Chapter 2

Approaches to Identifying Genes for Salinity Tolerance and the Importance of Timescale

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Abstract

Soil salinity reduces the ability of plants to take up water, and this quickly causes reductions in the rate of cell expansion in growing tissues. The slower formation of photosynthetic leaf area in turn reduces the flow of assimilates to the meristematic and growing tissues of the plant. Later, salt may exert an additional effect on growth. If excessive amounts of Na^+ or Cl^- enter the plant it may rise to toxic levels in the older transpiring leaves. This injury, added to an already reduced leaf area, will then further limit the flow of carbon compounds to meristems and growing zones in leaves. This chapter analyses the various plant responses over time, to provide a conceptual framework on which the different approaches to gene discovery can be based. Knowledge of the physiological processes that are important in the tolerance response, and the time frame in which they act, will enable further progress in understanding of the molecular regulation of salt tolerance.

Key words: Gene expression, salinity, salt tolerance, Na^+ accumulation.

1. Introduction

Salinity affects about 6% of the world's land area, much of which is important to agriculture. Over 800 million ha of land throughout the world are salt affected either by salinity or by the associated condition of sodicity (1). Most of this salinity, and all of the sodicity, is natural. However, a significant proportion of recently cultivated agricultural land has become saline due to clearing of natural vegetation or irrigation. Of the current 230 million ha of irrigated land, 45 million ha are salt affected (1). Irrigated land is only 15% of total cultivated land, but as irrigated land has at least twice the productivity of rain-fed land, it produces one-third of the world's food.

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Yield of essential food and forage crops is limited by soil salinity in many of these regions, so genetic improvements in salt tolerance are essential to sustain global food production. The ability to grow and reproduce in saline soil differs widely between species, due to differences in the ability to control salt uptake from the soil and to compartmentalise it effectively at the cellular level (2). Yet, even in the most salt-tolerant species, growth rates are greatly reduced by salinity, due to the osmotic stress of the salt in the soil. Maintaining turgor through osmotic adjustment is essential to counter the osmotic stress. Turgor maintenance through osmotic adjustment is a mechanism that contributes to the salt tolerance of halophytes (3).

The genetic control of the tolerance response is complex. There are a number of transporters involved in the regulation of Na^+ uptake and transport (2), and a number of signalling pathways involved in the growth response to the perturbation of the osmotic stress outside the roots (4). However, it is difficult to distinguish critical genes that determine the tolerance or susceptibility of the plant, from those 'downstream' of the perturbation, the so-called housekeeping genes. Genes controlling the level of reactive oxygen species are an example of these housekeeping genes (2).

This chapter aims to distinguish the osmotic and salt-specific parts of the response, so that candidate genes can be more clearly discerned. Growth reductions are predominantly due to the osmotic stress, but in species that have a high rate of salt uptake, or cannot compartmentalise salt effectively in vacuoles, salt-specific effects develop with time, impose an additional stress on the plant through failing capacity to produce photoassimilate, and give rise to the category of 'salt-sensitive'. This chapter does not focus on individual genes, but on the general process in which they are operating. These are basically the maintenance of cell turgor and volume, the control of ion transport, and the control of cell growth by hormone action or by carbon supply. Individual genes that are important in the tolerance mechanism have been covered in several recent reviews (2, 5, 6). This chapter attempts to construct a whole plant framework and to suggest the time and place in which gene expression should be investigated. An understanding of the tissue- or cell-specific nature of the response, and the whole plant phenotype associated with individual gene action, is essential in the identification of important genes.

2. Growth Processes at Different Timescales

Table 2.1 summarises the sequence of events in a plant when exposed to salinity. In the first few seconds or minutes, cells lose water and shrink. Over hours, cells regain their original turgor and

Table 2.1
The effect of salinity on plant growth at different timescales and the cellular and metabolic events involved. The species indicated as 'sensitive' have poor exclusion by roots or inadequate compartmentation within leaves, although this would occur in all species if the salinity was high enough

	Observed effect on growth	Cellular events	Metabolic events
Seconds to minutes	Instant reduction in leaf and root elongation rate then rapid partial recovery	Shrinkage of cell volume then restoration due to regaining turgor	Osmotic adjustment
Hours	Steady reduced rate of leaf and root elongation	Changed rheology of cell wall	Signalling pathways
Days	Reduced rate of leaf emergence; increase in root:shoot ratio	Cell production rate and primordia development inhibited	Signalling pathways and carbohydrate supply
Weeks	Reduced branch or tiller formation	Apical development program altered	Signalling pathways and carbohydrate supply
Weeks – sensitive species	*Old leaves die*	*Na^+ and/or Cl^- accumulates excessively in cells*	*Ion toxicity in mature leaves*
Months	Altered flowering time, reduced seed production	Reproductive development program altered	Signalling pathways and carbohydrate supply
Months – sensitive species	*Plant dies before maturity*	*Inadequate capacity for assimilate production to support further growth*	*Carbohydrate deficit*

volume but cell elongation rates in growing tissues are reduced, leading to lower rates of leaf and root growth. Over days, lower rates of leaf and root growth can be seen. Over weeks, gross changes in vegetative development are apparent, such as reduced formation of lateral shoots, and over months changes in reproductive development. In salt-sensitive species, which are often those less able to control the uptake and internal transport of salt, leaves die prematurely and the plant may be unable to produce sufficient photosynthate to complete its life cycle.

The different responses occur over different timescales, from changes in water relations that occur as soon as the roots encounter a saline soil solution to complex controls at the whole plant level involving long-distance signalling and the supply of assimilates. Salt toxicity affects growth in the longer term. Salt toxicity occurs in plants growing in salinities too high for them to adequately control the uptake of salt by roots, its transport

to leaves, and compartmentalisation of the salt within cells. Salts accumulate in the older transpiring leaves over time, and if reaching toxic concentrations will inhibit growth of the younger leaves by reducing the supply of carbohydrates to the growing cells.

2.1. Timescale of Seconds to Minutes – The Transient Phase

Cell expansion rates change suddenly in both leaves and roots. With a sudden change in salinity there are rapid, virtually instantaneous, shrinking of cells in both roots and leaves and cessation of cell expansion. Several minutes after the initial decline of leaf and root growth, a gradual recovery is observed that may take 30 minutes or more before reaching a new steady rate. This response has been recorded for both roots and leaves in many different species (7–9) and shown for barley in **Fig. 2.1**.

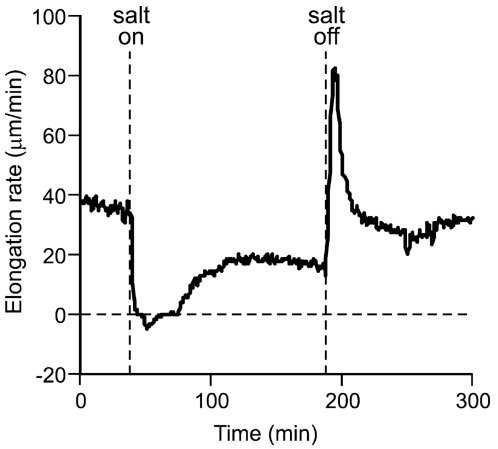

Fig. 2.1. Effect of changes in soil salinity (75 mM NaCl) on leaf elongation rate of a barley leaf. *Vertical broken lines* mark the times at which the soil solution was changed. Adapted from (7) [http://www.publish.csiro.au/journals/fpb; DOI: 10.1071/PP99193].

These rapid and transient changes in growth are due to changes in cell–water relations alone. Evidence for this comes from experiments in which leaf water status was maintained as the soil was made saline by a pressurisation technique, which prevented a drop in leaf water status and completely prevented the transient changes shown in **Fig. 2.1** (9). A water relations explanation is supported by the findings that different osmotica produce the same rapid and transient decline (10). In roots also, there are rapid and transient reductions in growth rates, which are due to changed water relations, as indicated by the similar effect of non-ionic osmotica (10).

2.2. Timescale of Hours – The Recovery Phase

Leaf growth recovers to a large extent within one or more hours after a sudden change in salinity, settling down to a new steady rate that is considerably less than the original one (**Fig. 2.1**). The time taken to recover and the new steady rate depend on the

concentration of the salt solution (7). Other osmotica have the same effect (10).

Root growth, in contrast to leaf growth, recovers remarkably well. With moderate levels of osmotic stress the recovery is essentially complete within 1 h, but with a larger osmotic shock this may take 24 h (10). In a study with maize roots comparing the effects of a salt shock versus a gradual increase, Rodriguez et al. (11) found that salt had no effect on root growth at concentrations up to 100 mM NaCl (about 0.5 MPa) as long as the concentration was increased gradually. The deleterious effects of a salt shock are presumably associated with the plasmolysis of root cells that would ensue if there was a single-step increase in osmotic pressure of more than 0.4 MPa (as the turgor of root cells is about 0.4 MPa), and the plasma membranes would take some time to repair (10).

The new steady rate of growth is unlikely to be determined by salt toxicity, as other osmotica such as KCl and mannitol have the same effect. It is also unlikely to be due to water relations or a hydraulic message from the roots, as turgor is regained in roots (7) and probably also in leaves. Complex signalling pathways are regulating root and leaf growth.

2.3. Timescale of Days – The Adjustment Phase

Over the timescale of days, in addition to a reduced rate of leaf expansion, there is a reduced meristematic activity, with delayed emergence of new leaves and lateral buds. Leaf growth is often more affected than root growth, resulting in an increased shoot:root ratio. This is an adaptive response as reduced leaf area would lessen the depletion of soil water and therefore the rate at which the salt concentration in the soil rises. Plants exclude at least 90% of the salt from the solution they take up (12), which results in a concentration of the salt around the roots, so a reduction in water use is beneficial in the long term.

The slower growth rate is not due to water relations, as shown by the fact that the leaves have regained turgor (13), and that leaf expansion of plants in saline soil does not respond to an increase in leaf water status (14). Photosynthesis of expanded leaves is reduced due to stomatal closure (15, 16); however, growth does not seem to be limited simply by carbon supply as carbohydrate status of the plant is still high (14). This means that the slower growth is regulated by signalling pathways, which includes the transmission of a message from the roots to the shoots.

Salt-specific effects are unlikely at this stage, as it is unlikely that salt ever builds up to toxic concentrations in the growing cells themselves. For instance, in the rapidly elongating tissue of leaves of wheat grown in 120 mM NaCl, Na^+ averaged only 15 mM and Cl^- averaged 50 mM (17). Fricke (18) found no correlation between the rate of barley leaf elongation and Na^+ concentrations in the growing zone. The rapid expansion of the growing

cells would keep the salt from building up to high concentrations. As long as it can be sequestered in rapidly expanding vacuoles, salt uptake at this stage of cell development is advantageous for osmotic adjustment.

Antioxidant activity is increased in order to maintain reactive oxygen species (ROS) at the levels required for their role in various signal transduction cascades. Leaves have surplus capacity to produce antioxidants to prevent ROS reaching toxic levels, the latter occurring only in a controlled oxidative burst that signals programmed cell death (19).

2.4. Timescale of Weeks – The Phase of Rapid Vegetative Development

After a week, developmental changes appear in the shoot. A marked reduction in the number of lateral shoots can occur. For example, the number of tillers of wheat growing in 150 mM NaCl was reduced by two-thirds (20), which accounted for the two-thirds reduction in leaf area. Leaf anatomy changes, so that leaves are smaller in area but thicker, resulting in a higher concentration of chlorophyll per unit area. Leaves are often visibly greener. For this reason, photosynthetic rate per unit area may be little affected (15), although photosynthetic rate per leaf and certainly per plant is reduced.

The mechanism by which salinity affects these developmental processes is unknown. It is unlikely to be by an effect of Na^+ or Cl^- in the meristematic tissues themselves, as concentrations there are probably quite low (20). Lateral bud development is influenced by the supply of carbohydrate, as elevated CO_2 increased the number of tillers of wheat plants in saline soil and reversed the effect of salinity (20). However, the carbohydrate status of the growing tissues was not reduced by salinity (20) indicating that signalling pathways are regulating the growth rate to keep a positive carbon balance between source and sink, i.e. between supply and demand.

Extra changes are seen in sensitive species. In this timescale of weeks, the sensitive genotypes show marked injury in older leaves. It is due to salts accumulating in transpiring leaves to excessive levels, exceeding the ability of the cells to compartmentalise salts in the vacuole. Salts then rapidly build up in the cytoplasm and inhibit enzyme activity. Salts eventually build up to high concentrations in the transpiring leaves and cause premature senescence.

In very sensitive species, leaves die at a fast rate. The rate at which they die becomes the crucial issue determining the survival of the plant. If new leaves are continually produced at a rate greater than that at which old leaves die, then there is enough photosynthetic surface for the plant to produce flowers and seeds. However, if the rate of leaf death exceeds the rate at which new leaves are produced, then the proportion of leaves that are injured starts to increase. There is then a race against time to initiate

flowers and form seeds while there are still an adequate number of green leaves left to supply the necessary photosynthate.

This is illustrated in an experiment with two wheat genotypes with contrasting rate of Na^+ transport to leaves, and resultant contrast in salt tolerance in the long term (21). A period of a month elapsed during which growth rates of both genotypes were equally reduced by salinity, even though leaf injury appeared on one more than the other. After a month, the growth rate of the injured genotype started to slow down, and within 2 weeks many individual plants had died (**Fig. 2.2**). This experiment indicated that the major effect on growth was osmotic, but after time, salt toxicity exerted an additional effect.

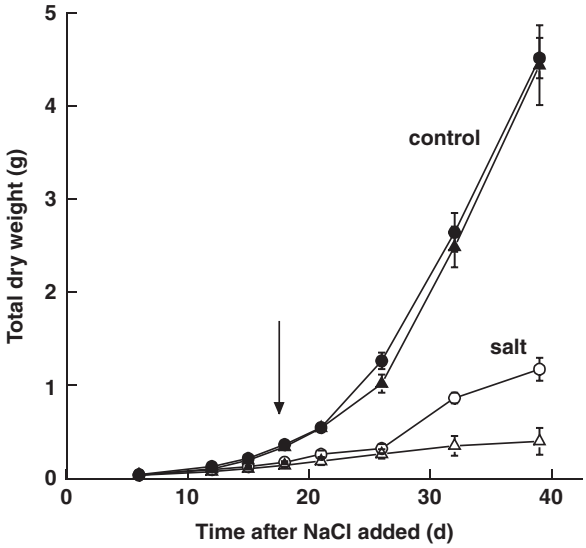

Fig. 2.2. Two accessions of the diploid wheat progenitor *Triticum tauschii* in control solution (*closed symbols*) and in 150 mM NaCl with supplemental Ca^{2+} (*open symbols*). *Circles* denote the tolerant accession and *triangles* the sensitive one. The *arrow marks* denote the time at which symptoms of salt injury could be seen on the sensitive accession; at that time the proportion of dead leaves was 10% for the sensitive and 1% for the tolerant accession. Adapted from (21) [http://www.publish.csiro.au/nid/102/paper/PP9950561.htm].

2.5. Timescale of Months – The Reproductive Phase

After a month there can be obvious effects of salinity on the development of reproductive organs. Salinity reduced the number of florets per ear in barley and wheat and altered the time of flowering (22).

The mechanisms by which salinity might affect the formation of reproductive organs are not clear. Similar phenomena occur under drought. It is probable that it is controlled by signalling pathways that affect the expression of genes switching on developmental programs, again influenced by the supply of carbohydrate but not wholly determined by it. A salt-specific effect is

an unlikely cause of altered reproductive development in the relatively salt-tolerant species like wheat and barley; Na^+ and Cl^- are present in the reproductive primordia (22), but at concentrations too low to affect metabolism. In tomato, another relatively salt-tolerant species, microanalysis of Na^+ concentrations and carbohydrate concentrations in various floral tissues at critical times indicated that the floral abortion was more likely due to the supply of carbohydrates to the inflorescence than to accumulation of ions to toxic levels (23).

In salt-sensitive plants like rice, in which salt has built up to excessive levels in leaves and the vacuoles can no longer contain the incoming salt, significant amounts of salt can be transported in the phloem to the reproductive organs. High levels of Na^+ were found in pollen and stigmas of rice grown at 50 mM NaCl, and stigmatic receptivity was reduced as well as pollen viability (24). These authors concluded that the high degree of sterility was probably due to Na^+ toxicity in the reproductive tissues. This may be peculiar to rice and explain why the yield of rice is particularly sensitive to salinity: the grain yield was only 10% of controls, whereas the straw weight was 80% of controls (24).

In summary, there is a two-phase growth response to salinity. The first phase of growth reduction is quickly apparent and is due to the salt outside the roots. It can be called a water stress or osmotic phase. The second phase of growth reduction, which takes time to develop, results from internal injury. It is due to salts accumulating in transpiring leaves to excessive levels, exceeding the ability of the cells to compartmentalise salts in the vacuole. The osmotic stress affects growing leaves and salt toxicity affects old leaves.

3. Measurement of Salt Tolerance

Salt tolerance is usually assessed as the percent biomass production in saline versus control conditions over a prolonged period of time. Screening methods that avoid the need to grow controls are desirable, as a large amount of space is needed to grow controls under optimum light levels and to obtain sufficient replication as the environmental influences on growth rate are large (25). When comparing landraces versus cultivars, or wild types versus mutants, there is likely to be large genotypic differences in height or leaf area, and space is needed to prevent shading of smaller genotypes by larger ones.

Specific screening methods that avoid the need for comparison with plants in control conditions are summarised in **Table 2.2**. These methods have been used to screen natural vari-

Table 2.2
Techniques used to screen large numbers of genotypes for salinity tolerance in glasshouses or controlled environments. Comments indicate whether a control (non-saline) treatment is necessary, particular advantages of the technique that relate to its experimental feasibility, whether the responses measured are due to the osmotic or the salt-specific effect of the salinity treatment, and how long the treatment needs to be imposed. Avoiding the need to grow controls plants is a major advantage

Technique[a]	Controls needed	Advantages	Osmotic or salt-specific effect	Length of treatment (weeks)
Screening techniques for tolerance to moderate salinity (50–150 mM NaCl)				
Measurements of growth				
Root elongation	Yes	Can be used with very young seedlings	Osmotic	1
Leaf elongation	Yes	Not destructive	Osmotic	2
Biomass	Yes	More likely to relate to field	Both	4
Yield	Yes	Most likely to relate to field	Both	16
Measurements of injury				
Leakage from leaf discs	No*	Not destructive	Either	3–4
Chlorophyll content	No*	Not destructive and quick (using hand-held meter)	Either	3–4
Chlorophyll fluorescence	No*	Not destructive	Either	3–4
Specific traits				
Na^+ exclusion	No	Not destructive, and a single easy analysis	Salt-specific	1–2
K^+/Na^+discrimination	No	Not destructive	Salt-specific	1–2
Cl^- exclusion	No	Not destructive	Salt-specific	1–2
Screening techniques for tolerance to high salinity (200–300 mM NaCl)				
Germination	Yes	Very large numbers easily handled	Osmotic	1
Survival	No*	Limited experimental period, as can adjust the salinity. Highly tolerant genotypes stand out	Either	2–8, depending on salinity

Reproduced from (25) with kind permission of Springer Science and Business Media.
*Assumes all genotypes under control conditions have no leakage, the same leaf chlorophyll concentration per unit area, fluorescence parameters typical of healthy plants, and 100% survival.
[a]Not listed are photosynthesis, transpiration efficiency, osmotic adjustment, enzyme activity, gene expression, compatible solutes, ABA, ethylene, as these are not feasible screening techniques. These measurements can be made on only small numbers of genotypes at one time.

ation, but can also be useful for screening mutant populations if the numbers can be reduced to a feasible level. Destructive harvests can be replaced by non-destructive harvests if an automated imaging system is available (26).

Survival is sometimes used as an index of salt tolerance, when dealing with large numbers of genotypes. Survival can be measured as the percent of plants alive after a given period of time at a given salinity. Alternatively, if there is a range of salinities, survival can be measured as the salinity at which 50% of the plants have died. The drawback of this index is that it gives little idea of how well a plant can actually *grow* in saline conditions.

Realistic experimental design should avoid large osmotic shocks, avoid salt-induced Ca deficiency, optimise ambient conditions especially if controls are grown, and ensure roots are not constrained by small pots that are waterlogged at the base.

It is not possible to be prescriptive about the length of time that plants should be grown before genotypic differences in salt tolerance can be seen. The second phase will start earlier in plants that are poor excluders of Na^+, such as lupins or beans, and when salinities are higher. It will also start earlier when root temperatures are higher. For plants such as rice that are grown at high temperatures, 10–15 days in salinity is sufficient to generate genotypic differences in biomass that correlate well with differences in yield (27).

4. Identifying Important Genes

Genes so far identified as being important in salt tolerance fall under the categories of ion transporters, compatible solutes or osmolytes, and transcription factors involved in growth regulation. Genes that regulate ion transport in salt-affected plants are listed in Table 3 of Munns (12) and summarised in Munns and Tester (2). Genes that synthesize osmoprotectants are listed in Table 2 of Chinnusamy et al. (6) and Table 4 of Munns (12). Signalling pathways involved in hormonal transduction and likely to be regulating cell growth under abiotic stress are reviewed by Xiong et al. (4).

Various approaches have been used successfully to identify candidate genes for salinity tolerance (**Table 2.3**):

1. A trait-based approach is built upon knowledge about function and forward genetics using a specific phenotype and Mendelian genetics. This approach has been successful in the discovery of sodium transporters of the HKT family from QTLs (*Q*uantitative *T*rait *Lo*ci) for Na^+ exclusion, namely, *SKC1*, *Nax1*, *Nax2*, and *Kna1*. The phenotype is the Na^+

Table 2.3
Different approaches for identifying genes for salinity tolerance. The first approach is based on physiological and mechanistic understanding, the next two assume no prior physiological knowledge. Candidate genes used in the last approach are based on prior knowledge of the gene function in other organisms or the outputs from approaches 1, 2, and 3

	Starting point	Material	Method	Result	Next step
1.	Knowledge of important trait	Natural variation	Phenotype	QTL	Fine mapping and cloning
2.	Molecular genetics	Mutant population	High-throughput screen	Candidate gene	Go to 4
3.	Technical advances in 'Omics'	Contrasting genotypes or treatments	Transcriptomics (microarray), proteomics, metabolomics	Candidate gene or metabolic pathway	Go to 4
4.	Candidate gene	Overexpression and null transformants	Phenotype	Proof of function	Cross with high yielding cultivar

concentration in a given leaf after a given time in salinity. In rice, fine mapping of the *SKC1* locus yielded the sodium transporter OsHKT1;5 (28). In wheat the *Nax1* locus yielded the sodium transporter TmHKT1;4 (29), the *Nax2* locus revealed TmHKT1;5 (30), and the *Kna1* locus revealed TaHKT1;5 (30).

2. High-throughput mutant screens based on clever assays have discovered new and important genes, the most significant being the plasma membrane Na^+/H^+ antiporter SOS1 (31) and the Na^+ transporter AtHKT1 (32).
3. 'Omics' approaches require no prior knowledge about traits or phenotypes. Salinity-related changes in gene expression (transcriptomics), protein levels (proteomics), or metabolite concentrations (metabolomics) can be detected (33) using genotype or treatment comparisons. Comparisons can be made between closely related genotypes with known differences in salinity tolerance, e.g. *Arabidopsis thaliana* versus *Thellungiella halophila* (34), transgenics with overexpression of a candidate gene (35), or different treatments with the same genotype (36). Comparison of different abiotic stresses (salt, cold, dehydration) can reveal salt-specific effects. Studies done at short periods of time after a sudden exposure to NaCl concentrations of over 50 mM, such as 1 or 3 h, are unlikely to reveal useful information as the cells are still recovering from the shrinkage (*see* **Fig. 2.1**).

4. Functional or candidate gene-based approach uses knowledge of the function of a metabolite or transporter in halophytic species, particularly microorganisms or model systems. Notable examples are the K^+ transporter HKT1 (37), the vacuolar Na^+/H^+ antiporter NHX1 (38), the Na^+-ATPase PpENA (39), and the osmoprotectant glycine betaine (40).

Tissue selection is a critical part of experimental design, particularly for the 'omics' approaches (12). Genes involved in growth regulation can be best detected by comparing growing versus non-growing tissues. The growing zones in roots and in leaves of monocotyledonous species have a very well-defined growing zone, and the different tissues that are affected by salinity in cereal leaves are well known (17, 41).

5. Conclusions

Salinity can affect growth in a number of ways. First, the presence of salt in the soil reduces the ability of the plant to take up water, and this quickly causes reductions in the rate of leaf and root elongation. This is the first phase of the growth response, due to the osmotic effect of the salt in the soil solution, and produces a suite of effects identical to those of water stress caused by drought. Later, there may be an additional effect on growth; if excessive amounts of salt enter the plant they will eventually rise to toxic levels in the older transpiring leaves. The reduced photosynthetic capacity of the plant will reduce the amount of assimilate transported to the growing tissues, which may further limit growth. This is the second phase of the growth response and is the phase that clearly separates species and genotypes that differ in the ability to tolerate saline soil.

Many genes are important in adapting plants to grow and yield well in saline soil. The concern about future food shortages makes it imperative to better understand the genetic control of salt tolerance and to use this knowledge to increase the salt tolerance of important crops and pasture species. Various approaches can be taken to discover genes for salinity tolerance. An understanding of the physiological mechanisms in which the genes operate will accelerate their application for improving salt tolerance of crops.

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