil layer, and growing plants have very little exposure to salt stress in the initial stages of development. During the growing season of a summer annual crop such as wheat or barley, with warm temperatures and evaporation/transpiration of soil water, NaCl moves to the upper soil layers and generates increasing salt stress. Plants, therefore, are gradually exposed to the increasing salt stress. This natural salinity increase is a slow and progressively one. In the field, plants rarely experience sudden 'jumps' in soil salinity.

Halophytes, extremely salt-tolerant plant types, are typically native plants growing in costal beach areas or near salinized lakes. They are exposed to particularly strong salt stress, but do not experience salt shock because they germinate and grow in a consistently high-salt environment with very high natural salinity but with little variability. Nevertheless, there is one case when salt shock for plants can be found in agricultural areas. This is following a tsunami, when a sudden large mass of seawater floods cultivated land. After the major tsunami in Japan in March 2011, all affected rice plants died, with the exception of only a few surviving seedlings which appeared to tolerate the osmotic shock and were termed 'diehard rice' (http://www.yomiuri.co.jp/dy/national/T110728005424.htm).

## Salt stress versus salt shock

To study the effects of salt stress on plants, scientists apply NaCl or seasalts to plants grown in the laboratory, growth chamber, greenhouse, or other facilities. Hydroponics (with or without supporting agents, aerated, flood-drained, or passive) is the most popular tool for studying salt stress, because NaCl application can be easily controlled (reviewed in Shavrukov *et al*., 2012). However, some researchers prefer to use soil mixtures (Ayarpadikannan *et al*., 2012; Chakraborty *et al*., 2012). Experiments with salinity are extremely variable, depending on the species being studied, purpose of the research, and even personal preference. However, one point remains particularly crucial for interpreting the results of experiments. How is salt applied to the plants: gradually or suddenly, in a single step?

Generally, the application of salt stress involves gradual application of NaCl, usually of 25 or maximum 50 mM increments of NaCl, twice daily, until a final, predetermined salt concentration is reached (Gorham *et al*., 1987, 1990, 1991; Shah *et al*., 1987; Gorham, 1990; Forster *et al*., 1994; Ellis *et al*., 1997; Munns *et al*., 2000; Munns and James, 2003; Huang *et al*., 2006; James *et al*., 2006; Shavrukov *et al*., 2006, 2009; Byrt *et al*., 2007; Roshandel and Flowers, 2009). Various researchers refer to this method as 'progressive imposition' (Almansouri *et al*., 1999), 'salt acclimation' or 'gradual step acclimation' (Rodriguez *et al*., 1997; Sanchez *et al*., 2008), or 'salt-adapting' (Baisakh *et al*., 2006). Ideally, salt application must be made as smoothly as possible, for example, 2.083 mM NaCl every hour to reach 50 mM NaCl by the end of first day (24 hours). However, this 'ideal' type of gradual salt application is technically difficult. The more gradual the application of NaCl, the more closely reactions of plants will reflect those expected for salt stress in saline field environments.

In contrast, salt shock occurs when plants are suddenly transferred from normal growth solution (without NaCl) into solution containing high concentrations of NaCl. The main component of salt shock is osmotic shock or plasmolysis, especially in root cells (Munns, 2002), when the cell protoplast shrinks and detaches from the cell wall, as has been observed in barley after transfer to 200 mM NaCl (Pritchard *et al*., 1991). Desperate attempts by the cells to maintain equilibrium between external and internal water content results in the leakage of cell solution into open spaces between the cell wall and plasma membrane. These apoplastic solutes, containing high concentrations of $Na^+$, can freely flow through the open spaces in root cells and be transported to the shoot with minimal control by the plant. There is consequently rapid activation of many genes, in response to osmotic shock and damaged plasma membrane in root cells and to ionic stress in shoot cells. The mechanism of osmotic shock is universal for all plant species because it results from the physical–chemical effects of loss of cell turgor as described above. Plants from different species, regardless of their level of salt tolerance, will only differ in the degree of damage to the plasma membrane during plasmolysis and in how quickly normal structure and function of affected cells is restored.

It has been reported that young wheat plants suffer osmotic shock and plasmolysis at 150 mM NaCl, when salt is applied in a single step. Older plants will suffer osmotic shock at lower levels, down to 100 mM NaCl (Pritchard *et al*., 1991). Therefore, it can be assumed that, when applied in a single application, levels of NaCl higher than 100–150 mM NaCl, in general, will cause plasmolysis. Salinity levels ranging between 50 and 100 mM NaCl can be assumed as intermediate between osmotic stress and osmotic shock, while application of 50 mM NaCl or less will not cause plasmolysis but only osmotic stress, where many plants can manage with moderated adjustments of cell turgor and osmolarity.

Plants exposed to severe salt shock can experience cell death. Apoptosis-like cell death was reported in barley roots under very strong salt shock (500 mM NaCl, Katsuhara, 1997), and salt shock with lower salt concentrations (150–200 mM NaCl) caused death of rice plants (Kawasaki *et al*., 2001; Zou *et al*., 2012), which was not reported at these levels when NaCl was applied gradually. This observation illustrates the principal difference between salt stress and salt shock.

## Gene expression in the response to salt stress and salt shock

Modern techniques and tools now enable researchers to study the expression of genes in response to different stresses, including salinity. Groups of highly up- or downregulated genes responding to stress are identified using microarray techniques and EST analyses (reviewed in Xiong and Zhu, 2002; Jamil *et al*., 2011). Some researchers select particular 'genes of interest' for investigation, based on their known or hypothesized function, involvement in stress responses in other species, or genetic links to the stress. Following is an overview of selected reports of gene expression profiles of plants exposed to salt stress or salt shock.

Only a few studies have used gradual exposure of the plants to NaCl to minimize the risk of osmotic shock and plasmolysis. For example, three increments of 80 mM NaCl daily were used in experiments with perennial ryegrass, *Lollium perenne* (Hu *et al*., 2012), and the reported changes in antioxidant gene expression were correctly described as salt-stress responsive. Another way to avoid salt shock is to expose plants to relatively low levels of salt in a single application. For example, Roshandel and Flowers (2009) applied 50 mM NaCl in one application to rice plants prior to cDNA synthesis and gene expression analysis. This concentration of NaCl would not be enough to cause salt shock.

Unfortunately, some reports do not recognize the differences between salt stress and salt shock and even use the two terms interchangeably when describing responses within the same experiment (e.g. Kawasaki *et al*., 2001). Abogadallah (2010) concluded correctly that an observed significant loss of water in leaves of clover plants following exposure to 200 mM NaCl was caused by 'osmotic shock'. Similarly, leaves of cotton plants were 'partially dehydrated and then recovered gradually' after transferring plants in a single step to nutrient solution containing 150 mM NaCl (Zhang *et al*., 2011) indicating to osmotic shock. However, the genes described in these studies were erroneously reported to be responsive to 'salt stress'.

*Arabidopsis* is a plant species relatively sensitive to salinity (Jamil *et al*., 2011). Dipping roots of seedlings into a beaker containing 300 mM NaCl (Tang *et al*., 2011) would definitely cause salt shock and plasmolysis. Consequently, transgenic *Arabidopsis* plants expressing *JcDREB*, a transcription factor from the woody plant *Jatropha curcas*, showed rapid (0.5 h) induction of expression of the transgene. However, the authors did not acknowledge the induction was a response to strong osmotic shock rather than salt stress. In the same study, the expression of *JcDREB* responded similarly when plants were exposed to 20% PEG 6000, simulating drought stress. This clearly indicates that the factor common to both experiments was very strong osmotic shock (Tang *et al*., 2011).

Highly upregulated expression of some genes in response to osmotic shock can be registered within minutes after sudden exposure of plants to salinity. Three *OsHSP* genes, encoding heat-stress proteins, were rapidly (within 5–15 minutes) and strongly expressed not only in rice roots but in leaves and leaf

sheaths (Zou *et al*., 2009). The results of this study suggest that plasmolysis can be involved in the genes expression with primarily osmoregulation rather than salt stress itself, where high expression of the *OsHSP* genes remained for 24 hours (Zou *et al*., 2009).

Many genes identified in experiments involving salt shock are directly related to osmotic shock responses. Cell turgor maintenance, accumulation of soluble sugars, other osmolytes, and water balance are the most important processes of osmotic adjustment and they are controlled by genes with osmotic function (reviewed in Munns, 2005). For example, significantly higher contents of soluble sugars and proline were measured in salt-shocked transgenic tomato plants that were overexpressing *SlERF1* transcription factor compared to wild type (Lu *et al*., 2011). In another study, a water channel protein and a TIP aquaporin (tonoplast intrinsic protein), two genes related to water balance in the plant cell, were among seven genes identified as being responsible for ion transport and homeostasis following salt shock (Ayarpadikannan *et al*., 2012). In contract, genes encoding potassium channels and potentially playing an important role as ion antiporter were induced only weakly in the same experiments (Ayarpadikannan *et al*., 2012).

If plants are exposed to salt with a single, large addition of NaCl, but the expression of genes is analysed only after several days (long-term exposure), then it is assumed that the plants will, to some degree, recover from osmotic shock and begin to respond to the ionic phase of salt treatment. But how does plasmolysis affect the development of plant tolerance to ionic stress? For example, in *Arabidopsis*, 100 mM NaCl application in a single step should cause osmotic shock. An ethylene perception gene, *ERS1*, was highly upregulated in mutants compared to wild type after 4 and 7 days of salt shock but did not differ after 11 days (Cela *et al*., 2011). In this study it remains unclear whether the expression of *ERS1* responded to osmotic adjustment in the first minutes, hours, or days, or to the ionic component of the NaCl stress.

A number of reported studies using sudden (single-step) salt application were selected (Table 1). These studies are representative of the majority of published research, which led Munns (2005) to conclude that all gene expression studies were investigations of salt shock. However, in this review it is suggested that low levels of NaCl application (25–50 mM NaCl) may be described as mild salt stress because these concentrations should not cause plasmolysis in root cells. Intermediate salt levels

**Table 1.** Selected reports employing different levels of salinity where NaCl was applied in a single step, and reported associated genes
The choice of NaCl concentration results in salt stress (25–50 mM NaCl), intermediate salt stress/salt shock (80–100 mM NaCl), or salt shock (150–300 mM NaCl) treatments and leads to discovery of different genes associated with these treatments..

| NaCl concentration (mM) | Species | Gene name | Gene function and reported changes in expression | Reference |
|---|---|---|---|---|
| 25 and 50 | Mustard (*Brassica juncea*, *B. campestris*) | *SOS1, SOS2, SOS3, AtNHX1* | Plasma membrane $Na^+/K^+$ antiporter, protein kinase, calcium-binding protein, vacuolar $Na^+/K^+$ antiporter, all increased after 24 h in roots and/or in leaves | Chakraborty *et al*. (2012) |
| 50 | Rice | *PRP*, *SAG*, *HSPC025* | Proline-rich proteins, cell-wall protection, 0.5 h in shoots; senescence-associated genes, regulatory processes, and cellular signal transduction, 8 d in shoots; heat-shock proteins, protein stabilizing, 8 d in shoots | Roshandel and Flowers (2009) |
| 50 | Rice | *CDPK* | Signal transduction pathways through phosphorylation, 7 and/or 24 d in roots and/or in leaves | Wan *et al*. (2007) |
| 80 | Rice | *HSP90* | Heat-shock proteins, molecular chaperones, folding, assembling, and transporting proteins, 1 and 3 d in roots and leaves. | Liu *et al*. (2006) |
| 100 | *Arabidopsis* | *ERS1* | Ethylene perception gene, ethylene signal transduction, increased after 4–7 d in leaves | Cela et al. (2011) |
| 100 | Rice | *OsHSP23.7*, *OsHSP71.1*, *OsHSP80.2* | Heat-shock proteins, molecular chaperones, folding, assembling, and transporting proteins, increased after 5 min – 24 h in different tissues | Zou *et al*. (2009) |
| 150 | *Arabidopsis* | *AtSKIP* | Transcription factor, transcriptional pre-initiation, splicing, and polyadenylation, increased after 6–12 h in different tissues | Lim *et al*. (2010) |
| 200 | Rice | *OsHsp17.0*, *OsHsp23.7* | Heat-shock proteins, molecular chaperones, and folding, assembling, and transporting proteins, 24 h for survival test | Zou *et al*., 2012 |
| 200 | Rice | *OsAPx2*, *OsAPx8* | Ascorbate peroxidase, salt-inducible genes, 24 hours in leaves | Asano *et al*. (2012) |
| 200 | Tomato | *SlERF1*, *LEA*, *P5CS*, *DREB3–1*, *ltpg2* | Transcription factor for signal transduction, stress-related genes, 10 d in leaves | Lu *et al*. (2011) |
| 200 | Egyptian clover (*Trifolium alexandrinum* L.) | *NHX1*, *H⁺-PPase*, *H⁺-ATPase*, *HRD* | Vacuolar $Na^+/K^+$ antiporter, proton pumps, transcription factor, 2–6 h in leaves and roots (*NHX1*); but downregulated after 6 d (*NHX1*, *H⁺-PPase*) or after 6 h in roots (*H⁺-ATPase*) | Abogadallah (2010) |
| 300 | Barley | *USP* | Universal stress protein, 9 and/or 27 h in roots and/or in leaves | Li *et al*. (2010) |
| 300 | Carrot (*Daucus carota* L.) | *DcHsp17.7* | Cell viability and membrane stability under heat stress, 5 h in leaves | Song and Ahn (2011) |
| 300 | *Arabidopsis* | *JcDREB* | Transcription factor, 0.5–24 h in leaves (dipping roots of seedlings in salt) | Tang *et al*. (2011) |

(80–100 mM NaCl) may or may not induce osmotic shock, and high levels of salinity (150–300 mM NaCl) cause salt shock and plasmolysis in root cells (Table 1). Despite the variability in NaCl application, all studies listed in Table 1 were originally reported as salt stress experiments.

## Comparison of gene expression in parallel experiments using gradual and sudden (single-step) salt application

The ideal way to distinguish between plant responses that are specific to salt shock and salt stress is to conduct side-by-side experiments, one with a single application of NaCl (salt shock) and one with gradual NaCl application (salt stress). Only a few such studies have been done, and the findings relating to gene expression from these are summarized graphically in Fig. 2. Large numbers of genes are expressed within a few minutes to several hours following the imposition of a salt shock treatment (Fig. 2A). These genes are mainly associated with the initial defence of plant cells against plasmolysis/salt shock, including processes such as signal transduction pathways, osmoregulation, and water loss. In salt shock-treated plants, the second (ionic) phase occurs early and there is overlap of changes in gene expression relating to ionic and osmotic stress responses. There are also relatively small numbers of genes identified as being associated with the ionic component of salt stress response (Fig. 2A). In contrast, following incremental application of salt, plants respond more smoothly to the osmotic phase of salt stress, with fewer genes associated with this phase (Fig. 2B). Genes showing altered expression are primarily responsible for osmotic adjustment and osmolyte production. The second, ionic phase of salt stress occurs slightly later, with minimum overlap of changes in gene expression between genes associated with the ionic and osmotic phases of salt stress. There are a large number of genes identified as being associated with the ionic phase, many of which are involved in responses to toxic cellular concentrations of $Na^+$ ions. Examples of studies that support the contrasting patterns of gene expression changes illustrated in Fig. 2 are provided below.

### *Experiments with rice*

Two studies using rice clearly demonstrate how choice of treatment can affect the gene expression changes observed in response to salt. In the first study, 150 mM NaCl was added in a single application to growth solution, and root tissue from rice (cv. Pokkali) was sampled at different time points following salt application for microarray analysis (Kawasaki *et al*., 2001). After 15 minutes following the salt shock treatment, only a few transcripts were highly upregulated. The two genes most represented in the instantaneous and early response clusters were: (1) glycine/serine-rich protein (*GRP*) and (2) calcium-dependent protein kinase (*CDPK*), which are involved in strengthening of cell walls and early stages in signal transduction, respectively, both components of immediate responses to osmotic shock/plasmolysis. Transcripts of *GRP* and *CDPK* genes were still highly expressed after 1 hour of salt treatment but returned to normal levels after 3 hours. Profile analysis of GRP and CDPK transcripts clearly indicated that rice plants respond to osmotic shock/plasmolysis between 15 minutes and 1–3 hours. A cascade of other defence-signalling genes with upregulated transcripts was registered between 3 and 24 hours. Among them were transcripts for a ribosomal 40S protein and elongation factor 1α, which play similar roles in defence signalling, and Osr40c1 (40-kD protein), which is involved in preventing water loss and the reducing the rigidity of cell walls. These genes are also considered to be indicative of osmotic shock/plasmolysis, and the total number of upregulated transcripts during this stage was estimated to be as much as 29. Based on the results, the restoration of damaged plasma membranes took from 24 hours to several days following initial exposure to salt.

In the same study, the number of upregulated transcripts which were likely to be related to the ionic phase of salt shock was only 13 (Kawasaki *et al*., 2001). The authors observed upregulation of glutathione *S*-transferase and ascorbate peroxidase, both involved in defence against reactive oxidative species, with highest transcript levels reached after 24 hours of salt application. However, metallothionein, which is also has an antioxidative function, and also water channel proteins (aquaporins) were

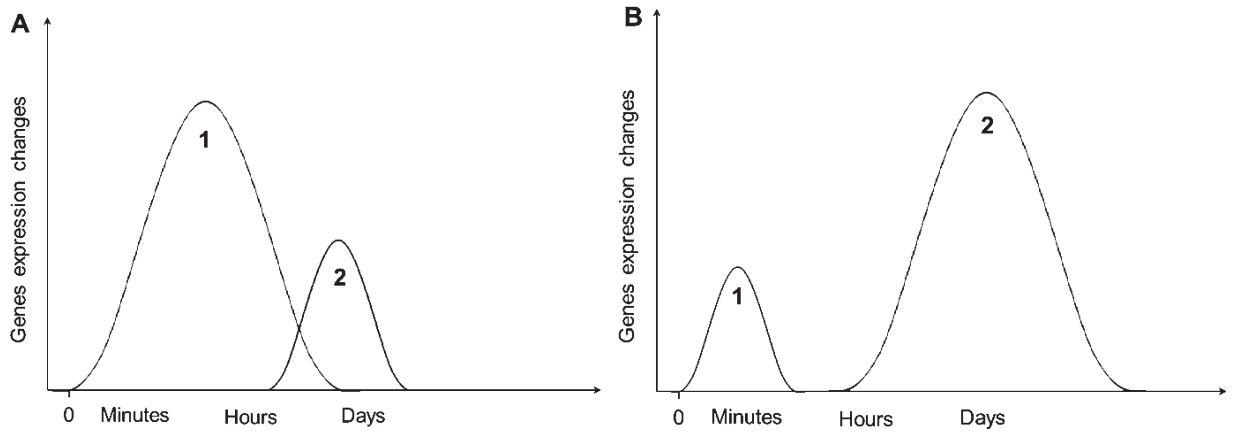


**Fig. 2.** Schematic representation of expected gene expression changes in experiments following either salt shock (A) or salt stress with incremental NaCl application (B). Curves 1 and 2 indicate changes in gene expression relating to the osmotic and ionic phases of salt shock/stress, respectively. '0' on the X-axis is the time of first salt application.

significantly upregulated only after 7 days of salt application, indicating that this length of time is required for complete recovery of salt-shocked rice plants. While it is possible that more than 13 transcripts may be related to ionic stress, it is interesting to note that no ion transporters or other genes responsible for $Na^+$ accumulation, relocation, or detoxification were reported in this study. Strong osmotic shock can greatly affect observations of changes in gene expression, such that well-documented salt stress responsive genes will not be identified.

In the second study, step-wise additions of salt to the same final concentration, 150 mM NaCl (with 50 mM NaCl increments daily) were made to rice cv. Horkuch (Lisa *et al*., 2011). The authors describe Horkuch as being similarly tolerant of salinity to cv. Pokkali, and here it is assumed that the gene expression profiles obtained in this study should be comparable to those from the first study, with differences attributable to the method of NaCl application employed. Despite analysis of hundreds of transcripts, only two genes, glutathione *S*-transferase and metallothionein, were reported as being upregulated in both studies (Kawasaki *et al*., 2001; Lisa *et al*., 2011). Looking specifically at genes involved in osmotic adjustment and the osmotic stress response phase of salt stress, in the first study, both *GRD* and *CDPK* genes were rapidly upregulated in response to strong osmotic shock/plasmolysis (Kawasaki *et al*., 2001). In contrast, in the second study, two osmotic-adjustment genes, arginine decarboxylase and late embyogenesis abundant protein Lea14-A, were reported as responding to salt stress (Lisa *et al*., 2011). Interestingly, both these genes showed increased expression only after 24 hours of the commencement of salt application, suggesting that these rice plants were effectively coping with osmotic stress. There were four salt-responsive genes identified in the second study that were specifically related to the ionic phase of salt stress, all of which were ion transporters (Lisa *et al*., 2011). The expression of three separate subunits of vacuolar ATPase increased dramatically after 24 hours of exposure to NaCl. These control the pumping of protons into the vacuole, which are required for $Na^+$ antiporters. The fourth upregulated gene encodes a PHO-like protein for phosphorous transport and showed 2–3-fold increased activity after the same period (Lisa *et al*., 2011).

These two studies illustrate clearly that experiments causing salt shock lead to the identification of genes that are primarily responsible for defence reactions against osmotic shock/plasmolysis (Munns, 2005). Such types of exposure to salt almost never happen in natural conditions and, therefore, conclusions about suitable genes or genetic material to use for improving salinity tolerance are not sound. In contrast, experiments with controlled application of salt to induce salt stress rather than shock are more likely to lead to the identification of genes and genotypes important in the reactions of plants to salt stress in real environments.

### *Halophyte species:* Spartina alterniflora *Loisel*

In a study with a halophyte plant species, a 10% solution of a commercial seasalts mix (equivalent to 445 mM NaCl) was added in a single application to *Spartina alterniflora* plants grown in hydroponics, and 14 upregulated cDNAs were selected as playing major roles in tolerance to salinity stress (Baisakh *et al*., 2006). However, at least 10 of the listed genes are directly related to osmotic regulation and not to the management of toxic $Na^+$ ions (Baisakh *et al*., 2006). Quite different results were observed when salt was applied incrementally, with an initial addition of 1% of seasalts mix (equivalent to 44.5 mM NaCl) for 1 week followed by exposure to 10% of seasalts mix (equivalent to 445 mM NaCl). While the design of this experiment was not perfect (only two steps of salt application with a big difference between NaCl concentrations), differences in gene expression were still observed. The authors reported that none of the previously identified and selected cDNAs showed an increase in expression levels, and they concluded that in the second experiment, the plants had become salt-adapted during the first week of lower salt exposure (Baisakh *et al*., 2006). However, these results are an illustration of the differences in gene expression responses to salt shock (first experiment) and salt stress (second experiment). The majority of the identified genes in the second experiment were related to the osmotic rather than ionic phase of salt stress and, therefore, it is supposed that these genes reached maximum expression after the first application of salt and remained steady following the second application.

### *Model legume plant:* Lotus japonicus

Rubio *et al*. (2009) reported results from experiments with both gradual application (salt stress) and single-step application (salt shock) to a final concentration of 150 mM NaCl. The expression levels of four from five superoxide dismutase (SOD) genes, encoding antioxidative enzymes, were not different from control plants in the experiment with graduate salt application. In contract, the same four *SOD* genes were significantly upregulated following sudden NaCl application, indicating that these genes are directly involved in the reaction of plants to osmotic shock/plasmolysis, but are not involved in responses to salt stress itself (Rubio *et al*., 2009). Examination of expression profiles of other antioxidative genes, *GPX* (glutathione peroxidase), revealed that *GPX4* also responded to osmotic shock. Two genes, *GPX1* and *GPX6*, were strongly upregulated by both types of salt application, and they are probably involved in both osmoregulation and ionic stress. Three other genes (*GPX2*, *GPX3*, and *GPX5*) did not show any differences in expression in either experiment, and thus are unlikely to control plant reactions to either salt stress or salt shock. This paper (Rubio *et al*., 2009) clearly demonstrates that salt shock and salt stress elicit very different responses in plants. It also shows that conducting salt shock and salt stress experiments side-by-side is a very powerful way to dissect out genes that are involved in the osmotic and ionic phases of salt stress.

Changes in gene expression using the same model legume plant (*L. japonicus*) and the same gradual salt stress treatment (150 mM NaCl) were also analysed over a longer time (16 days) (Sanchez *et al*., 2008). The authors found that the most responsive genes were either related to transcription and signalling pathways (17% of the total number of genes responsive to NaCl), including members of the transcription factor families AP2/ERF and MYB, membrane and cytoplasmatic receptor-like kinases and other classes of kinases, or were transport-related genes (8% of genes). These genes are not likely to be involved

in the reaction of plants to osmoregulation but are clearly expected to be responsive to ionic changes. A defence-related functional group of genes (6% of total upregulated genes) were also identified, including dehydration-responsive genes, such as *LEA* and *LEA*-like genes (Sanchez *et al*., 2008). These results support the proposed hypothesis that graduate NaCl application causes fewer changes in expression of genes related to osmotic stress, but many more changes in ion toxicity-related genes.

## Comparison of gene expression in parallel experiments with low and high concentrations of salt application

There are a small number of studies where plants were exposed in one step either to low or to high concentrations of NaCl in independent experiments conducted side-by-side. In many examples, salt shock was still likely to have occurred, especially if 150 mM NaCl was chosen as a low level of 'salt stress' (Pritchard *et al*., 1991). However, such experiments are still valuable for interpretation, as a comparison between gene responses to low and high incidences of plasmolysis. Compared to the corresponding high-salt experiments, those experiments with lower NaCl applications induced less osmotic stress and the reaction of plants is considered to have been relatively more related to the ionic phase of salt stress, especially if gene expression was assessed after one or several days following salt application.

Only one well-documented paper was found where side-by-side experiments were conducted, in tolerant C3 model species *Pancratium maritimum* L (Abogadallah, 2011), and the results are discussed here in the context of this review. Different levels of gene expression were reported encoding three photorespiratory enzymes (glycine decarboxylase complex – H-protein, GDS-H; serine:glyoxylate aminotransferase, SGAT; and glutamine synthetase 2, GS-2) following moderate (150 mM NaCl) and severe (300 mM NaCl) sudden salt shocks. In this study, 150 mM NaCl would be high enough to have partially caused plasmolysis in root cells, but the responses to salt shock would have been more moderate than following application of 300 mM NaCl. Genes encoding two photorespiratory enzymes, GDS-H and SGAT, were highly expressed following application of 150 mM NaCl compared to controls (without salt) but had reduced expression following severe salt shock (300 mM NaCl). These two enzymes are less likely to be related to osmotic shock but are more likely involved in the reaction of plants to toxic levels of $Na^+$ ions. The third gene, encoding enzyme GS-2, had increased expression at 150 mM NaCl and even higher expression at 300 mM NaCl. This gene particularly reacted to osmotic phase of both salt shock and salt stress. In this study, gene expression profiles were not identical in experiments with lower and higher salt applications.

## Conclusion

The following are several practical suggestions for researchers studying the genetics of salinity tolerance in plants.

(1) Experiments employing gradual or single applications of salt must be clearly described, and correctly identified with either salt stress or salt shock. Researchers may use any method of salt application, but they must bear this in mind when interpreting and reporting results.
(2) Plasmolysis (osmotic shock) must be taken into consideration in experiments where plants encounter salt shock. Total gene expression profiles, and expression changes in particular genes of interest, are very likely to be different following treatment with the same NaCl concentration applied to induce either salt stress or salt shock.
(3) Researchers with experimental preferences to salt shock should think about a preliminary test with 'differential display', where both salt stress and salt shock can be carried out side-by-side for their comparison. The timescale of the treatment should extend from minutes (e.g. 30 minutes) to days (e.g. 1 week), for identification of genes with short- and longer-term responses.
(4) Expression of genes responsive to salt stress can be studied using significantly lower levels of salt. For the majority of plants, 50–100 mM NaCl is sufficient. However, for salt-sensitive plants, including *Arabidopsis* and the legume crop chickpea, even lower concentrations will be necessary, while halophyte species will require higher levels of salt.
(5) Some researchers worry about the slow process of salt application when simulating salt stress, especially in experiments where samplings are made in minutes or hours following the first salt application. However, the reaction of plants to salt stress should be as smooth as possible. Lower levels of NaCl can be applied in a single step with minimal risk of plasmolysis. An alternative may be to apply frequent doses of salt (for example, 6–8-times per day) to raise salt levels more rapidly.

In conclusion, it is hoped that the question of which genes are being studied in salt stress/shock experiments, is answered in this paper. If a researcher applies salt stress with incremental and/or low concentrations of NaCl, the expression of salt stress-responsive genes can be described correctly. Salt shock, in contrast, following sudden addition of NaCl cause plasmolysis/osmotic shock, and consequently the gene expression profile will be very different. If researchers want to study and create novel salt-tolerant plants, salt stress rather than salt shock is to be studied in preference. Salt stress in experiments must simulate what occurs in natural environments. The reaction of plants to salinity is very complicated, involving of hundreds of genes and, therefore, 'limited success for commercial utilization under field saline conditions' (Jamil *et al*., 2011) has been enjoyed so far. This has been confounded by the lack of distinction made between genes responsive to the osmotic and ionic components of salt exposure, and by many examples of studies inadvertently employing salt shock treatments. In contrast, with the recent success of the application of the *Nax2* gene for the improvement of grain yield in durum wheat by up to 25% in salinized field conditions is an excellent example of results that may be expected when having a clear scientific understanding of the components of salt stress and how to achieve salt stress experimentally (Munns *et al*., 2012). To make more real progress in this area, we need to clearly understand, distinguish differences. and primarily use salt stress rather than salt shock in experiments relating to salinity tolerance in plants.

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