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TECHNIQUES FOR MOLECULAR ANALYSIS

# Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status

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**Summary**

**The abiotic stresses of drought, salinity and freezing are linked by the fact that they all decrease the availability of water to plant cells. This decreased availability of water is quantified as a decrease in water potential. Plants resist low water potential and related stresses by modifying water uptake and loss to avoid low water potential, accumulating solutes and modifying the properties of cell walls to avoid the dehydration induced by low water potential and using protective proteins and mechanisms to tolerate reduced water content by preventing or repairing cell damage. Salt stress also alters plant ion homeostasis, and under many conditions this may be the predominant factor affecting plant performance. Our emphasis is on experiments that quantify resistance to realistic and reproducible low water potential (drought), salt and freezing stresses while being suitable for genetic studies where a large number of lines must be analyzed. Detailed protocols for the use of polyethylene glycol-infused agar plates to impose low water potential stress, assay of salt tolerance based on root elongation, quantification of freezing tolerance and the use of electrolyte leakage experiments to quantify cellular damage induced by freezing and low water potential are also presented.**

**Keywords: Arabidopsis, stress quantification.**

## Introduction

Abiotic stress limits crop productivity (Araus *et al.*, 2002; Boyer, 1982), and plays a major role in determining the distribution of plant species across different types of environments. Abiotic stress and its effects on plants in both natural and agricultural settings is a topic that is receiving increasing attention because of the potential impacts of climate change on rainfall patterns and temperature extremes, salinization of agricultural lands by irrigation, and the overall need to maintain or increase agricultural productivity on marginal lands. In the field, a plant may experience several distinct abiotic stresses either concurrently or at different times through the growing season (Tester and Bacic, 2005). Some common examples of the abiotic stresses a plant may encounter include a decreased availability of water, extremes of temperature including freezing, decreased availability of essential nutrients from the soil (or conversely the build-up of toxic ions during salt stress), excess light (especially when photosynthesis is restricted) or increased hardness of the soil that restricts root growth.

Several abiotic stresses are united by the fact that at least part of their detrimental effect on plant performance is caused by disruption of plant water status. This can occur through decreased availability of water in the environment during drought, altered ion content and water uptake caused by salinity or cellular dehydration caused by formation of extracellular ice during freezing stress. Consequently, this paper focuses on these three stress factors: drought, salinity and freezing. In designing laboratory experiments to study plant responses to these stresses, the method used to impose the stress, the severity and duration of the stress, the parameters to be measured and how the observed responses of the plant fit into an overall strategy for resisting the stress are all important considerations. Evaluating the

 

stress responses of mutants and transgenic plants is often the most challenging type of experiment because the objective is to evaluate whether the plant's overall performance under stress has been altered. This is a much broader question than the measurement of more narrowly defined parameters such as changes in gene expression or metabolite levels. Genetic studies can also be challenging because the number of lines to be tested can demand a relatively high level of throughput, thus constraining the type of experiments that are feasible.

In the case of crop plants, it is ultimately the yield of genetically altered plants under specific field conditions that will determine whether or not a specific gene or metabolic or signaling pathway is of technological importance. The challenge of abiotic stress research is to bridge the gap between such agronomic or ecophysiological experiments and the basic research in Arabidopsis and other model organisms that is elucidating the molecular mechanisms by which plants sense and respond to abiotic stress. It is in this gap that the focus of this paper lies. Our goal is to discuss relevant ideas and provide examples that will be of assistance in designing experiments that are suitable for genetic studies and rapid screening while still being relevant to stress conditions in the real world. We first describe some basic principles of the responses of plants to altered water status. This background is then used to introduce experiments designed to examine responses to low water availability and to discuss the role of altered water status in salinity and freezing stress, and the methods used to impose these stresses and evaluate plant resistance. Finally we discuss some examples of the types of techniques useful in quantifying the extent of cellular and tissue damage caused by abiotic stress treatments.

## Drought and low water potential

Although altered water status is a factor in a number of abiotic stresses, it is of most obvious importance in drought. Drought can be most simply defined as a period of below normal precipitation that limits plant productivity in a natural or agricultural system (Boyer, 1982; Kramer and Boyer, 1995). In the field, drought can cause a number of plant stresses including temperature, light and nutrient stresses. However, the stress component that defines drought is a decrease in the availability of soil water. This decreased water availability can be quantified as a decrease in water potential ($\psi_w$, Kramer and Boyer, 1995). Mathematically, $\psi_w$ is the chemical potential of water divided by the partial molar volume (Kramer and Boyer, 1995); thus, the free energy of water, as well as the turgor of plant cells, can be expressed in units of pressure and a straightforward assessment of the direction of water movement in the soil/plant system can be made. Decreased $\psi_w$ (decreased free energy of the water) makes it more difficult for the plant to take up water, and this in turn elicits a range of responses that allow the plant to avoid water loss, allow water uptake to continue at reduced $\psi_w$ or allow the plant to tolerate a reduced tissue water content. An overall picture of these responses must include changes in water fluxes and water relations at the whole plant and the cellular levels.

### *Avoidance and tolerance of low $\psi_w$*

To understand the responses of plants to low $\psi_w$ at the level of the organism and cell it is useful to consider the stress avoidance/stress tolerance terminology proposed by Levitt (1972), a modified version of which is presented in Figure 1. In most cases, the plant's first response is to avoid low $\psi_w$. Tissue $\psi_w$ and water content are maintained close to the unstressed level by increasing water uptake or limiting water loss such that the rates of water loss and water uptake remain balanced. Such a balance is achieved in the short term mainly by stomatal closure. In the longer term, changes in root and shoot growth, leading to an increased root/shoot ratio, tissue water storage capacity and cuticle thickness and water permeability are also of potential importance. Of these, changes in root growth to maximize water uptake are of the greatest importance for crop plants.

In the case of mild water stress or water stress of a limited duration, avoidance mechanisms by themselves can be sufficient to maintain plant performance (Kramer and Boyer, 1995). Under such conditions, modifications such as increased root growth or decreased stomatal conductance have the potential to increase crop productivity. The trade-off in this case is the lost photosynthesis caused by reduced stomatal $CO_2$ uptake or a shift of resources into root growth at the expense of photosynthetic and reproductive tissue. Furthermore, these mechanisms for avoiding water loss do not themselves offer any protection from the effects of low $\psi_w$ if the stress becomes more severe and the plant is no longer able to maintain a balance between water uptake and loss. In cases where low $\psi_w$ cannot be avoided by altering water uptake and water loss, additional mechanisms become important in maintaining plant function.

## Dehydration avoidance

When transpiration is minimized, as is likely to be the case when stomata are closed because of stress, the $\psi_w$ of the plant will equilibrate with that of the water source (in most cases this is the soil $\psi_w$). Thus, when soil water content and $\psi_w$ are low, $\psi_w$ of the plant tissue must also decrease, either through water loss or by adjustments made by the plant to achieve a low $\psi_w$ while avoiding water loss. Such adjustments are termed 'dehydration avoidance' (Figure 1). The main mechanisms of dehydration avoidance are accumulation of solutes and cell wall hardening.

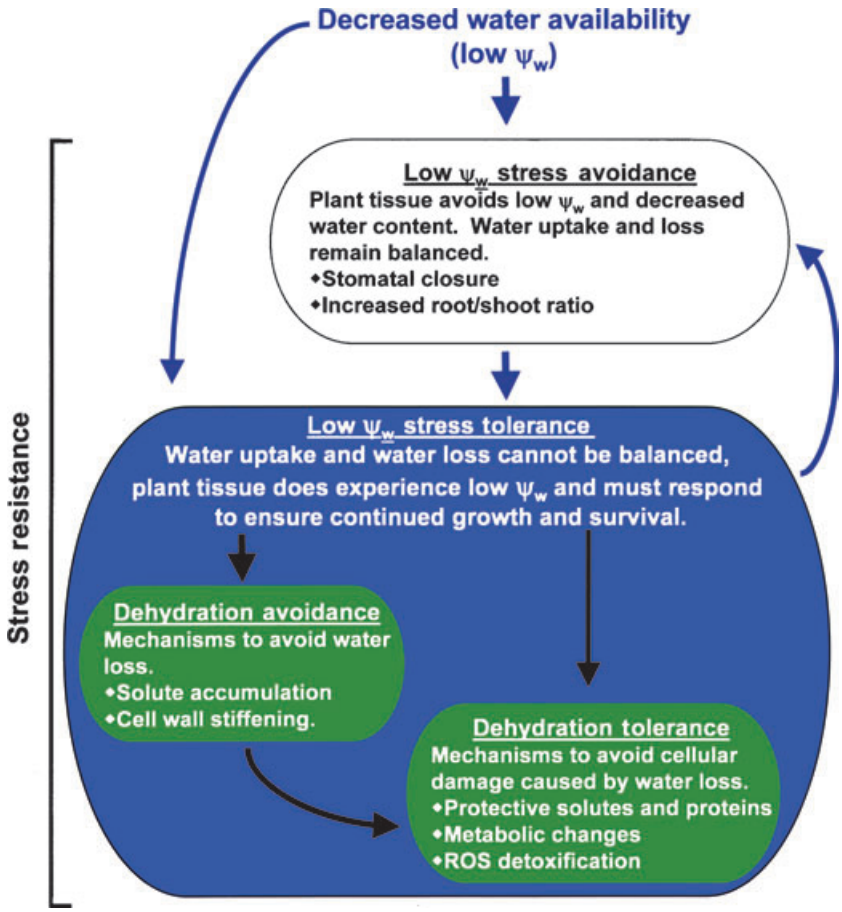


**Figure 1.** Conceptual diagram of the stress tolerance/stress avoidance model of low-$\psi_w$ responses.
In low-$\psi_w$ stress avoidance the plant balances water uptake and water loss to avoid an effect of the stress on tissue $\psi_w$ or water content (essentially, the stress is kept outside the plant tissue). If this cannot be achieved and the plant tissue does experience low $\psi_w$ (the stress becomes internalized to the plant tissue), stress responses occur that maintain a high water content despite a decreased $\psi_w$ (dehydration avoidance) or tolerate a reduced water content (dehydration tolerance). We use the term 'stress resistance' in cases where it is not possible or not desirable to refer to a more specific mechanism. The diagram is based on the stress avoidance/stress tolerance terminology of Levitt (1972).

Whether water will flow into or out of a plant cell is dependent on the $\psi_w$ gradient between the cell and its surroundings. The $\psi_w$ of a walled cell, such as a plant cell, is governed by the equation: $\psi_w = \psi_s + \psi_p$ (Figure 2) where $\psi_s$ is the osmotic potential and $\psi_p$ is the pressure potential (turgor pressure). For a cell to take up water from the soil, or other growth medium, it must have a lower $\psi_w$ than the water source. An example of $\psi_w$, $\psi_s$ and $\psi_p$ values which could occur in a plant cell that is fully hydrated and exposed to a relatively high external $\psi_w$ is presented in Figure 2(a).

At a given $\psi_w$, a higher $\psi_p$ can be achieved by accumulating solutes inside the cell, thus lowering $\psi_s$. The accumulation of additional solutes in response to low $\psi_w$ is termed osmotic adjustment (Zhang *et al.*, 1999). Osmotic adjustment refers to the active accumulation of additional solutes in response to low $\psi_w$ (after the effect of reduced water content on the concentration of existing solutes has been factored out). Examples of plant cells exposed to low external $\psi_w$ are presented in Figure 2(b). The top cell in Figure 2(b) did not alter its solute content in response to decreased external $\psi_w$; the solute concentration inside the cell did increase, but this was solely a result of decreased water content. In contrast, the middle cell in Figure 2(b) did accumulate additional solutes in response to low $\psi_w$ and this allowed the cell to maintain its original water content and volume. In reality, of course, solute accumulation and water loss can both occur in the same tissue and it is necessary to measure both change in volume (experimentally, change in volume is often approximated as the change in relative water content, Figure 2) and change in solute content to calculate the extent of osmotic adjustment. This can be done by calculating $\psi_{s100}$ [the osmotic potential at 100% relative water content (Babu *et al.*, 1999)] as shown for the examples in Figure 2(b).

It is important that the solutes accumulated to prevent water loss do not themselves interfere with cellular function. Thus, many plants accumulate one or more types of compatible solutes, such as proline or glycine betaine, in response to low $\psi_w$, salinity, freezing and other abiotic stresses that alter water status. These and other similar solutes are termed compatible solutes because they can accumulate to high levels without interfering with metabolism (Yancey *et al.*, 1982) and may also have other protective properties. Osmotic adjustment and accumulation of compatible solutes can be an important factor in drought tolerance in the field (Kramer and Boyer, 1995; Morgan, 1984, 1991), and engineering of increased synthesis of compatible solutes is one approach that has been taken to

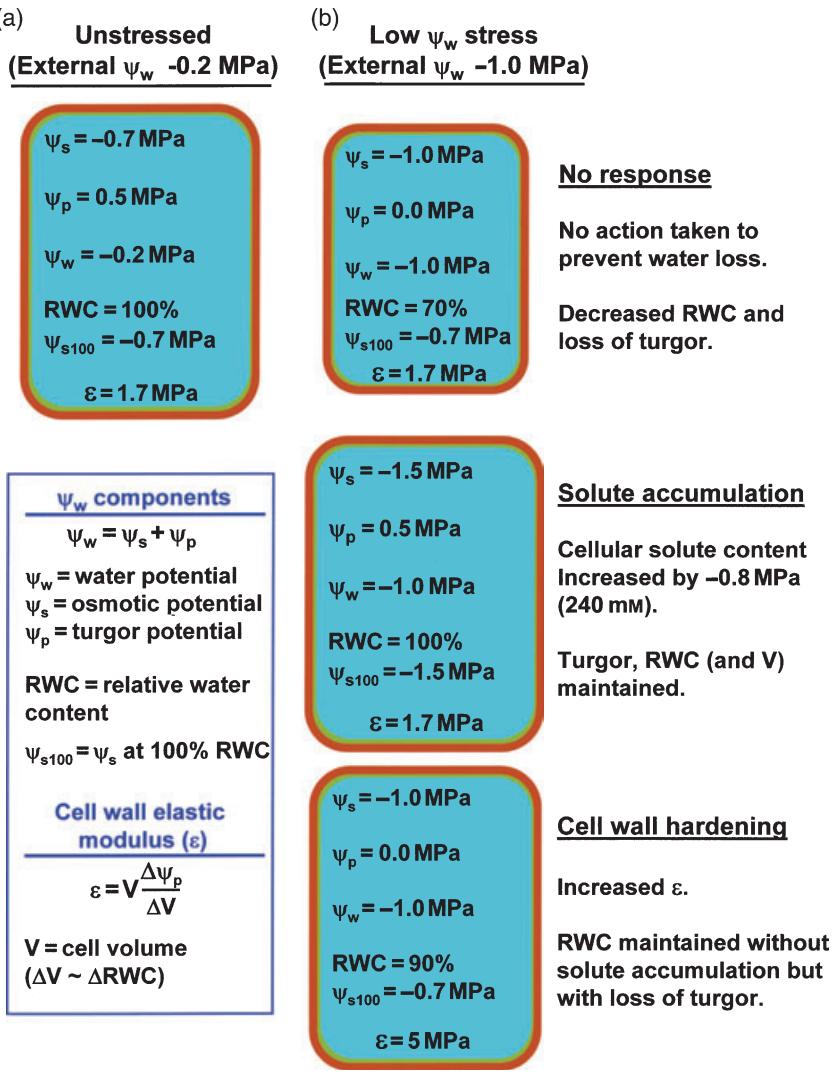


**Figure 2.** (a) Possible values of $\psi_w$, $\psi_s$ (solute content), $\psi_p$ (turgor), relative water content and cell wall extensibility values for a plant cell exposed to an external $\psi_w$ (–0.2 MPa) typical of unstressed conditions.
(b) Examples of the alterations in values of water relations, water content and cell wall extensibility after exposure to reduced external $\psi_w$ (–1.0 MPa) for three scenarios: no response (top), solute accumulation (middle) and adjustment of cell wall extensibility (bottom). Box: definition of water relation terms.

increase abiotic stress tolerance in plants (Apse and Blumwald, 2002; Bohnert and Shen, 1999). The trade-off in this case is that increased accumulation of compatible solutes can be energy and resource intensive for the plant, and, in cases of severe stress where soil water content is largely depleted, may have only a small effect on water uptake (Kramer and Boyer, 1995).

The properties of cell walls also play an important role in several abiotic stress responses, including dehydration avoidance. The deformability of the cell wall can be quantified by the elastic modulus of the cell wall, $\varepsilon$ (Figure 2). Simply stated, $\varepsilon$ is the pressure change required to cause a unit change in cell volume (Kramer and Boyer, 1995; Murphy and Ortega, 1995). When $\varepsilon$ is low, the cell wall deforms readily; thus a loss of water will cause a large change in volume but a small change in turgor because the cell wall shrinks and continues to squeeze the cytoplasm. The high turgor will cause $\psi_w$ to remain high, thus allowing further water loss from the cell. In contrast when $\varepsilon$ is high, a small loss of water causes little change in volume of the cell, but a rapid decrease in turgor and $\psi_w$ that allows the cell to avoid further water loss. This can be seen by comparing the top cell in Figure 2(b), which has a relatively high $\varepsilon$, with the bottom cell in Figure 2(b). Because of the high $\varepsilon$, the bottom cell is able to largely avoid dehydration even in the absence of solute accumulation. The trade-off of this strategy is that a rigid cell wall and loss of turgor prevent any further expansion of the cell. Thus, increasing $\varepsilon$ to avoid water loss is a strategy that is largely confined to non-growing tissues. Also, barring any increase in $\psi_w$ of the water source, solute accumulation is still required for this cell to lower its $\psi_w$ and take up water.

### Dehydration tolerance

As low-$\psi_w$ stress becomes more severe, it becomes increasingly difficult for the plant to avoid dehydration and mechanisms to tolerate reduced water content become

important. The most dramatic examples of dehydration tolerance are 'desiccation-tolerant' plants that can recover from a fully air-dried state (Oliver *et al.*, 2000; Vicre *et al.*, 2004). When fully dehydrated, these plants are in a metabolically dormant state that is in many ways similar to seed dormancy. Tolerance to severe dehydration is also a critical factor in freezing tolerance (see below). At the molecular level, seed dormancy, freezing tolerance, the vegetative dormancy experienced by desiccation-tolerant plants and the dehydration responses in less tolerant species have many similarities. However, most mesophytic plants (including almost all crop plants) lack the ability to enter a dormant state to tolerate complete desiccation and thus cannot recover from a severe (approximately 50% or greater) decrease in water content. These plants instead attempt to tolerate lesser degrees of water loss while maintaining metabolic activity.

Most of the dehydration tolerance mechanisms studied to date function primarily to protect cellular structure from the effects of dehydration. Several types of protective proteins, most notably dehydrins and other late-embryogenesis-abundant (LEA) proteins, are well known to accumulate in response to decreases in tissue water content either in response to abiotic stress or during seed development (Close, 1997). Although the function of many dehydrins and LEA proteins is not fully understood, at least part of their function is to act as chaperones that protect protein and membrane structure (Bravo *et al.*, 2003; Hara *et al.*, 2001). Compatible solutes can also protect protein and membrane structure under dehydration (Hincha and Hagemann, 2004). Another aspect of dehydration tolerance, and of tolerance to other abiotic and biotic stresses, is the control of the level of reactive oxygen species (ROS) or limitation of the damage caused by ROS. The sources of ROS under stress, mechanisms of ROS detoxification and the role of ROS in stress signaling are all active areas of current research and have been extensively studied and reviewed (Apel and Hirt, 2004; op den Camp *et al.*, 2003; Chen and Gallie, 2004; Corpas *et al.*, 2001; Foyer and Noctor, 2003; Hung *et al.*, 2005; Jiang and Zhang, 2003; Kwak *et al.*, 2003; Laloi *et al.*, 2004; Milla *et al.*, 2003; Moller, 2001; Mori and Schroeder, 2004; Pastori and Foyer, 2002; Shin and Schachtman, 2004).

### An integrated response

The consideration of avoidance versus tolerance mechanisms provides a valuable framework for designing experiments and interpreting the effects of low $\psi_w$. Our understanding, however, of the molecular and cellular events that occur when plants are exposed to low $\psi_w$ has increased greatly in the years since Levitt (1972) and others proposed the ideas of avoidance and tolerance of low $\psi_w$. With this increased understanding, it has become clear that many of the molecular events initiated by low $\psi_w$ do not fit exclusively into one of the avoidance or tolerance categories shown in Figure 1. For example, accumulation of a compatible solute such as proline may play a role in dehydration avoidance by increasing the cellular solute content and thus maintaining a higher water content. At the same time, accumulation of proline has been proposed to play a role in dehydration tolerance by protecting protein and membrane structure, regulating redox status or acting as a scavenger of ROS (Hare *et al.*, 1998; Hincha and Hagemann, 2004; Smirnoff and Cumbes, 1989; Verslues and Sharp, 1999). Likewise, the dehydrin proteins may also act as 'hydrophilins', proteins that bind water and thus could have a role in retaining water (dehydration avoidance) in addition to a role in protecting cellular structures (dehydration tolerance) (Close, 1997). Also, mechanisms that promote continued root growth at low $\psi_w$, such as osmotic adjustment in the growing region of the root (dehydration avoidance), may allow roots to penetrate deeper into the soil and take up more water, thus contributing to avoidance of low $\psi_w$.

It should also not be assumed that stress avoidance and tolerance occur in a linear progression in time after the stress begins or in a linear progression from responses initiated by mild stress to those initiated by severe stress. For example, some decrease in water content and turgor is likely to be required to trigger accumulation of abscisic acid (ABA) (Creelman and Zeevaart, 1985; Pierce and Rashke, 1980) which then causes stomatal closure to prevent further decrease in water content. Also, dehydration tolerance mechanisms such as accumulation of dehydrin and LEA protein may be initiated before significant dehydration occurs as a way of preparing the plant for any further decrease in water content. Rather than attempting to classify the various stress responses at a molecular level, a consideration of tolerance and avoidance mechanisms is most useful in clarifying the appropriate types of experiments, the interpretation of the data and the terminology used to establish the role of a particular molecular event in the plant's integrated response to low $\psi_w$ and other abiotic stresses.

Given the overlapping functions of many low $\psi_w$ responses, it is perhaps not surprising that these responses are controlled by a complex regulatory network. This network responds to both external stimuli, such as loss of turgor or reduced water content, and internal stimuli, such as production of ROS, sugar sensing and various hormonal stimuli, that reflect the metabolic and developmental status of the plant (Verslues and Zhu, 2005). Although many of the molecular components involved in this regulation remain uncharacterized, ABA is well known to be a key regulatory factor in controlling responses to many types of abiotic stress, including low $\psi_w$. Abscisic acid accumulates in response to abiotic stress and regulates the processes involved in all of the aspects of the low-$\psi_w$ response discussed above: ABA-regulated stomatal conductance and

root growth (Schroeder *et al.*, 2001; Sharp and LeNoble, 2002) are important in avoidance of low $\psi_w$; ABA-induced accumulation of compatible solutes can be crucial for dehydration avoidance (Ober and Sharp, 1994) and ABA-regulated synthesis of dehydrins and LEA proteins is important for dehydration tolerance (Sivamani *et al.*, 2000; Xu *et al.*, 1996). Thus, at the level of the organism, it seems that a main function of ABA is to coordinate the various aspects of low-$\psi_w$ response. A key aspect of understanding low-$\psi_w$ response as a whole is a better understanding of the upstream sensing and signaling that control ABA accumulation and downstream signals that modulate the response to ABA (Verslues and Zhu, 2005; Zhu, 2002). The current state of knowledge of perception of ABA, regulation of growth by ABA, ABA-dependent signal transduction and ABA-regulated gene expression have been reviewed (Bray, 2002; Finkelstein *et al.*, 2002; Sharp and LeNoble, 2002; Zhu, 2002).

### The homeostasis and protection model

The avoidance/tolerance model has been most commonly used to describe low-$\psi_w$ responses at the levels of the whole plant and the cell. In addition to this model, molecular-level responses and responses to other abiotic stresses are often discussed in terms of homeostasis and protection or damage repair (Zhu, 2001; Figure 3). These homeostatic mechanisms include ion homeostasis, which is likely to be a dominant factor in determining salt tolerance, and osmotic or water homeostasis, which is similar to the dehydration avoidance mechanisms discussed above and likely to be a dominant factor in the low-$\psi_w$ response. Protection and repair mechanisms are largely the same as the dehydration tolerance mechanisms described above. These protective mechanisms are important for all abiotic stresses but may play the dominant role in tolerance of the severe dehydration caused by freezing.

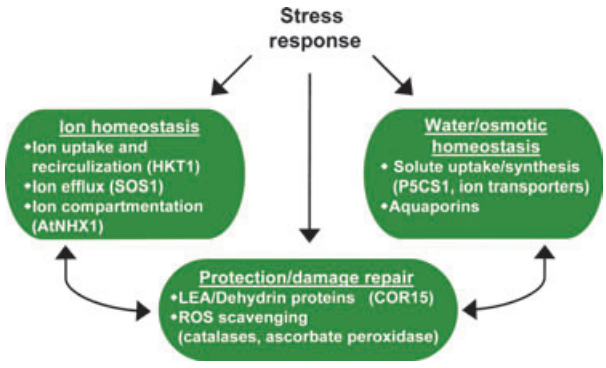


**Figure 3.** Homeostasis and protection/damage repair model of the abiotic stress response.
Mechanisms of ion homeostasis and water/osmotic homeostasis attempt to restore the cellular ion or water content to levels similar to those present under unstressed conditions. Protection and damage repair mechanisms attempt to prevent or repair cellular damage caused by altered ion or water content under stress. Some examples of genes involved in each class of response are also shown. Arrows indicate interaction between these stress response mechanisms.

A number of genes have been suggested to be involved in different aspects of homeostasis or damage prevention under abiotic stress (Figure 3). Some examples are genes for the $Na^+/H^+$ antiporter SOS1 (Shi *et al.*, 2000), the $Na^+$ influx transporter HKT1 (Rus *et al.*, 2001, 2004) and the tonoplast $Na^+/H^+$ antiporter AtNHX1 (Apse and Blumwald, 2002), all of which are known to be important determinants of salt tolerance because of their role in ion homeostasis. Water/osmotic homeostasis (dehydration avoidance) likely depends on the action of genes for solute synthesis [such as *P5CS1* in Arabidopsis (Strizhov *et al.*, 1997; Yoshiba *et al.*, 1999)] and a number of channels and carriers for uptake and compartmentalization of inorganic solutes, especially $K^+$. Aquaporins may also have a role in water and osmotic homeostasis by facilitating water movement; however, the precise role of aquaporins in abiotic stress responses remains undefined. One example of a protective protein for which a mechanism of action has been proposed is COR15. COR15 preserves membrane structure by preventing formation of the hexagonal phase and membrane fusion (Steponkus *et al.*, 1998). Regulatory proteins, for example ICE1 (Chinnusamy *et al.*, 2003) and DREBs/CBFs (Shinozaki *et al.*, 2003), are critical for the induction of protective responses.

Overall, while general measurements of plant performance such as growth and photosynthesis are applicable to many types of abiotic stress experiments, consideration of the mechanisms involved, either in the avoidance/tolerance terminology or in terms of homeostatic and protective mechanisms, will often suggest a more defined hypothesis about the mechanisms by which a particular genetic change may affect the stress response. These hypotheses can then be used to design more targeted experiments to quantify the particular stress resistance mechanisms of greatest interest. Some common experimental designs and the aspects of stress avoidance, stress tolerance and homeostasis they address are discussed in more detail below.

### Experimental techniques for evaluating the low-$\psi_w$ response

Here we describe some basic experimental designs (Figure 4) that are suited to the evaluation of mutants and transgenic plants: a number of lines can be tested in a fairly high-throughput manner and relatively little specialized equipment or apparatus are required. Given this starting point, Arabidopsis is used as the example plant. However, the principles illustrated and, to a large extent, the experimental techniques described, are applicable to other plants as well. Particular attention is paid to consideration of which of the aspects of low-$\psi_w$ response discussed above is tested by each type of experiment.

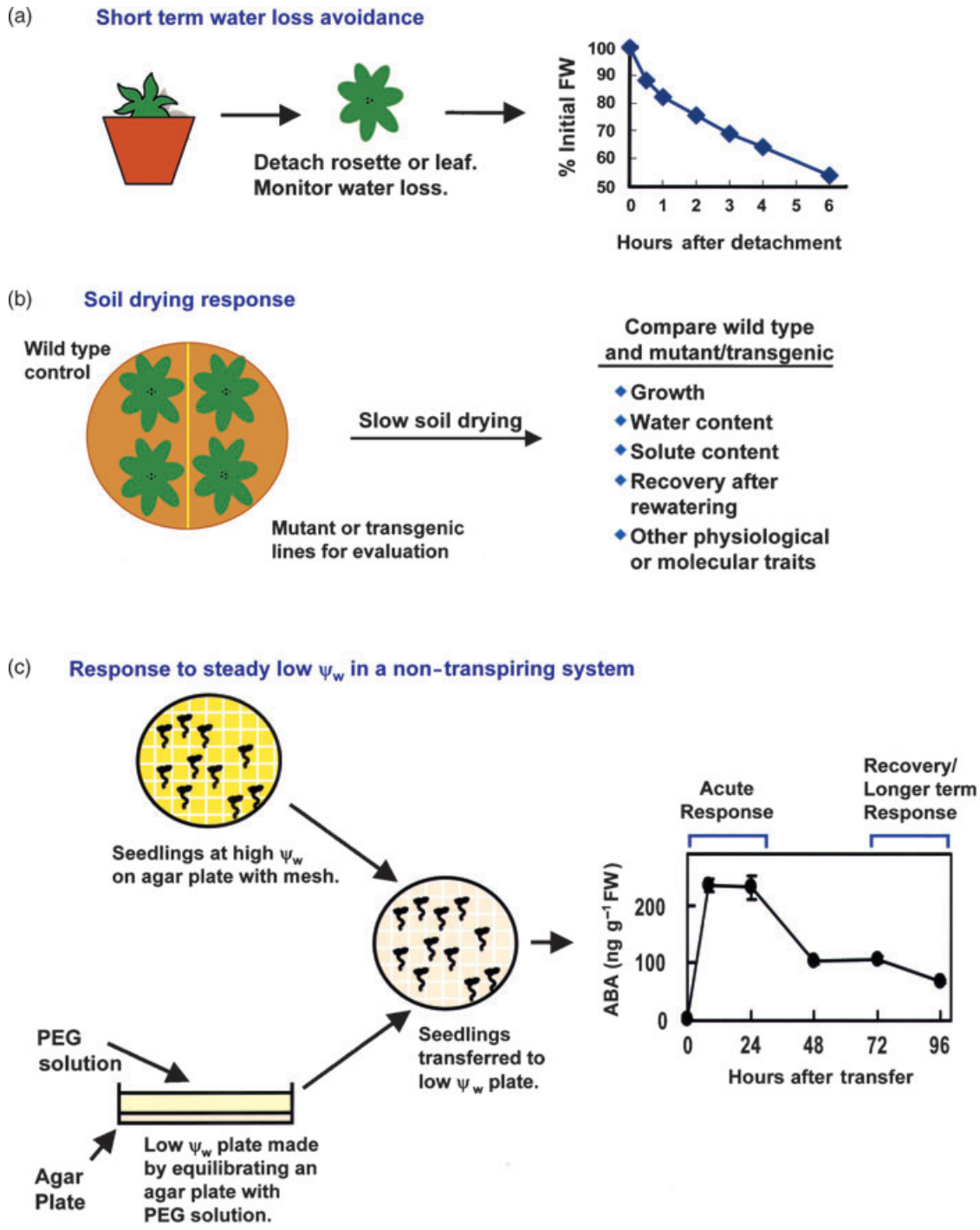


**Figure 4.** Three types of experiments used to evaluate low-$\psi_w$ responses.
(a) Short-term avoidance of water loss using detached leaves or rosettes. The graph shows a typical result for decrease in fresh weight over time after detachment.
(b) Soil drying of pot-grown plants.
(c) Imposition of constant low $\psi_w$ under non-transpiring condition using PEG-infused agar plates. Preparation of PEG-infused plates is described in detail in Protocol S1 of the Supplementary Material. The graph shows the typical pattern of ABA accumulation over time after transfer of 5- or 7-day-old seedlings from high $\psi_w$ (−0.25 MPa) to low $\psi_w$ (−1.2 MPa) using media without sugar. The acute response is the response from 0 to approximately 24 h after transfer. Recovery and longer-term responses can be seen after 72 or 96 h or longer exposure to low $\psi_w$.

*Leaf water loss*

Perhaps the easiest experiment to perform is to simply remove the aerial portion of the plant (or an individual leaf) from the roots and measure the decline in fresh weight over time (Figure 4a). The experiment should be set up under controlled temperature, light and humidity conditions that allow a gradual decline in leaf water content to be observed. A decline to 50% water content over the course of 6 to 8 h is typical in Arabidopsis (Figure 4a). The rate of water loss is largely determined by stomatal conductance; thus, experiments on leaf water loss measure avoidance of low $\psi_w$ and are typically not applicable to investigation of tolerance mechanisms. In addition to leaf water loss experiments, measurements of leaf conductance and direct microscopic observation of stomatal apertures in leaf epidermal strips (see for example Leymarie *et al.*, 1999) can be performed. Rates of leaf water loss can also be estimated based on leaf temperature. Thermal imaging has been used to isolate Arabidopsis mutants with altered stomatal regulation and stress avoidance (Merlot *et al.*, 2002; Wang *et al.*, 2004) and at the field level to estimate plant water status (Cohen *et al.*, 2005).

Because stomatal conductance is controlled in large part by ABA, measurements of leaf water loss are often most useful as an indicator of altered accumulation of or sensitivity to ABA. Mutants deficient in ABA and many (although not all) mutants with altered ABA sensitivity exhibit altered leaf water loss. In our laboratory, leaf water loss experiments are followed by, or performed concurrently with, other tests of ABA accumulation and response. These include the effect of ABA on seed germination and seedling growth and ABA-dependent gene expression and stress-induced accumulation of ABA. In many cases, these parameters are measured using the polyethylene glycol (PEG)-infused agar plate system described below. Measurements of ABA-responsive seed germination have been described in numerous studies (see for example Finkelstein, 1994) and typically involve plating seed on media containing ABA at a range of concentrations and scoring either emergence of radicles or the formation of green cotyledons over a period from 1 to 10 days after the end of stratification.

*Soil drying*

Soil drying experiments using pot-grown plants are typically done by removing the water supply and measuring some aspect(s) of plant growth, survival and water status after a fixed period of soil drying. Such soil drying experiments can at first seem quite straightforward but often turn out to be one of the most difficult types of experiment to interpret. This is because the severity of stress experienced by the plant is not determined directly by the investigator but rather by the plant itself based on the rate at which it depletes the available soil water. This can lead to confusion if the severity of the stress is not quantified by measuring leaf or soil $\psi_w$ or if steps are not taken to ensure that the genotype of interest is exposed to the same severity of stress as a wild-type control.

An example of one of the complexities of soil drying experiments is the evaluation of mutants or transgenic plants with decreased stomatal conductance or decreased growth and leaf area. When water is withheld and the condition of the plants assessed after a given time, plants that have reduced stomatal conductance or reduced leaf area can be expected to deplete soil water more slowly (avoidance of low $\psi_w$) and may exhibit delayed wilting compared with wild-type plants. Such delayed wilting has been used to label such plants as stress or drought tolerant when instead the transgenic plant has avoided low-$\psi_w$ stress by using the available water more slowly. In general, to establish whether a particular genetic modification leads to tolerance of low $\psi_w$, it must be shown that the stress response under study differs in plants exposed to the same severity of stress (same $\psi_w$) and that this difference leads to a desirable change in phenotype. A better-defined use of the term 'tolerance', as well as other terms related to the low-$\psi_w$ response, could do much to clarify the literature on this topic.

These difficulties can be overcome in two ways. The first is by quantification of leaf and/or soil $\psi_w$ during the drying cycle. This can be combined with control of humidity levels or partial rewatering of some plants to ensure that the comparisons of stress response are made only between plants exposed to the same $\psi_w$ (see for example: Sharp *et al.*, 2000; Thompson *et al.*, 2004). Partial rewatering can also be used to extend the time for which the plants are exposed to low $\psi_w$, thus allowing physiological and molecular responses to low $\psi_w$ be examined in more detail. These experiments are particularly relevant to more detailed evaluation of crop species (Sharp *et al.*, 2000; Thompson *et al.*, 2004) and numerous other studies where parameters such as osmotic adjustment and leaf growth have been evaluated in a number of crop species (see for example Babu *et al.*, 1999; Puliga *et al.*, 1996).

In the case of Arabidopsis, however, repeated measurements of leaf or soil $\psi_w$ during the drying cycle are laborious and require a quantity of material that may be difficult to obtain. For genetic studies, where a mutant or transgenic plant is being compared with a wild type, the easiest way to ensure a valid comparison while avoiding extensive measurements of $\psi_w$ is to grow the wild-type plant in the same pot as the genotype under evaluation (Figure 4b). Thus the roots of both genotypes will grow into the same soil and be exposed to the same $\psi_w$ even if one genotype uses water more quickly than the other. This approach can be combined with measurement of soil $\psi_w$ at the end of the drying cycle to quantify the final severity of the stress.

The rate of soil drying is a key factor in these experiments. A very rapid rate of soil drying allows little time for slow responses such as solute accumulation or cell wall modification to occur and can cause many important aspects of the low-$\psi_w$ response to be overlooked. Using a sufficiently large and deep pot will avoid this situation. The soil type [we typically use a well-aerated potting mix such as Metro-mix 350 (Sungrow Horticulture, Bellevue, WA, USA): similar potting mixtures are also available from other suppliers], humidity, temperature and light intensity will also affect the rate of drying and these factors must be adjusted empirically for any given set of conditions. As a rule of thumb, leaf water content should decline by no more than 30–40% over a 10–12-day period after the cessation of watering.

Several measurements of response to low $\psi_w$ can be used in conjunction with soil drying experiments. A general indication of plant performance can be obtained through measurements of growth (shoot fresh and dry weights, leaf area and root mass after soil removal), efficiency of water use or photosynthetic performance. Measurement of leaf relative water content and solute content and calculation of osmotic adjustment have been performed for many crop species (Babu *et al.*, 1999; Zhang *et al.*, 1999) and allow the capacity for dehydration avoidance to be accessed. If dehydration tolerance is the main interest, then measurements of plant survival after severe stress and measurements that quantify cellular damage such as loss of chlorophyll content, electrolyte leakage and ROS-induced damage (see below) can be performed.

### *Low-$\psi_w$ treatment using PEG-infused agar plates*

Many studies of low-$\psi_w$ stress have used osmotica to lower the $\psi_w$ of plant growth media. This approach has many advantages: $\psi_w$ can be controlled precisely and reproducibly and a large number of treatments can be performed quickly. Osmoticum treatment does, however, bring up its own set of potential problems that become apparent when osmoticum treatment is compared with soil drying. In most cases, when soil water content decreases water is withdrawn from both the cell wall and the protoplast resulting in cytorrhysis, a process where both the cell wall and protoplast shrink (Oertli, 1985). This contrasts with the response to low molecular weight solutes such as mannitol that are often used to lower $\psi_w$. In this case the solute freely penetrates the pores of the cell wall and causes plasmolysis; a loss of water from and decrease in volume of the protoplast while the volume of the cell wall remains unchanged. Because it is not a part of the typical soil drying response and may cause cellular damage that is perceived and responded to differently from water loss caused by soil drying, plasmolysis should be avoided in studies of low $\psi_w$ or salinity (Munns, 2002).

Experimentally, a cytorrhytic rather than plasmolytic low-$\psi_w$ treatment can be imposed using solutions containing a high-molecular-weight solute such as PEG of molecular weight 6000 or above. Polyethylene glycol of this molecular weight range cannot enter the pores of plant cells (Carpita *et al.*, 1979; Oertli, 1985) and thus causes cytorrhysis rather than plasmolysis. Polyethylene glycol is also a better choice for imposing low $\psi_w$ than the often used solute mannitol because mannitol has been shown to be taken up by plant cells and can cause specific toxic effects on growth (Hohl and Schopfer, 1991; Verslues *et al.*, 1998). An example of the toxic effects of mannitol and a similar solute melibiose are shown in Figure 5. For maize primary roots, transfer to a −1.6 MPa solution of mannitol or melibiose had less initial effect (0–10 h) on root growth than transfer to a −1.6 MPa PEG solution (Verslues *et al.*, 1998). This is consistent with mannitol and melibiose being taken up by the roots, thus leading to less initial loss of turgor and less initial growth inhibition. After 48 h, however, PEG-treated roots had recovered and resumed steady-state growth, albeit at a reduced rate [root growth of the unstressed control at this time was approximately 4 mm $h^{-1}$ (Verslues *et al.*, 1998)] while growth of the mannitol or melibiose roots had stopped. This clearly demonstrates that mannitol, and other low-molecular-weight solutes, have toxic effects that can obscure the low-$\psi_w$ response. In experimental systems such as PEG-infused agar plates (Protocol S1 in the Supplementary Material accompanying this article) where there is low transpiration, root damage is avoided, and the roots are not

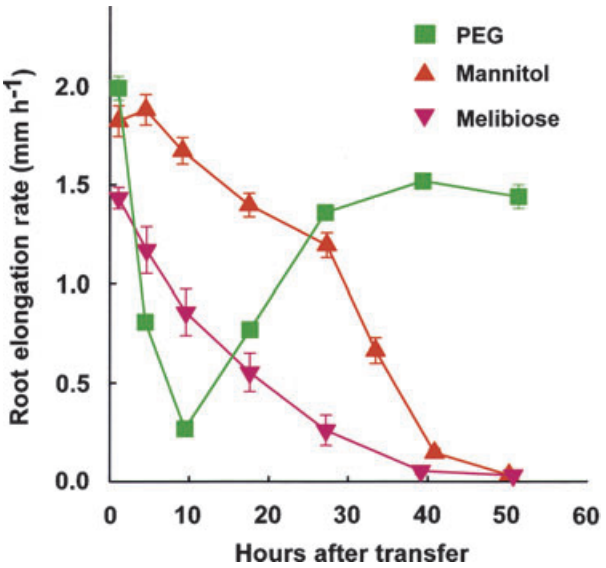


**Figure 5.** Rates of primary root elongation in maize seedlings transferred from wet vermiculite to −1.7 MPa solutions of either PEG, mannitol or melibiose.
In all cases, solutions were oxygenated to prevent root hypoxia (see Verslues *et al.*, 1998 for methods). Rates of root elongation in seedlings transferred to high-$\psi_w$ (no added solute) solution increased to approximately 4 mm $h^{-1}$ by 50 h (data not shown). Thus, PEG treatment caused a reduction of approximately 60% in the steady-state root elongation rate but mannitol or melibiose of the same $\psi_w$ completely stopped root elongation by 50 h. Data are from Verslues *et al.* (1998) and Verslues (1997).

subjected to hypoxic conditions by submergence in PEG solution. Polyethylene glycol is the best solute that we are aware of for imposing a low-$\psi_w$ stress that is reflective of the type of stress imposed by a drying soil (Verslues and Bray, 2004; Verslues *et al.*, 1998; van der Weele *et al.*, 2000).

In addition to the choice of solute used to impose the low $\psi_w$ stress, our experience, and that of others (van der Weele *et al.*, 2000), shows that for many types of measurements, it is better to use media without sugar, or with a low level of sugar (0.5% or less). This is because sugar is well known to affect ABA responses (Finkelstein *et al.*, 2002). Also, the addition of high a high level of sucrose itself can induce an osmotic response (the $\psi_s$ of a 3.0% sucrose solution is approximately –0.2 MPa). Thus, seedlings in 'control media' containing a high level of sucrose can already be experiencing a low level of osmotic stress. This causes a high baseline level for many low-$\psi_w$ responses. For example, ABA levels of more than 300 ng $g^{-1}$ fresh weight (FW) have been reported for Arabidopsis seedlings on MS media with 3% sucrose (Ruggiero *et al.*, 2004) whereas we routinely observe ABA levels of 1 to 4 ng $g^{-1}$ FW in a half-strength MS medium without sucrose (Verslues and Bray, 2004). This high baseline and the possibility that sugar from the medium can accumulate in the plant tissue and reduce the water loss caused by further decreases in $\psi_w$ means that many low-$\psi_w$ responses can be difficult to detect in high-sugar media.

A system of using PEG-infused agar plates to impose low $\psi_w$ has been described by van der Weele *et al.* (2000) and a modified version of this procedure is in use in our laboratory. A detailed protocol for the preparation and use of PEG plates is included as Supplementary Material with this article (Protocol S1). This system has the advantage of being able to easily make plates of a range of $\psi_w$ without the complications that arise from using low-molecular-weight solutes. Another advantage is that as long as steps are taken to prevent drying of the plates use of PEG-infused plates allows the imposition of a constant $\psi_w$ over time. Because $\psi_w$ is constant and transpiration minimal in the PEG-infused plate system, avoidance of stress is not an issue; the seedlings must equilibrate with the $\psi_w$ of the agar over time. Thus, the PEG plate system is ideal for studies of dehydration avoidance and mechanisms of dehydration tolerance. Measurements of growth, water and solute content, hormone accumulation and stress-regulated gene expression are examples of specific traits that can be quantified.

Seeds can be plated directly onto PEG-infused plates and seed germination and growth measured. However, in many cases the more useful experiment is to plate seeds on unstressed media (typically half-strength MS without sugar) and transfer them to PEG-infused plates after 5–7 days of growth (Figure 4c). To facilitate transfer of seedlings between plates, seed can be plated on a mesh overlaid on the original agar plate and transferred by moving the mesh and seedlings to the PEG-infused plate (Verslues and Bray, 2004; van der Weele *et al.*, 2000). For $\psi_w$ of –0.7 MPa or below, this transfer leads to rapid dehydration of the seedlings (Verslues and Bray, 2004). This loss of water in turn causes a number of rapid stress responses including high levels of ABA accumulation (Figure 4c) and, similar, to other systems, rapid induction of a number of stress- and ABA-regulated genes (P. E. Verslues and J.-K. Zhu, unpublished). These events, which we refer to as the 'acute' phase of the low-$\psi_w$ response (Figure 4c) have been the focus of most studies of low-$\psi_w$ response at the molecular and genetic levels. This acute response is followed by longer-term responses, such as solute accumulation and osmotic adjustment (Verslues and Bray, 2004) and changes in root and shoot growth (van der Weele *et al.*, 2000) indicative of an adjustment to and recovery from the effects of the reduced $\psi_w$. These recovery and longer-term responses are also important aspects of the low-$\psi_w$ response to be investigated by molecular and genetic studies. The PEG-infused plate system is in many ways (imposition of a constant low $\psi_w$ with minimal transpiration) similar to the dry vermiculite system that has been used to study low-$\psi_w$ responses of seedlings of maize and other crop species (Sharp *et al.*, 1988, 2004).

## Salt stress

### *Similarities and differences in salt stress, low $\psi_w$ and other abiotic stresses*

High salt causes several types of plant stress including altered nutrient uptake, especially of ions such as $K^+$ and $Ca^+$, accumulation of toxic ions, especially $Na^+$, osmotic stress and oxidative stress. Since NaCl is the major component of most saline soils, our usage of the terms salinity and salt stress here refers to stress caused by high levels of NaCl. Salt stress differs from the low $\psi_w$ imposed by soil drying or a high-molecular-weight solute in that a major factor causing long-term injury in salt stress is the ionic imbalance and toxicity caused by excess $Na^+$ rather than the effects of salt on $\psi_w$ (Huh *et al.*, 2002; Munns, 2002). Munns (2002) refers to several studies reporting that rapid responses to salt (responses that occur within a few hours of application of salt) often resemble responses to low $\psi_w$ imposed using non-ionic solutes. However, longer-term responses that occur over a time frame of days to weeks are more salt specific. This is also consistent with our isolation of several *salt overly sensitive* (*sos*) mutants that are hypersensitive to salt but not to non-ionic osmotic stress and regulate a relatively small number of ion transport processes and genes specifically involved in tolerance of salt stress (Gong *et al.*, 2001; Shi *et al.*, 2002; Wu *et al.*, 1996; Zhu, 2000; Zhu *et al.*, 1998).

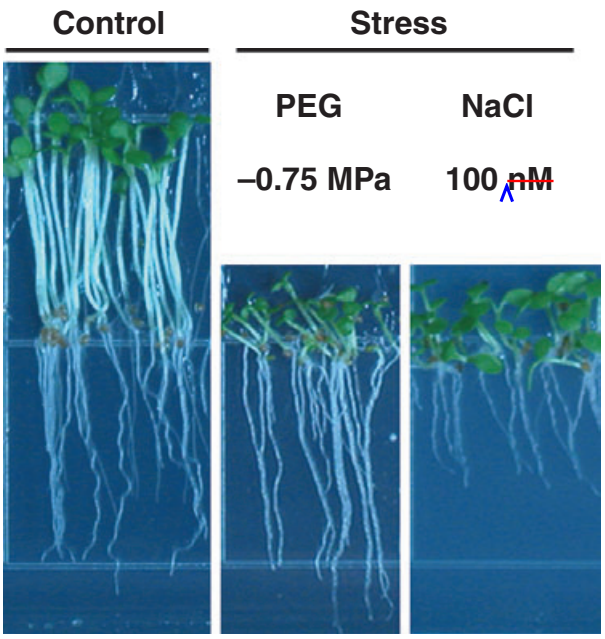


**Figure 6.** Growth of Arabidopsis seedlings on control (half-strength MS medium with 0.5% sucrose, $\psi_w = -0.30$ MPa) and PEG-infused or salt-containing plates, both having $\psi_w = -0.75$ MPa. Seeds were plated on each medium, stratified at 4°C for 3 days and seedlings grown for 7 days on vertically oriented plates before photographs were taken.

A simple example to illustrate some of the possible differences between low $\psi_w$ and salt stress is shown in Figure 6. Arabidopsis seedlings were germinated and grown on low-$\psi_w$ PEG-infused agar plates or salt-containing agar plates. The low-$\psi_w$ and salt treatments used were of the same $\psi_w$ (–0.75 MPa) and caused a similar amount of total inhibition of seedling growth. As previously observed (van der Weele *et al.*, 2000), low $\psi_w$ caused a large inhibition of shoot growth with root growth being relatively unaffected or even slightly increased. The relative maintenance of root growth at low $\psi_w$ is a well established response to low $\psi_w$ (Hsiao and Xu, 2000) and is the result of regulation of growth by ABA and other factors (Sharp and LeNoble, 2002).

In contrast, seedlings grown in agar plates with 100 mM NaCl had a greater inhibition of root growth, most likely caused by direct toxicity of $Na^+$. In this case, shoot growth was inhibited equally or slightly less than root growth, most likely because the rate of transpiration in the plates is too low to cause a build-up of high levels of $Na^+$ in the shoot. As has been previously suggested (Munns, 2002; Zhu, 2003), factors that affect the uptake and distribution of $Na^+$ within the plant can have a predominant role in the response to salt stress. Thus, while in a broad sense salt and low $\psi_w$ both have the same effect of inhibiting growth and causing cellular damage, the specific changes involved can be different and can be influenced by the choice of experimental system (in this example, the amount of sugar in the medium and the absence of transpiration to carry salt to the shoot can both alter the phenotype). Microarray analysis of salt- and dehydration-treated plants has also indicated substantial differences between the gene expression profiles elicited by these stresses (Seki *et al.*, 2002). One consideration for both low-$\psi_w$ and salt stress experiments should be to identify factors that are specific to, or more important to, one type of stress and those that may be shared and are of similar importance to salt, low $\psi_w$ and other abiotic stresses.

Here again, the concepts of homeostasis and of tolerance versus avoidance are useful. Salt injury can be avoided by maintaining proper ion homeostasis. This can be done by excluding salt from the cytoplasm, either through reducing salt uptake by the roots, activating salt export or by compartmentalizing the salt in the vacuole (Munns, 2002; Zhu, 2003). Under conditions of transpiration, blocking salt transport from the roots to the shoot is also critically important. Several lines of evidence suggest that the SOS signaling pathway, by regulating $Na^+$ and $K^+$ transport at both the plasma membrane and tonoplast, has a major role in maintaining ion homeostasis and thus avoiding salt injury (Zhu, 2002, 2003). Also, HKT1 is a major determinant of salt tolerance through its role in $Na^+$ uptake and transport of $Na^+$ within the plant (Liu *et al.*, 2000; Rus *et al.*, 2001). It is these ion homeostasis mechanisms that are most likely to be specifically important in the salt stress response and of lesser importance in responses to other abiotic stresses.

Other salt responses are important for tolerating the deleterious effects of high cytoplasmic levels of salt accumulation or of dehydration. To the extent that they have been characterized, the mechanisms for tolerating accumulation of salt in the plant tissue are closely related to the mechanisms of tolerating dehydration caused by low $\psi_w$ or freezing. These mechanisms can include accumulation of compatible solutes and proteins and ROS detoxification. It is in these tolerance mechanisms that many of the commonalities between salt, low $\psi_w$ and freezing can be found.

### *Experimental techniques for evaluating salt stress response*

Salt stress can be imposed by irrigating soil-grown plants with saline solutions or by transferring seedlings or plants to salt-containing media. One important consideration is that plasmolysis should be avoided whenever possible (Munns, 2002). For pot-grown plants this can be done by adding salt gradually or in steps of 50 mM or less separated by time for the plant to adjust. Pots should be periodically rewatered with the same saline solution to keep the salt concentration in the soil at a constant level. Similar to the soil drying experiments described above, it is advisable to grow the genotype being tested in the same pot as a wild-type control to ensure that they are exposed to the same salt concentration. Another concern is that the nutrient content of the

media should be sufficient such that addition of salt does not cause nutrient deficiency by decreasing the activity of other ions, particularly calcium (Cramer *et al.*, 1986; Reid and Smith, 2000). Salt treatment can also be performed by incorporating NaCl into agar plates. Seeds can then be germinated directly on the salt-containing media or transferred to the salt stress plates. For salt-treated plants or seedlings, a number of traits can be measured to quantify the salt response. These most often include measurements of growth and survival to assess the overall level of salt resistance.

### *Root and shoot growth, stomatal conductance and photosynthesis*

The effects of salt can be quantified through effects on growth (root fresh or dry weight, leaf area and leaf expansion and time of flowering and seed yield) and stomatal conductance and photosynthetic gas exchange. The specific experiments to be performed depend on the trait of greatest interest and the feasibility of the experiments for the number of genotypes to be tested. It must be noted that such experiments cannot determine whether any differences observed are caused by altered ion homeostasis (for example altered shoot $Na^+$ accumulation or $K^+/Na^+$ ratio) or altered tolerance to $Na^+$ accumulation. To answer this question it is necessary to also quantify tissue ion content and/or ion uptake (see below).

The most extensive experience of our laboratory is in rapidly screening Arabidopsis lines for altered root growth under salt stress using a root bending assay (see Protocol S2 in the Supplementary Material). This method was employed to identify *sos* mutants of Arabidopsis (Liu and Zhu, 1998; Wu *et al.*, 1996). In this method, seeds are plated on control media (typically MS or half-strength MS) and grown for approximately 4 days on vertically oriented plates. Seedlings are then transferred to plates containing NaCl (50–200 mM) and the plates inverted so that the roots point upward. In seedlings that continue to grow after transfer to salt-containing media, the roots will acquire a curled appearance as they grow downward. The advantage of this method is that it allows the extent of root growth to be checked rapidly without having to mark the position of the root apex. For salt stress, root bending assays have typically been done in media with high levels of sucrose (up to 3%), as high sucrose stimulates root growth and makes it easier to find mutants with inhibited root growth. Although agravitropic mutants will also not exhibit root bending, they can be easily recognized by continued upward root growth.

### *Salt-induced leaf damage*

An example of a quick method to measure salt-induced damage is by leaf disk assay (Sanan-Mishra *et al.*, 2005; Singla-Pareek *et al.*, 2003). Leaf disks from leaves of a similar age from test plants and an appropriate wild-type control are floated in NaCl solution and the extent of bleaching and chlorophyll loss determined. In comparing different genotypes, this technique eliminates any effect of altered root to shoot ion transport and allows a more focused assessment of the ability of the tissue to tolerate $Na^+$ accumulation.

### *Tissue ion content and uptake*

A complete investigation of the effect of a particular genetic change on the salt stress response should include a quantification of the accumulation of ions in plant tissue. Bulk tissue levels of $Na^+$ and other ions of interest can be quantified by straightforward methods such as atomic absorption spectroscopy. Potassium is of particular interest, as maintaining $K^+/Na^+$ selectivity is critical for salt tolerance (Zhu, 2003). If altered $K^+$ levels are observed, analysis of $K^+$ uptake can be performed by quantification of radioactive $^{86}Rb^+$ uptake (Wu *et al.*, 1996). Comparison of accumulation of, and growth responses to, $Na^+$ and other ions such as $Li^+$, a toxic $Na^+$ analog, and $Cs^+$, another toxic ion, can differentiate between a specific effect on $Na^+$ transport and more general effects on ion uptake (Zhu *et al.*, 1998; Protocol S2, Supplementary Material). In general, such measurements can address the question of whether a genetic change alters the ability of the plant to avoid salt-induced damage by keeping tissue $Na^+$ levels low while maintaining uptake of other critical ions. Such analysis of the *sos1*, *-2* and *-3* mutants had implicated these loci in the control of $K^+/Na^+$ ion homeostasis well before the identities of the mutated genes were known (Zhu *et al.*, 1998).

### *Germination*

Seed germination assays can provide a quick assay of salt response but must be interpreted with caution. A high rate of germination under salt stress is not well correlated with salinity tolerance at later developmental stages (Almansouri *et al.*, 2001; Kurth *et al.*, 1986; Saleki *et al.*, 1993). In agar media with high sucrose, seed germination and initial growth can occur in the presence of relatively high levels of salt but is normally blocked by accumulation of ABA. This is supported by the observation that several mutants that block ABA synthesis have increased germination under saline conditions (Gonzalez-Guzman *et al.*, 2002; Ruggiero *et al.*, 2004). We have also observed similar increased germination and growth when the ABA-deficient mutant *aba2-1* is germinated on salt- or PEG-infused plates containing 3% sucrose (P.E. Verslues and J.-K. Zhu, unpublished). Under most conditions, this inhibition of germination and early seedling growth by ABA is an adaptive response; it allows the plant to delay the start of growth and, importantly, transpirational water loss, until conditions are more

favorable. Screens that have looked for mutants with enhanced germination and early seedling growth under salt stress in non-transpiring conditions have predominantly found ABA-deficient or ABA-insensitive mutants (Gonzalez-Guzman *et al.*, 2002; Quesada *et al.*, 2000, 2002; Ruggiero *et al.*, 2004; Saleki *et al.*, 1993; Werner and Finkelstein, 1995). Whether or not such ABA-deficient mutants should be described as salt tolerant should be carefully considered. In addition, it is important to determine the ABA content and ABA sensitivity of any genotypes that exhibit altered germination under saline (or low-$\psi_w$) conditions before attempting to interpret their role in stress tolerance.

### *Freezing*

The general term 'cold stress' can be divided into two related phenomena; chilling stress and freezing stress. Chilling stress occurs at temperatures lower than the plant's normal growth temperatures but not low enough to cause ice formation (Levitt, 1972). Chilling is damaging primarily because of membrane leakiness caused by an inability to increase membrane fluidity to accommodate the lower temperature. Such chilling-sensitive plants are also highly sensitive to freezing stress (Guy, 2003). Here we will focus on freezing stress as it is intrinsically related to dehydration caused by low $\psi_w$. In the case of freezing, it is the formation of ice crystals in the extracellular space that dehydrates the cell. Thus, the dehydration tolerance mechanisms discussed above are also relevant to tolerance of freezing stress. For example, the constitutively freezing tolerant mutant *eskimo1* (*esk1*; Xin and Browse, 1998) has increased total solute accumulation and increased accumulation of the compatible solute proline, traits that are also likely to make *esk1* more resistant to low-$\psi_w$-induced dehydration (to our knowledge, however, this has not been tested). Increased tolerance to dehydration, salt and freezing has been reported in plants overexpressing DREB (dehydration response element binding) transcription factors which leads to enhanced expression of a wide range of stress responsive genes (Liu *et al.*, 1998; Kasuga *et al.*, 1999). In addition to the tolerance of freezing itself, the ability to increase chilling and freezing tolerance by first exposing the plant to a short duration of a less severe low-temperature treatment is an area of active investigation.

To understand the methodology used to impose freezing stress, it is important to understand the mechanism of ice formation and its harmful effects on the cellular environment. Freezing injury is caused by the formation of ice in and around cells. The temperature at which ice begins to form depends on the presence of ice nucleators. In most situations, epiphytic bacteria found on plant leaves provide sites for ice nucleation (Lindow *et al.*, 1982). Plant cells and cell walls may also have intrinsic ice nucleation sites but these are not as efficient and the specific cellular structures that can nucleate ice formation have not been identified (Ashworth and Kieft, 1995). Consequently, sterile leaf disks can be supercooled (cooled below freezing without ice nucleation) to −8°C whereas leaves colonized by bacteria will nucleate ice formation at approximately −2°C (Lindow *et al.*, 1982). Experimentally, a constant ice nucleation temperature can be imposed by incubating plants with ice chips.

After initiation of ice formation, subsequent nucleation occurs on the surface of the ice crystal itself. In addition to its effects on dehydration avoidance (Figure 2) the composition and structure of the cell wall provides the plant with an opportunity to control the location of ice nucleation sites in the tissue. At the whole-plant level, ice first forms in the large vessels of the xylem in leaves and stems, in substomatal cavities and in intercellular spaces (Levitt, 1980). The large diameter of xylem vessels favors ice formation, and their dilute sap has a higher freezing point than other solutions in the plant. Once ice forms it will spread throughout the vessels and into the extracellular spaces of other tissues. However, the ice crystals cannot penetrate an intact plasma membrane to inoculate the cytoplasm. Thus ice formation in the extracellular space decreases the $\psi_w$ of the extracellular space, leading to movement of water out of the cells and cell walls until equilibrium of $\psi_w$ across the membrane is re-established. This is similar to the cytorrhytic dehydration described above for plants in drying soil, although the extent of dehydration is likely to be more severe during freezing stress. Thus, freezing stress causes damage primarily by dehydrating and collapsing cells, disrupting tissue structure by the formation of large ice crystals and causing large fluxes of water across cellular membranes during freezing and thawing.

## Experimental procedures for imposing freezing stress

Since the formation of ice is so important for freezing tests, factors affecting ice formation should be considered carefully when laboratory freezing tests are performed. These issues have been discussed in detail by Gusta *et al.* (2003) and are reviewed briefly here.

### *Ice nucleation*

Under controlled conditions of plant growth naturally occurring ice nucleators are generally absent and therefore it is important to incubate the plant tissues with ice chips (which act as nucleating agents).

### *Nucleation temperature*

The temperature at which nucleation is started is important because prolonged supercooling results in non-freezing equilibrium (Olien, 1974), resulting in explosive ice growth and formation at unfavorable sites. This indicates that temperature until which supercooling should be done is very important for determining the $LT_{50}$ (the

temperature at which 50% lethality occurs). An example of this was observed in *Solanum acaule* where the $LT_{50}$ of leaves was determined to be −7°C, if nucleation was started at −1°C. However, when leaves were supercooled to −2°C, followed by ice nucleation the $LT_{50}$ was observed to be −3°C (Rajashekar *et al.*, 1983). Before beginning freezing experiments, it is advisable to review the freezing stress literature for a particular species to determine if an optimal ice nucleation temperature has been established.

### *Intactness of the cuticle*

The cuticle acts as a barrier to ice formation inside the plant tissue (Wisniewski and Fuller, 1999). Damage to the cuticle, such as mechanical damage or damage caused by pathogen infection, can result in lesions through which ice crystals can grow and can skew the results of freezing tests. Also, when excised tissues are used for freezing stress, the cut surface provides an excellent opportunity for ice to enter the conducting vessels. Because of this, such an assay may not accurately reflect the whole-plant response where such an easy route for ice entry is not available.

### *Cooling rate*

The rate of cooling is another important criterion to be considered in artificial freezing tests. A rapid rate of cooling can result in non-uniform cooling across the plant tissue and rapid freezing that does not mimic the natural freezing process. If the rate of cooling is too slow, it may be more difficult to detect differences in freezing tolerance.

Many freezing protocols have been developed for a number of plant species. The Supplementary Material to this paper includes a protocol (Protocol S3) suitable for Arabidopsis. It can be adapted for other species and conditions with consideration of the factors outlined above.

## Quantifying abiotic stress-induced cellular damage

Severe levels of low-$\psi_w$, salt or freezing stresses cause cellular damage, and quantifying the extent of this damage can be an important component in testing the effect of a specific genetic modification. Often, the extent of stress-induced damage is measured by testing the percentage of plants that survive and recover after undergoing a stress treatment and then being transferred back to unstressed conditions. Such survival tests can be done quickly, and in many cases are sufficient to detect differences between genotypes. However, such tests are a relatively crude measure of the stress response and can miss differences in cellular damage that are significant but do not change the ability of the whole plant to recover after release of the stress. In many cases the particular gene, protein or cellular component under study will suggest specific methods for quantifying the damaging effects of low $\psi_w$. In other cases, assays of electrolyte leakage, ROS accumulation and ROS-induced chemical damage can be good indicators of the degree of cellular damage. Electrolyte leakage allows relatively quick assessment of the intactness of cell membranes. Detailed procedures for measuring electrolyte leakage in freezing and low-$\psi_w$ treated tissue are included with the Supplementary Material accompanying this article (Protocol S4).

Accumulation of ROS and ROS-induced damage can serve as an indicator both of structural damage to cells and of metabolic dysfunction. However, when interpreting the effects of ROS accumulation, the increasing recognition that ROS accumulation is an important aspect of abiotic stress signaling must be kept in mind. Total ROS accumulation can be assayed using the non-fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) that is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The non-fluorescent $H_2$DCFDA can diffuse readily into cells but becomes trapped after interaction with ROS molecules and oxidation to DCF. The DCF can then be detected by confocal scanning fluorescence microscopy (Mazel *et al.*, 2004). Superoxide can be specifically detected by nitroblue tetrazolium (NBT) staining (Lee *et al.*, 2002). Hydrogen peroxide can be detected either by staining using 3′,3′-diaminobenzidine (Lee *et al.*, 2002) or by quantitative assay of tissue extracts using the hydrogen peroxide-specific dye Amplex Red (Shin and Schachtman, 2004). The most common measure of ROS-induced damage is lipid peroxidation. Lipid peroxidation can be estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malonaldehyde (Heath and Packer, 1968). It has been observed that some salt-tolerant germplasms have less peroxidative damage than more sensitive genotypes (Shalata and Tal, 1998).

## Conclusions

There is an increasing availability and ease of generation of genetically modified lines in Arabidopsis, and other model organisms, that either increase (overexpression or ectopic expression) or decrease (mutants, gene knockouts and RNA-interference lines) the production of certain gene products. Thus, the genetic resources available for the investigation of abiotic stress resistance have increased dramatically in the last few years and are likely to continue to do so. This has led to the emergence of what has been termed the 'phenotype gap' (Miflin, 2000), where the identification of useful phenotypes and applications has increased at a much slower pace than the increase in molecular and genetic data. It is hoped that this paper will stimulate thinking about the best methods to use to translate the increasingly available molecular and genetic resources into identification and better understanding of the phenotypes associated with abiotic stress resistance.

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## Supplementary Material

The following supplementary material is available for this article online:

**Appendix S1.** Protocol I: Preparation of PEG-infused plates for low water potential treatment.

**Appendix S2.** Protocol II: Evaluation of salt tolerance in Arabidopsis seedlings by measuring root elongation.

**Appendix S3.** Protocol III: Monitoring plant survival by whole plant freezing tests.

**Appendix S4.** Protocol IV: Electrolyte leakage test.

This material is available as part of the online article from http://www.blackwell-synergy.com

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