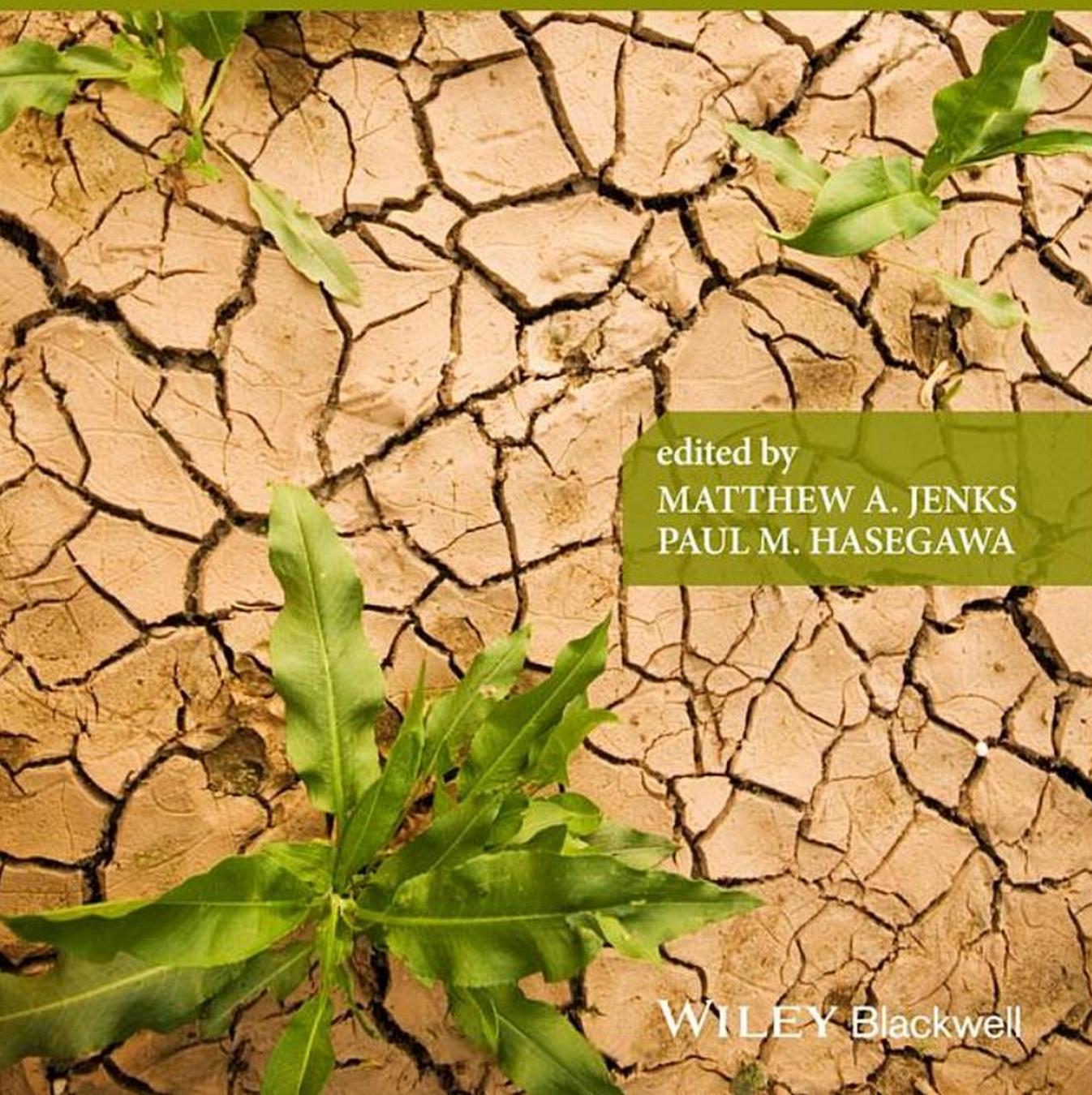


SECOND EDITION

# PLANT ABIOTIC STRESS



edited by  
MATTHEW A. JENKS  
PAUL M. HASEGAWA

WILEY Blackwell

## **Plant Abiotic Stress**



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Second Edition

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## Preface

Since the last edition of *Plant Abiotic Stress*, insight has come from various research programs that now shines new light on the determinants of plant adaptation to environmental stress. While there are many sources of plant stress, this book will focus as in the first edition on the inanimate components of the environment associated with climatic, edaphic, and physiographic factors that substantially limit plant growth and survival. Categorically, this book places a focus on plant abiotic stresses caused by flooding, drought, salinity, non-optimal temperatures, and poor soil nutrition. Discussions of plant abiotic stress that originate from climate change, and its potential impacts on crop production, are also included in these chapters.

The greatest cause of reduced yield in annual crops worldwide is the combined impact of abiotic stress. For example, the threat of water scarcity to crop production worldwide is increasing as continued overutilization of aquifer-based irrigation by farmers continues unabated, a condition that is now posing a serious threat to the long-term sustainability of many regional agricultural systems. With increasing irrigation in arid and subarid zones comes increased salinization of field soils, a condition already having dramatic negative impacts on crop yield in many parts of the world. Another major threat is temperatures that are too high, too low, or too erratic for efficient crop production, much of this due to changes in climate. Degradation of field soils by increasingly intensive cultivation to satisfy growing world demand for agricultural products is compounding soil degradation and directly limiting crop yield. Although better field management practices can improve production efficiency, there can be no doubt that new crops with increased resistance to drought, salinity, sub- and supra-optimal temperatures, poor soil nutrient status, and other stresses, like flooding and global climate change, are necessary to meet future food, fiber, and biomass needs globally.

The advent of new technologies for the efficient identification of genetic determinants involved in plant stress adaptation, fostered especially by the use of molecular genetics and high throughput transcriptome, proteome, metabolome, and ionome profiling, as well as the use of genome-wide association and other molecular mapping tools, has improved our understanding of the mechanisms plants use to tolerate abiotic stress and revealed new opportunities for creating improved stress-tolerant crops. This book seeks to summarize the large body of current knowledge about the diverse mechanisms that confer or

influence plant stress tolerance, placing special emphasis on the cellular aspects of plant response whose expression is common across diverse environments. Leading scientists involved in plant abiotic stress research worldwide provide a comprehensive treatise to these major stress factors having an impact on world crop production. The material presented in this book emphasizes fundamental genetic, epigenetic, physiological, biochemical, and ecological knowledge of plant abiotic stress, which may lead to novel applications for improving crop performance in stressful environments.

*Matthew A. Jenks and Paul M. Hasegawa*

# **1 Flood tolerance mediated by the rice SUB1A transcription factor**

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## **1.1 Introduction**

Over one billion people, 15% of the world's population, live in extreme poverty. Most of these people live on farms and barely produce enough food for themselves and their families. The most economic and effective method for improving farm productivity is the planting of high-yielding and more resilient varieties that thrive on these farms. Varieties that are resistant to diseases and/or tolerant of environmental stresses can have dramatic and positive impacts on the lives of the very poor worldwide.

Rice is the staple food for more than half of the world's population. Flooding is a major constraint to rice production in South and Southeast Asia, where the majority of the world's rice farmers live. Each year, 25% of the global rice croplands are inundated by flash floods, which are unpredictable and can occur several times a year. Although rice is grown in flooded soil, most rice cultivars die within a week of complete submergence, causing yield losses ranging from 10% to total destruction (Mackill et al., 2012). These losses disproportionately affect the rice farmers in the world, where 70 million people live on less than \$1 a day.

Compounding the challenges facing rice production are the predicted effects of climate change. As the sea level rises and glaciers melt, low-lying croplands will be submerged and river systems will experience shorter and more intense seasonal flows, as well as more flooding. Most of the coastal rice production areas in the tropics and subtropics are vulnerable to such conditions, especially low-lying deltas along the coastlines of South, East, and Southeast Asia. Rice production in these deltas is the major agricultural activity. These areas include the Mekong and Red River deltas of Vietnam, the Ayeyerwaddy Delta of

Myanmar, and the Ganges-Brahmaputra Delta of Bangladesh. These deltas provide between 34% and 70% of the total rice production in these countries, and any reduction in rice production due to increases in the frequency of flooding will have serious consequences on food security (Wassmann et al., 2009). This is a challenge especially in places like Bangladesh, Eastern India, Vietnam, and Myanmar, where people get about two-thirds of their total calories from rice. Large areas of Bangladesh and India already flood on an annual basis and are likely to flood even more frequently in the future, leading to a substantial loss of agricultural land. In Bangladesh and India alone, 4 million tons of rice, enough to feed 30 million people, is lost to floods each year.

Thus, an important goal for improving the rural economy and livelihood in these vulnerable countries is to develop rice varieties that can survive flooding. Because most of the world's poorest people get their food and income by farming small plots of land, the availability of rice varieties with enhanced tolerance to flooding is expected to make a major difference in food security for these farmers.

Although rice can withstand shallow flooding, most rice varieties will die if completely submerged for more than a few days. There are a few rice landraces that can survive prolonged submergences, and these are of great interest to rice breeders. For example, the ancient Indian rice landrace, FR13A, has poor grain and yield qualities but is unusual in its ability to endure complete submergence for over 14 days.

FR13A has been known to farmers in Orissa, India, since the 1950s. For over 40 years, breeders at the International Rice Research Institute (IRRI) tried to use FR13A as a donor parent to introduce the submergence tolerance trait into varieties that would be useful to rice farmers. Although submergence tolerant varieties were developed, they were not widely adopted. The main reason is that because breeding was carried out with relatively crude genetic tools based mainly on visual selection, the resulting varieties lacked many of the traits desired by farmers in the major rice-growing areas of Asia. With lack of knowledge on the exact genes needed to confer submergence tolerance, the breeders unknowingly dragged in undesirable traits along with the submergence tolerance trait, which reduced yield and grain quality.

Over the last 15 years, we collaborated with Dave Mackill at the International Rice Research Institute and other researchers and breeders to carry out detailed genetic analyses of submergence tolerance in rice. Our long-term goal was to understand the underlying molecular mechanisms controlling submergence tolerance and generate tools that breeders could use to develop rice varieties with high yields and good grain quality that are tolerant to submergence. The results of this team effort led to the identification of the *SUB1* locus and associated genes, development of rice "mega varieties" with submergence tolerance for farmers, and elucidation of the gene networks and physiological processes mediated by *SUB1*.

## 1.2 Isolation of the rice *SUB1* locus

In early genetic studies, rice submergence tolerance derived from FR13A had been shown to have a relatively high heritability, with tolerance being partially to completely dominant (Haque et al., 1989; Mohanty and Khush, 1985; Mohanty et al., 1982; Sinha and Saran, 1988; Suprihatno and Coffman, 1981). The trait was also thought to be controlled by one or a few loci with major effects and loci with smaller, modifying effects. On the basis of these studies, we began to investigate submergence tolerance using an approach combining the power of molecular markers and quantitative trait locus (QTL) analysis. This initial study employed a population (DX18) of 169 F<sub>2</sub> plants and their resulting F<sub>3</sub> families that were derived from a cross between two breeding lines, PI613988 (japonica) and IR40931-26 (indica), the latter of which inherits strong submergence tolerance from FR13A. Kenong Xu and David Mackill demonstrated that a major QTL, *SUB1*, mapped between two restriction fragment length polymorphism (RFLP) markers (C1232 and RZ698) on rice chromosome 9 (Xu and Mackill, 1996). The *SUB1* QTL was supported with a logarithm of odds (LOD) score of 36 and accounted for 69% of phenotypic variation in the F<sub>2</sub> population, concluding that *SUB1* is critical for conferring submergence tolerance in rice. Simultaneously, other teams (Kamolsukyunyong et al., 2001; Nandi et al., 1997; Toojinda et al., 2003) also reported the strong phenotypic effect of the *SUB1* locus, confirming its effect as the major determinant of tolerance, besides few other minor QTLs.

Previously, the Ronald laboratory had successfully used an approach of “positional cloning” to isolate a rice gene, called *Xa21*, that conferred broad-spectrum resistance to a serious bacterial disease in Asia and Africa (Song et al., 1995). This experience encouraged us to take the same approach to isolate the *SUB1* QTL although it was challenging because a QTL for an important agronomic trait had never before been isolated from a staple crop species, and the rice genome had not yet been sequenced.

We first carried out fine mapping of the *SUB1* QTL to characterize the *SUB1* region with more markers in a large F<sub>2</sub> population (DX202) of 2,950 plants, which was derived from a cross between M202 (a widely grown japonica rice cultivar in California) and DX18-121 (a tolerant line from population DX18, see above). The resulting *SUB1* fine map comprised ten amplified fragment length polymorphism (AFLP) markers. Two of these markers co-segregated with *SUB1* and eight linked to *SUB1* within 0.2 cM (Xu et al., 2000). The significance of this fine map is that it laid a foundation for physically mapping the *SUB1* locus on rice chromosome 9.

We then carried out physical mapping of the *SUB1* locus by identifying a set of five bacterial artificial chromosome (BAC) and 13 binary clones that overlapped each other and that entirely covered the *SUB1* region (Xu et al., 2006). The five BAC clones were obtained from the two BAC libraries constructed

from rice cultivars IRBB21 and Teqing, respectively. Both BAC libraries were publically available, but IRBB21 and Teqing do not carry the submergence tolerance trait. The 13 binary clones were achieved from a genomic library constructed from the submergence tolerance parental line IR40931-26 using a binary vector that could be used to directly engineer rice plants. By developing more markers from these BAC and binary clones and analyzing the expanded F<sub>2</sub> population DX202 of 4,022 plants, we were able to delimit the *SUB1* locus with a region of 182 kb between markers CR25K and SSR1A (Xu et al., 2006).

Complete sequencing of the 182 kb *SUB1* region revealed that the region encodes 13 genes, including 3 that contain ethylene response-factor (ERF) domains, which were designated *SUB1A*, *SUB1B*, and *SUB1C*. We found that the corresponding *SUB1* region in the sequenced genome of japonica rice Nipponbare (International Rice Genome Sequencing Project, 2005) spans only 142 kb and lacks *SUB1A*.

We next carried out an allelic variation survey of the *SUB1* genes in 21 varieties (17 indica and 4 japonica). We identified two *SUB1A*, nine *SUB1B*, and seven *SUB1C* alleles. The *SUB1A-1* and *SUB1C-1* alleles are specific to all six submergence tolerant accessions studied, including FR13A, Goda Heenati, and Kurkurapan, which are of independent geographic origins. However, there was no such correlation between a specific *SUB1B* allele and submergence tolerance.

Using gene expression analysis, we found that *SUB1A* was rapidly induced upon submergence in the submergence tolerant variety. In contrast, *SUB1C* was upregulated only in the intolerant variety, M202. The expression of *SUB1B* was low and constant in both submergence tolerant and intolerant varieties. These data suggested that *SUB1A* controlled the *SUB1*-mediated submergence tolerance response.

To functionally prove *SUB1A* as the very gene underlying the *SUB1* QTL, we created a construct containing the *SUB1A-1* full-length cDNA under the control of the maize Ubiquitin1 promoter (Christensen and Quail, 1996) to overexpress *SUB1A-1* in Liaogeng, a submergence intolerant japonica rice that also lacks *SUB1A*. Submergence screening of the resulting T<sub>1</sub> transgenic plants identified four independent T<sub>1</sub> families segregating for submergence. A detailed analysis of two of the four T<sub>1</sub> families showed a nearly complete correlation between high expression of the *SUB1A-1* transgene and submergence tolerance. We therefore concluded that *SUB1A-1* is sufficient to confer submergence tolerance to intolerant varieties, signifying the isolation of the *SUB1* QTL (Xu et al., 2006).

This work was significant because it represented the first isolation of a QTL with an important agronomic effect and revealed an important genetic mechanism with which rice plants can control tolerance to submergence. Isolation of *SUB1A* and the 180 kb of genetic sequence surrounding the gene set the stage for advanced marker assisted breeding at the IRRI (Neeraja et al., 2007; Septiningsih et al., 2009; Mackill et al., 2012).

### 1.3 Sub1 rice in farmers' fields

Initially, the IRRI group monitored the *SUB1* locus using markers closely linked with the gene. However, the availability of the sequences from BAC clone AP005907, which carried the sequences of the *SUB1* genes, soon facilitated the development of six more markers tightly linked to the *SUB1* QTL. This approach allowed for the transfer of the “donor” (Sub1) genetic region to be precisely monitored. The Sub1 donor FR13A variety carries many undesirable agronomic characters; therefore without knowledge on the precise location of *SUB1A* and the ability to select against other regions of the FR13A genome, these undesirable characteristics are dragged into the new variety along with *SUB1* (Neerja et al., 2007). Thus, with the availability of the *SUB1A* sequence and other sequences in the region, the *SUB1* locus could be precisely introduced into a wide range of recipient rice varieties favored by farmers, while at the same time minimizing the effects of “linkage drag” from the Sub1 donor. This work resulted in the introduction of *SUB1* into eight rice varieties popular in South and Southeast Asia. The first of these was the mega variety Swarna, which is grown on ca. 5 million hectares in India and on additional areas in Bangladesh and Nepal (Xu et al., 2006).

The new rice variety—called *Swarna-Sub1*—was tested in farmers’ fields in Bangladesh and India. In the absence of flooding both Swarna and Swarna-Sub1 yield 5–6 tons per hectare. However, in the presence of flooding, fewer plants of the Swarna rice crop survived (0–20% in most cases depending on floodwater conditions and duration; Das et al., 2009), whereas the Swarna-Sub1 rice flourished—80–95% of it survived. This enhanced survival means that farmers growing the Swarna-Sub1 variety gain a 1 to over 3 tons per hectare yield advantage following floods (Singh et al., 2009). Using this marker assisted breeding approach, the IRRI team has now generated and released several Sub1 varieties in six countries (Indonesia [4], Nepal [2], Myanmar [1], India [2], Bangladesh [2], and the Philippines [2]). In 2011, Swarna-Sub1 alone was estimated to have reached over one million farmers in South Asia (Mackill et al., 2012).

Over the last 5 years, our colleagues at IRRI have been working with India’s National Food Security Mission, the Ministry of Agriculture, the government of India, and with state governments, non-governmental organizations (NGOs), and public and private seed producers and breeders in India, Bangladesh, and Nepal to multiply and disseminate Swarna-Sub1 seeds and seeds of other released Sub1 varieties and to strengthen the existing seed systems. The supply will aid various states in South Asia that do not have enough seeds to distribute to farmers.

The Bill and Melinda Gates Foundation is now supporting a large program, called *Stress-Tolerant Rice for Africa and South Asia* (STRASA; [www.strasa.org](http://www.strasa.org)), that is assisting with the development and dissemination of Sub1 rice varieties in three countries (<http://irri.org/news-events/irri-news/bill-and-melinda-gates-visit-strasa-and-csisa-projects-at-icar-research-farms-in-patna-india>). STRASA was conceived as a 10-year project with the vision of reaching about 20 million farmers in

South Asia and Sub-Saharan Africa by 2017. By 2014, Sub1 varieties are predicted to be grown in over 5 million hectares (Mackill et al., 2012).

We initially introduced *SUB1* into a set of popular varieties including Swarna (also widely grown in Bangladesh and Nepal), Samba Mahsuri, and CR1009 (Savitri) from India; BR11 from Bangladesh; Thadkkham 1 (TDK 1) from Laos; and IR64 from IRRI-Philippines. More recently, *SUB1* has been introduced into Ciherang from Indonesia and PSBRc 18 from the Philippines. These varieties were chosen because they are popular among farmers and consumers in rainfed lowland areas, each covering between 1 and over 6 million hectares. The flood-tolerant versions of these high-yielding “mega varieties” are effectively identical to their intolerant counterparts but survive better after severe floods to yield well. The grain quality of all Sub1 lines developed so far is essentially identical to the conventional varieties, with the extra advantages of fast recovery and earlier maturity (by 10–15 days) than their non-Sub1 counterparts following submergence for various durations (Singh et al., 2009). Breeders predict that the most popular Sub1 varieties like Swarna-Sub1 and BR11-Sub1 will soon entirely replace the existing non-Sub1 versions and spread to other flood-prone areas all over these countries.

Introgression of *SUB1* into these varieties also facilitated the introduction of these varieties to regions where they were not known before; for example, Swarna-Sub1, which previously had only been planted in South Asia, has now been released in Indonesia, and Ciherang from Indonesia is in the final stages of release for flood-prone areas in Bangladesh and India.

We chose to introduce these popular varieties because they were well known to farmers, millers, and consumers, and therefore less time would be needed to evaluate and commercialize the new varieties. One difficulty with such success is that although there are now ample incentives for farmers to grow these mega Sub1 varieties like Swarna-Sub1 and BR11-Sub1, there is still little incentive to introduce additional rice varieties to enhance the overall genetic diversity of the rice planted in large areas as in India and Bangladesh. Breeders, geneticists, and agronomists know from past experience that monocultures can be vulnerable to other problems, such as yield stability. The issue is to balance the demand of farmers for high-yielding, high-quality, flood-tolerant rice varieties with the need to plant genetically diverse rice varieties to minimize possible future losses to pest and disease. For these reasons, IRRI decided to introduce *SUB1* into all varieties being bred for rainfed lowlands, and a considerable number of breeding lines are now being evaluated at target sites in Asia and Africa. In addition, breeding lines combining *SUB1* and drought tolerance as well as *SUB1* and salt tolerance have been developed and are being field tested. These new breeding lines are useful for areas experiencing both flash floods and drought as in most rainfed lowlands, as well as submergence and salt stress as in tropical coastal areas of South and Southeast Asia (Ismail et al., 2008). Substantial efforts are also being undertaken by national programs to incorporate

*SUB1* into additional local popular varieties as well as into new elite lines as in Vietnam, India, Bangladesh, and Thailand.

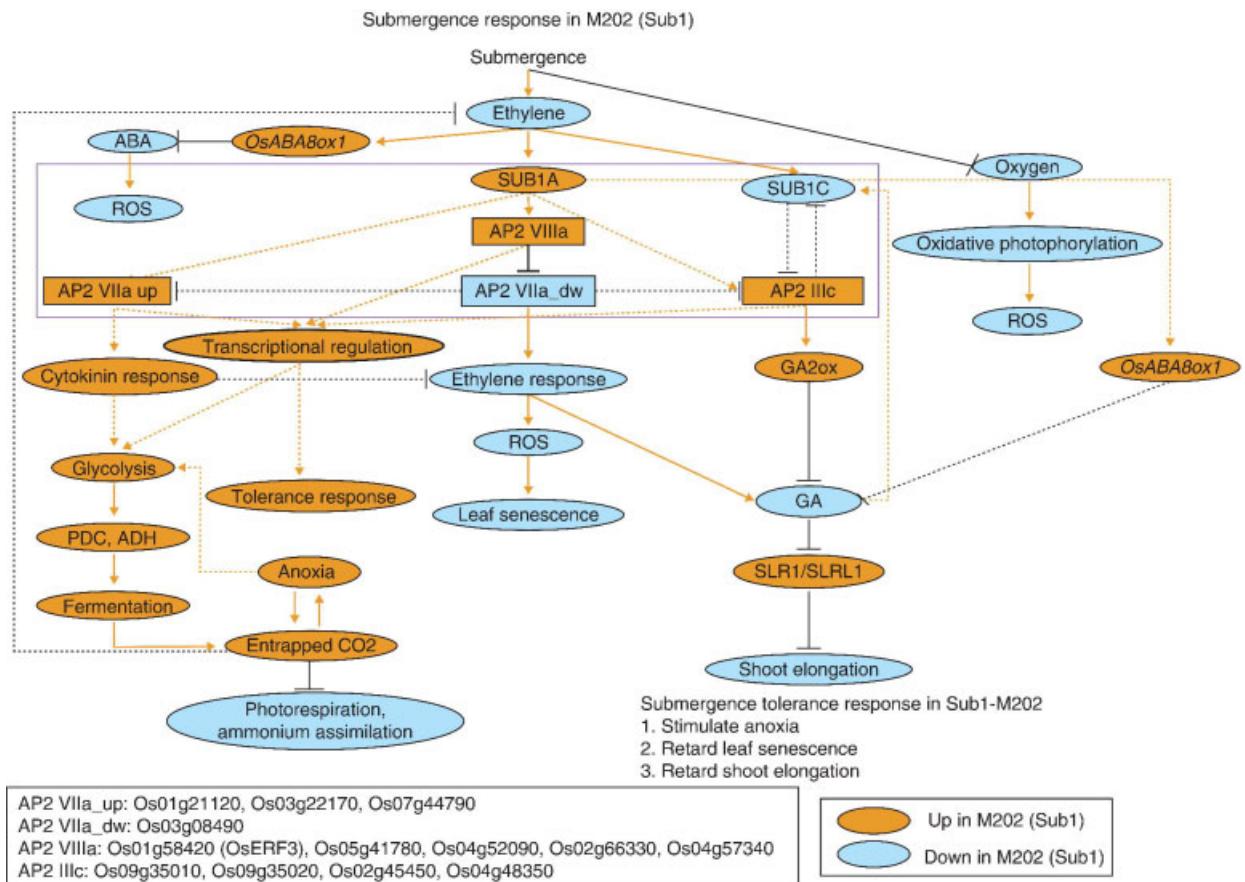
#### 1.4 The SUB1 effect

SUB1 exerts its effect by limiting gibberellic acid (GA)-activated elongation growth and ethylene-induced leaf senescence. Complete submergence restricts light intensity, slows O<sub>2</sub> and CO<sub>2</sub> exchange between shoot tissue and floodwater, and enhances the accumulation of ethylene due to increased synthesis and entrapment. Ethylene accumulation triggers chlorophyll degradation and leaf senescence (Ella et al., 2003) and causes excessive elongation of leaves and internodes of the submerged plants in an attempt to maintain contact with air. This is mediated through ethylene-induced suppression of abscisic acid (ABA) synthesis but enhanced synthesis and sensitivity to GA (Das et al., 2005). Reduced photosynthetic capacity during and following submergence, together with excessive growth during submergence, results in severe carbohydrate starvation and consequent death of the submerged plants. In collaboration with Bailey-Serres at the University of California-Riverside, we have demonstrated that SUB1A exerts its effect by limiting GA-activated elongation growth and conserving carbohydrates (Figure 1.1). The *SUB1* locus enables plants to endure complete submergence for prolonged periods due to activation of a “quiescence strategy” that conserves the shoot meristem and energy reserves until the flood subsides.

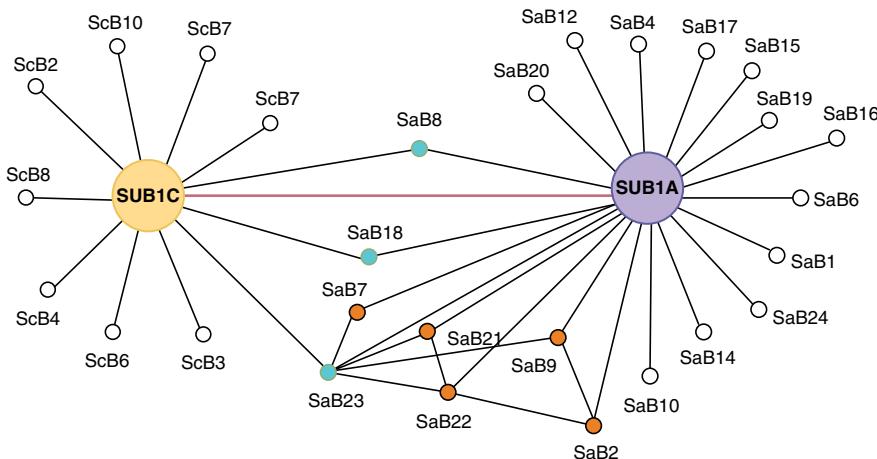
#### 1.5 The SUB1-mediated gene network

In addition to flooding, other environmental stresses such as drought, salinity, and heat stress are predicted to be increasingly problematic for farmers as the climate warms. For example, in Africa, three-quarters of the world’s severe droughts have occurred over the past 10 years (African Agricultural Technology Foundation, 2010). Losses to pests and diseases are also expected to increase over the next 50 years. Much of the losses caused by these pests, diseases, and environmental stresses, which already result in 30–60% yield reductions globally each year, occur after the plants are fully grown: a point at which most or all of the land and water required to grow a crop has been invested. Thus, there is a need to identify genes that confer robust tolerance to environmental stresses and diseases and to use this information to develop new varieties.

As part of this goal, we and others are using genomic, molecular-genetic, allelic diversity, and computational approaches to identify other genes and gene networks involved in tolerance to stress and devastating diseases. For example, we recently demonstrated the usefulness of transcriptomics and interactomics



**Figure 1.1** SUB1A-mediated submergence tolerance responses revealed by integrating omics tools (Jung et al., 2010). Orange boxes indicate events upregulated in M202(Sub1) after submergence, and blue boxes indicate events downregulated in M202(Sub1) after submergence. Several of the AP2/ERF TFs are associated with submergence tolerance response. For color details, please see color plate section.



**Figure 1.2** The rice SUB1A/SUB1C interactome. The interactome map represents 28 proteins identified from high-throughput Y2H screening using SUB1A and SUB1C as baits. Proteins in blue represent interactors with both SUB1A and SUB1C (Seo et al., 2011). For color details, please see color plate section.

approaches to identify genes and proteins that are part of the predicted rice SUB1A-mediated response network, and we have shown, through genetic analysis, that this approach efficiently identifies key genes regulating these biological pathways (Jung et al., 2010; Seo et al., 2011).

Transcriptional regulation plays a key role in development and response to abiotic stresses (e.g., SUB1A, CBFs, OsNAC5, dehydration-responsive element-binding proteins [DREBs], AP2/ERFs, WRKYs). In plants, regulation of gene expression is complex, with the majority of genes differentially expressed in different tissues and cell types. We have shown that a suite of 898 genes is differentially regulated during the SUB1A-mediated rice tolerance response (Jung et al., 2010). Notably, there are 16 genes encoding transcription factors (TFs) that are differentially regulated by SUB1A (Figure 1.2). Of these, ten AP2/ERF genes belong to the ERF subfamily and six to DREB.

Our results suggest that the *SUB1* locus regulates the ethylene response using AP2/ERF genes in the ERF subfamily and stress tolerance response with AP2/ERF genes in the DREB subfamily.

To further elucidate the rice submergence stress response pathway, we used an efficient and reliable yeast two-hybrid (Y2H) screening strategy (Jung et al., 2008) to identify proteins that interact with SUB1A and SUB1C (a negative regulator of submergence tolerance at the same locus as *SUB1A*; Xu et al., 2006). Several million transformants were screened using SUB1A and SUB1C as baits (Seo et al., 2011). Five binding proteins were chosen for further screening as baits in the Y2H to identify additional proteins in the stress response network (Figure 1.2).

We determined that SUB1A and SUB1C interact with 28 SUB1A and SUB1C binding proteins (SaBs and ScBs, respectively). These interactions were reconfirmed through secondary screenings. Seventy-five percent of the interactions were validated *in vivo*, using a bimolecular fluorescence complementation approach in rice protoplasts (Seo et al., 2011). The interactome includes several candidate TFs that interact with both SUB1A and SUB1C. One such protein is SAB18, a putative trihelix protein. We analyzed insertion alleles of SAB18 obtained from the POSTECH collection (Jung et al., 2008). Homozygous insertion mutants in SAB18 displayed enhanced tolerance to submergence. Lines overexpressing SAB18 did not show a submergence tolerance phenotype (Seo et al., 2011). These results suggest that SUB1A (a transcription factor) may serve as a component of large and/or changing complexes *in vivo* (Seo et al., 2011). It remains to be determined how the SUB1A-associated complex(s) is reorganized in response to submergence and drought stress.

The flood of data from functional genomics, comparative genomics, and proteomics approaches now allows diverse aspects of gene function to be assayed on a genome-wide scale. Because datasets from each technique are incomplete, error-prone, and limited in sensitivity, none of them are sufficient to fully describe the biological role for a particular gene. However, such datasets can be integrated to generate a more accurate and comprehensive view of gene function than is contained in any single dataset. Probabilistic integrated gene networks—graphical models in which linkages between genes indicate their likelihood to belong to the same biological process—provide such an approach. Such a network can guide phenotypic predictions in varied tissues and developmental contexts.

Integrated networks have proven successful for unicellular and multicellular organisms, accurately predicting gene functions and gene loss-of-function phenotypes, and are thus powerful tools for generating testable biological hypotheses. Using probabilistic gene networks, the Marcotte (Lee et al., 2004, 2008, 2010) group has successfully demonstrated proof-of-concept for yeast, worm (*C. elegans*), and mouse. Each network model is highly predictive of gene function and for organismal phenotype following gene perturbation. These results indicate that researchers can efficiently gain new functional knowledge by prioritizing genes for a given biological role based upon gene networks, then testing these candidates using available reverse genetics resources.

We have used these approaches to develop an experimentally validated genome-scale functional gene network of rice genes, named RiceNet, covering the majority of encoded rice proteins. We have leveraged RiceNet to identify networks of genes governing the XA21-mediated response (Lee et al., 2011) and demonstrated that RiceNet successfully predicts gene function in rice and maize. Thus, RiceNet is broadly useful for dissecting complex immune response pathways and is particularly useful for identifying gene function in other monocot species such as wheat and barley, for which species-specific networks have not yet been constructed.

We are now using RiceNet to generate subnetworks of genes that are predicted to control SUB1A-mediated pathways, which are important for tolerance to submergence and possibly to drought. The advantage of this approach is that it facilitates non-biased identification of key networks predicted to control a particular biological response. Instead of working on a single gene predicted to have a function, researchers can study entire networks, including genes that would not have been predicted to function in the network using standard genetic and proteomics approaches.

## 1.6 Conclusion

From the start, the Sub1 project was guided by the needs of small-holder farmers, adapted to local circumstances, and sustainable for the economy and the environment.

The Sub1 project revealed that it was possible to identify important agro-nomic traits, which were thought to be quite complex, identify genes underlying these traits, and use this knowledge of gene sequences and function to develop new crop varieties that can immensely benefit farmers. It is clear from our work and that of others that this type of approach will greatly accelerate the pace with which plant geneticists and breeders can develop new crop varieties and/or improve the resilience of existing popular varieties. This approach is now being extended to other abiotic stresses such as drought and salinity, where major QTLs were identified (Thomson et al., 2010; Mackill et al., 2012), and is also being used to combine tolerances of multiple abiotic stresses.

One of the important aspects of the Sub1 project was its highly collaborative nature. Specifically, the combination of molecular geneticists, physiologists, and breeders working together and freely sharing material greatly enhanced success of this project. For example, early on, prior to publication, our laboratories shared the *SUB1* genes and our Sub1 rice lines with other laboratories. This “open science” approach facilitated the breeding collaborations and also advanced our understanding of *SUB1* function.

The discovery of *SUB1* enabled the conversion of eight popular varieties into submergence tolerant types using marker assisted backcrossing; five of these have been commercialized in several countries in Asia and the rest are in advance stages of release. *SUB1* effectively works in all environments and genetic backgrounds, from crop establishment until flowering. *SUB1* has no observable effects in the absence of submergence but substantially improves survival and yield following transient complete flooding for 4–18 days in farmers’ fields. The positive impact of Sub1 varieties has been recognized in several countries, triggering enormous interest and additional resources by national programs to produce and distribute sufficient seeds of these varieties to all farmers in areas affected by flash floods.

The identification of genes that modulate *SUB1*-mediated tolerance to flooding will be useful for the development of “Sub1<sup>plus</sup>” varieties that have a higher and wider range of tolerance. Such enhanced tolerance will be needed as farmers face a future predicted climate of unusually heavy rainfall. Additional QTLs/genes need to be pyramided with *SUB1* for higher tolerance for areas encountering longer duration of flash floods. Several studies already identified few minor QTLs affecting submergence tolerance independent of *SUB1* (Nandi et al., 1997; Tooijindda et al., 2003; Septiningsih et al., 2012); however, exploring these QTLs is challenging because of their relatively smaller effects compared with *SUB1*. Furthermore, *SUB1* needs to be introgressed into varieties that tolerate partial stagnant floods common in most rainfed lowlands, where floodwater of 20–50 cm depth stagnates in the field for several months (Singh et al., 2011). None of the modern rice varieties developed to date, including Sub1 types, can withstand this type of flooding; however, recent efforts at IRRI have established the possibility of combining both *SUB1*-mediated tolerance to transient, complete flooding and tolerance to stagnant flooding.

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## **2 Drought tolerance mechanisms and their molecular basis**

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### **2.1 Introduction**

Drought limits plant productivity and distribution of plant species worldwide. Meteorologically, drought is a period of substantially reduced rainfall. Even small changes in water supply from the soil can exacerbate diurnal patterns where plants are prone to dehydration during the day when stomates are open and relative humidity is low but rehydrate at night when stomates are closed. As a drought period persists and soil drying becomes more severe, the plant will eventually become unable to maintain hydration even with the stomates closed. Thus, plants respond to drought both by rapid adjustments, particularly to stomatal aperture, throughout the course of a day and by longer-term adjustments to growth and dehydration-protective mechanisms. A drought period in the field may include additional stress factors that impact plant growth; however, the defining plant stress factor of drought is the decreased water availability and decreased water potential.

#### *2.1.1 The water potential concept*

Water potential is a measure of the free energy status of water relative to pure water at a reference state (water potential zero) of temperature pressure and position in gravity (Boyer, 1995; Kramer and Boyer, 1995). Water potentials are expressed in units of pressure to allow soil and plant water status to be easily integrated with turgor. Water that has a low free energy state, such as water that is bound to soil particles or in solution with a high solute concentration, has a water potential below zero. As water will only move down a gradient in potential energy, water uptake into a plant can only occur if the plant tissue

is at lower (more negative) water potential than the water source. Positive turgor pressures, which are required for cell expansion and support, increase the free energy of water (bring the water potential closer to zero). To decrease water potential while still having a positive turgor, plant cells accumulate large concentrations of solutes. The ability to accumulate additional solutes to respond to lower water potential of the soil is one factor in drought tolerance (see below). The overall importance of water potential is that it both allows us to understand the forces moving water into and out of the plant tissue and is the physical parameter that allows us to quantify and compare the severity of drought stress across different environments and different experimental systems. Thus the use of water potential is an essential part of drought research and readers are referred to previous publications for more thorough descriptions of the water potential concept and its use (Boyer, 1995; Kramer and Boyer, 1995; Verslues et al., 2006).

### 2.1.2 *Escape, avoidance, and tolerance strategies of drought response*

Stress physiologists have traditionally described plant drought responses as escape, avoidance, and tolerance mechanisms (Levitt, 1972; Ludlow, 1989). Escape mechanisms such as acceleration of flowering and seed set allow the plant life cycle to be completed before drought severity reaches a critical level. Avoidance consists of mechanisms to limit transpiration or promote water uptake such that the plant tissue does not experience reduced water potentials or tissue dehydration. Prominent avoidance strategies are stomatal closure to restrict water loss and increased root to shoot ratio to allow water uptake from a larger soil volume while reducing transpiring leaf area. In terms of water potential, drought avoidance mechanisms attempt to maintain a water homeostasis by conserving water and thus keep tissue water potential high and relatively little changed from plants with ample water supply. Avoidance can be a key factor in plant productivity under moderate drought and has been the subject of many physiological and molecular studies (see e.g., Kramer and Boyer, 1995, and references therein; Des Marais and Juenger, 2010). Indeed, the majority of papers describing the molecular mechanisms of drought and abscisic acid (ABA) signaling deal with avoidance, even though they often use the term *tolerance*. In these cases, the main phenotypes observed are typically changes in leaf water loss, stomatal density, or control of stomatal aperture. Particularly, the control of stomatal opening and closing is one of the most extensively studied signaling models in plants (Kim et al., 2010). Both stomatal regulation and stomatal density influence water use efficiency at the whole plant level (Yoo et al., 2009). Water use efficiency and related topics are covered in other chapters of this volume. This chapter will take a more focused view of drought tolerance mechanisms about which less is known at the molecular level.

### 2.1.3 *What is drought tolerance?*

Drought tolerance, rigorously defined, is the ability of the plant to cope with reduced water potential of the plant tissue. This reduced water potential may be accompanied by either small or large changes in tissue water content, depending on the severity of the stress and the plant species involved. Drought tolerance mechanisms allow the plant to function, or at least survive, at reduced water potential. Drought tolerance can be difficult to quantify experimentally, as it requires that some steps be taken to ensure that different plants being tested are exposed to the same severity of stress (i.e., the same water potential). The seemingly simple experiment of withholding water from potted plants for a predetermined amount of time is actually difficult to interpret, as varying transpiration rates and leaf areas can lead to very different rates of soil water depletion (Verslues et al., 2006; Boyer, 2010). At the end of such water withholding experiments there can be dramatic, and very photogenic, differences in appearance of different genotypes. Such differences may be referred to as drought tolerance but are more often differences in avoidance. Measurements of soil water potential or water content, or simply growing different genotypes under comparison together in the same container where they are fully interrooted, can ensure that all the plants are exposed to the same water potential (Verslues et al., 2006) and allow differences in drought tolerance to be measured with less ambiguity.

Most often, we are concerned about drought tolerance differences between plant genotypes. Many of these differences are essentially differences in plant response to low water potential, often referred to as phenotypic plasticity. Other differences in tolerance are determined by constitutive differences in morphology, cellular structure, or metabolism that are often referred to as drought adaptations. However, there is overlap between drought adaptation and phenotypic plasticity, as much of the variation between drought resistant versus less drought resistant plant varieties or species includes differences in the type or extent of phenotypic plasticity induced by drought. Defining drought tolerance is also complicated by the fact that there are widely varying ideas between different scientific disciplines of what are the most relevant parameters to use when assessing drought tolerance. Agronomists and biotechnologists view biomass production and yield as the ultimate phenotype that defines drought tolerance. Ecologists speak in terms of fitness and reproductive success as primary indicators of which plants are adapted to drought-prone environments. In molecular studies, the response under study can be reduced in scope to gene expression, accumulation of a key metabolite, or a key protein modification. The common theme is that a drought tolerance response contributes to continued growth or function at reduced water potential, as opposed to something that seeks to avoid that reduction in water potential.

#### *2.1.4 Responses to longer-term moderate water limitation versus stress shock and short-term response*

The gaps between scientific disciplines studying drought are also apparent in another way. Agronomists, whole plant physiologists, and ecologists are typically interested in how plants continue to grow and function under moderate levels of stress that occur over extended periods of time. This view of drought tolerance contrasts with the approach taken in many molecular genetic studies of model species where there has been an emphasis on rapid (a few hours or less) responses and on ability to survive a severe stress shock. Such experiments can have value in uncovering basic signal transduction mechanisms; however, there is then another step to determining which of the genes or mechanisms discovered in such short-term or severe stress experiments are of value for longer-term response to more moderate reductions in water potential and ultimately for yield maintenance or drought adaptation or acclimation (phenotypic plasticity) in natural settings.

The differences between short-term versus longer-term responses and between tolerance of moderate drought versus survival of severe dehydration have been illustrated by recent studies. Des Marais et al. (2012) analyzed transcriptional responses to a slowly imposed and carefully controlled low water potential stress and found a suite of responsive genes that differed substantially from the short-term transcriptional response to severe stress identified in several previous studies. One example was a prominent role of upregulated photosynthesis genes that may support increased root growth at low water potential. In a converse approach, Skirycz et al. (2011) took mutants of 25 “stress tolerance genes” identified from the literature as having increased survival after a period of water withholding. These mutants were subjected to a period of controlled drought stress using an automated weighing and watering system, and increase in leaf area measured. None of the mutants differed in growth response to drought (some had higher or lower growth under control that was also observed in the drought period). As the drought survival experiments used to select the 25 “stress tolerance genes” involved uncontrolled drying and lack of water status measurements, it was likely that differing rates of water depletion (drought avoidance) caused most of the survival differences. Thus, the experiment of Skirycz et al. (2011) indicates differences both in tolerance of moderate drought versus survival of severe dehydration and differences in drought avoidance versus drought tolerance. A similar conclusion can be drawn from Skirycz et al. (2010), which analyzed *Arabidopsis* responses to prolonged mild osmotic stress imposed by mannitol and found an important role of mitochondrial metabolism in sustaining growth and developmental stage-specific differences in gene expression. All of these studies demonstrated that examining moderate low water potential stress over longer time frames can bring out new aspects of the molecular responses to drought stress. To accomplish

this, a number of laboratories and companies have constructed automated plant weighing, watering, and imaging systems for *Arabidopsis* and other species (see, e.g., Granier et al., 2006).

Although all of the above-mentioned examples were studies of *Arabidopsis*, similar principles apply to nearly all crop plants. For such glycophytic plants, loss of more than 40% of cellular water leads to severe damage, and water potentials lower than  $-1.5\text{ MPa}$  represent a severe stress (Kramer and Boyer, 1995). Desiccation-tolerant plants can survive much lower water potentials and reduced water content close to complete dehydration by using special metabolic adaptations to enter a metabolically dormant state (Oliver et al., 2011). The adaptations needed for desiccation tolerance can be different than those needed for continued growth under more moderate water limitations (Moore et al., 2008). Thus, there is some uncertainty as to whether the adaptations of extremophile plants are useful in the practical improvement of crop species. All of our discussion in this chapter will be about glycophytic plants that are unable to shut down under severe drought and instead must maintain metabolic activity as well as possible at low water potentials.

### 2.1.5 Natural variation and next generation sequencing

Recent years have also seen increased interest in using natural variation to understand drought tolerance. While breeders have long used variation, new genomics tools are making it much more feasible to directly connect natural variation with underlying molecular mechanisms. For this type of study, *Arabidopsis* is the main model system because of the extensive genetic and genomic resources that have been developed for it. The geographic distribution of *Arabidopsis* covers a range of climates differing in the frequency, distribution, and variation of precipitation (Koornneef et al., 2004), and there is an increasing awareness that *Arabidopsis* accessions can differ dramatically in drought-associated traits (see below for discussion of proline accumulation as an example). Common garden experiments where accessions from different regions are grown together at the same site have shown that locally adapted accessions had better growth and seed set compared to accessions from different climate regions. These studies also identified genome variation associated with adaptation to differing climates (Fournier-Level et al., 2011; Hancock et al., 2011). Also, Des Maria et al. (2012) found substantial differences in drought-induced gene expression changes between accessions. Thus, local adaptation and differences in drought response exist within *Arabidopsis*. When combined with the extensive *Arabidopsis* genomic data and detailed phenotyping of drought-related traits, natural variation can be a tool to discover new loci under selection for drought adaptation and new types of allelic variation that cannot be generated using mutagenesis. An example of such variation in proline metabolism is discussed below. The falling cost of sequencing will

allow natural variation in other species to be examined in ways not previously possible and is also making traditional forward genetic screens more attractive by shortening the route from interesting phenotypes to identification of the underlying genomic variation (see, e.g., Austin et al., 2011).

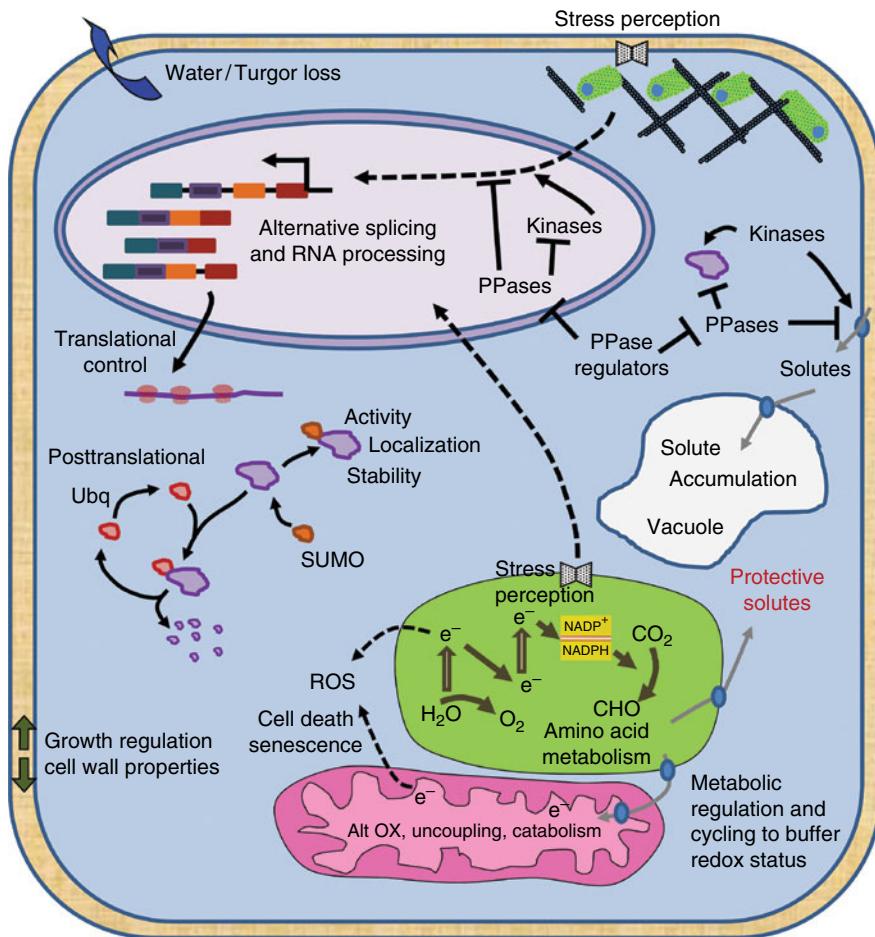
Overall, there is a great practical and scientific motivation to understand drought tolerance at the genetic and molecular level. This motivation seems set to increase because of climate change. We also have an increasing range of genomic and omics tools to identify and manipulate drought tolerance. Indeed, one of the great challenges of this research area is to bridge the “phenotype gap” between the accumulation of genomic information and connection of this information to drought tolerance phenotypes (Miflin, 2000). In the following sections of this chapter, we will discuss some important drought tolerance phenotypes for which there is a relative lack of information on the underlying molecular mechanisms. Conversely, we also discuss some regulatory mechanisms likely to be of importance in drought tolerance but that are only beginning to be characterized in detail. The goal of our laboratory, and many others in this field, is to continue to connect these two sets of information to form a better understanding of drought tolerance relevant to plant agriculture and ecology.

## 2.2 Some key drought tolerance mechanisms

There are many physiological factors that can affect drought tolerance, and the following sections are not a comprehensive review. Instead, we focus on some drought tolerance phenotypes (see Figure 2.1 for illustration) where there is a great potential for molecular genetics to uncover how these processes are regulated and better understand how they contribute to overall drought tolerance.

### 2.2.1 Osmoregulation/osmotic adjustment

One of the most basic requirements of plants, or other organisms, when confronted with decreasing water potential is the need to accumulate additional solutes inside the cell to prevent water loss and generate turgor (see Verslues et al., 2006, for further discussion of osmotic adjustment; Kramer and Boyer, 1995, for detailed background of plant water relations). Differences in osmotic adjustment do exist in plants (see, e.g., Morgan, 1984; Kiani et al., 2007; Izanloo et al., 2008; Bartlett et al., 2012) and in at least one case differences in osmotic adjustment have been evaluated for crop improvement (Morgan, 1991). Increased osmotic adjustment may be particularly valuable in controlled irrigation systems where plants are maintained at moderately reduced water potentials to allow growth to continue while enhancing water use efficiency. For more severe soil drying, some crop physiologists have debated whether greater osmotic adjustment is of value for crop improvement based on the fact that



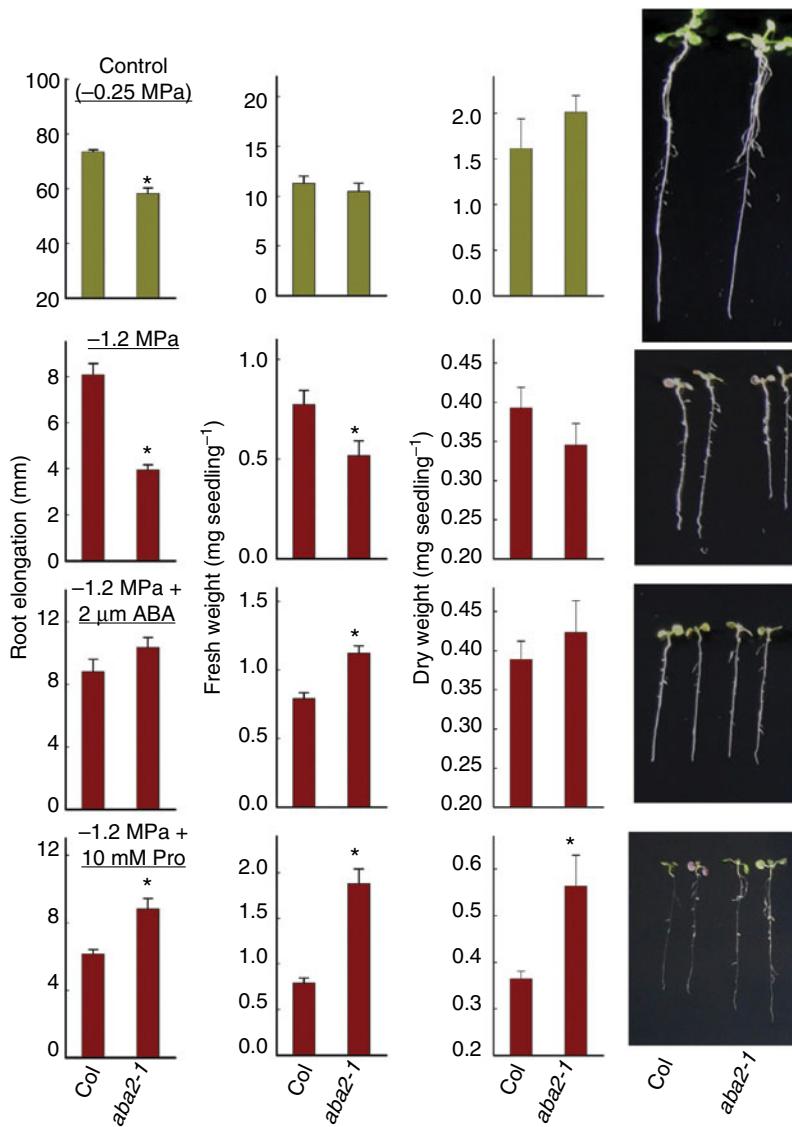
**Figure 2.1** Summary diagram of regulatory mechanisms and drought tolerance related cellular changes discussed in this chapter. Sensing and signaling of drought stress (top half of diagram) begins with an initial perception of water loss or loss of turgor that occurs via unknown mechanisms but may involve plasma membrane or organelle localized sensors and cytoskeleton changes. Downstream signaling involves the action of kinases and phosphatases (PPases), which can have opposing effects on a range of targets including transporters, transcription factors, and other proteins. Alternative splicing, selective translation, and protein modification by attachment of ubiquitin (Ubq) or small ubiquitin-like modifier proteins (SUMO) pathways generate additional changes in protein content and activity important for drought response. The regulator events lead to specific cellular changes related to drought tolerance (bottom half of diagram) including solute uptake and synthesis of protective solutes, changes in chloroplast and mitochondrial metabolism to buffer cellular redox status, and control of solute synthesis, as well as changes in cell wall properties. Adjustment to photosynthetic metabolism as well as mitochondrial alternative oxidases and uncoupling proteins act to dissipate reducing potential when necessary and prevent reactive oxygen species (ROS) formation. Even with this, the mitochondria and chloroplast are also sources of ROS, which, along with other specific signals, determine the time and extent of cell death and senescence during drought. For color details, please see color plate section.

water content and hydraulic conductivity of most soils decrease rapidly as water potential decreases (Munns, 1988; Serraj and Sinclair, 2002). In this view, increased osmotic adjustment may not be expected to allow substantially greater water uptake from drying soil. However, an elegant new analysis by Bartlett et al. (2012) found that the osmotic potential at full turgor, a measure of osmotic adjustment, is a strong predictor of ecological adaptation to dry environments. Likewise, osmotic potential at turgor loss was also strongly correlated with adaptation to dry environments and more influenced by osmotic potential than by cell wall elasticity. These analyses make a strong case for a critical role of cellular osmoregulation and osmotic adjustment in drought tolerance. Crop plants typically have fairly low solute contents (a high osmotic potential at full turgor; Bartlett et al., 2012). Increasing osmotic adjustment may be a viable strategy for plant improvement but would have to be coupled with the correct agronomic practices (such as the deficit irrigation strategy mentioned above) and correct drought-inducible regulation as high levels of osmotic adjustment likely impose a cost on the plant and may have a negative impact on productivity when water is plentiful.

We have very little knowledge of the molecular sensing and signaling mechanisms that control cellular osmoregulation and drought-induced solute accumulation and little knowledge of the genetics of osmoregulation in any plant species. A greater use of basic water relations measurement in molecular genetic studies would be a promising approach. In one example, studies of the proposed osmosensor protein AHK1 in *Arabidopsis* did not include water relations measurements needed to distinguish whether AHK1 affects osmoregulation and drought tolerance versus drought avoidance and leaf water loss (Tran et al., 2007; Wohlbach et al., 2008).

### 2.2.2 Regulated changes in growth

It is well known that low water potential restricts plant growth. What is perhaps less well appreciated is that for plants subjected to realistic severities of low water potential stress, decreased growth is not caused by physical or metabolic limitations but rather by regulatory events that are part of the plant's overall drought response strategy (Skirycz and Inze, 2010; Muller et al., 2011). An example of this is increased root to shoot ratio that is often observed in plants subjected to moderate drought stress. Such altered root to shoot ratio is regulated primarily by ABA, which restricts growth of the shoot while maintaining root growth (Munns and Sharp, 1993). A similar example in *Arabidopsis* is shown in Figure 2.2. When seedlings of either wild type or the ABA-deficient mutant *aba2-1* were transferred to low water potential media and held at a defined level of low water potential stress for 10 days, growth of *aba2-1* was clearly impaired compared to wild type with root elongation particularly affected (Sharma et al., 2011). Addition of ABA could restore root elongation,



**Figure 2.2** Regulated changes in growth at low water potential: stimulation of *aba2-1* by proline. Seedlings of Arabidopsis Columbia wild type or the ABA-deficient mutant *aba2-1* were transferred from control media to low water potential (-1.2 MPa) on polyethylene glycol-infused agar plates. Measurements of seedling growth (root elongation, fresh weight and dry weight) were performed 10 days after transfer. Transpiration is low in this experimental system, thus dehydration avoidance through stomatal closure has a relatively minor role. Because of this, *aba2-1* differed little from wild type in the high water potential control (top panels). At low water potential *aba2-1* was inhibited in root elongation, consistent with previous observations that ABA is required to promote root elongation at low water potential. Fresh weight and dry weight, which indicate shoot growth as well as root growth, were slightly decreased or unchanged. Adding a low level of ABA complemented the reduced growth *aba2-1*. However, adding the compatible solute proline had a more dramatic effect of increasing fresh weight and dry weight by 1.5 to nearly 2-fold (bottom panels). Thus, when the growth restraining effect of ABA was removed, added proline could greatly stimulate shoot growth at low water potential. Asterisks (\*) indicate significant differences between wild type and *aba2-1*. Pictures show representative seedlings from a series of replicated experiments. Figure is modified from Sharma et al., 2011. For color details, please see color plate section.

indicating that ABA is a promoter of root growth at low water potential (in contrast to its often observed inhibitory effect when applied to unstressed plants). Interestingly, application of the compatible solute proline could increase fresh weight and dry weight (which were primarily indicators of shoot growth because relative water content was little affected by the stress treatment used and transpiration was low), much more in *aba2-1* than in wild type (Figure 2.2). This indicated that in the wild type ABA was restricting the growth of the shoot and when ABA was restricted, a metabolic change (applying proline) could stimulate growth to a much greater extent.

Such regulated inhibition of growth during drought is adaptive in reducing leaf area and thus reducing transpiration and water use of the plant (to avoid dehydration). However, in cases where the shoot is the harvestable part of the plant it may be desirable for the plant to be less conservative in restricting growth at the onset of drought. Clearly, we cannot use ABA-deficient plants for such purpose because of the many functions of ABA in development and stress responses. What would be of interest is to uncouple the decreased growth from other drought responses so that growth continues in the early stages of drought or under moderate stress conditions. This would not be beneficial in longer-term or more severe drought; however, if the plant is harvested before the drought progresses, or agronomic/irrigation practices ensure that the water potential stays in a moderate stress range and does not decline further, the overall result may be beneficial (Skirycz and Inze, 2010). Currently, there is limited knowledge of which regulatory genes could be directly targeted for such a strategy, as genes identified based on survival of severe dehydration may not affect growth under moderate drought severity (Skirycz et al., 2011). One idea is that protein phosphatases, some of which are negative regulators of ABA signaling (see below), may also be negative regulators of growth during drought stress. Stress-induced changes in cell wall properties may also be important.

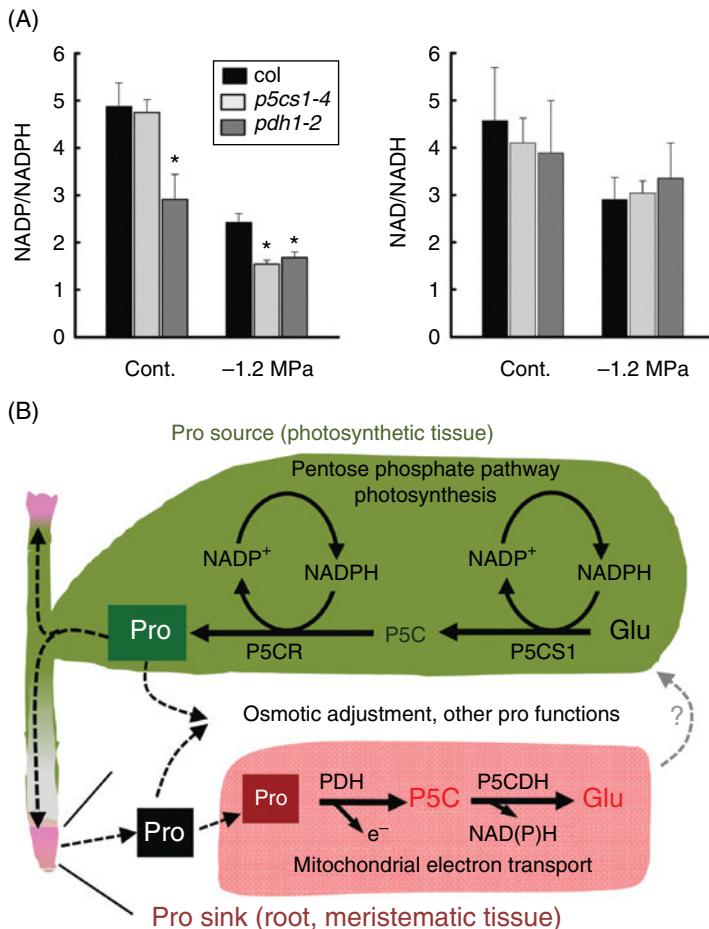
### 2.2.3 Redox buffering and energy metabolism

Much attention has been focused on reactive oxygen scavenging and detoxification as a way to withstand severe stress. However, it is perhaps less well appreciated that adjustments to metabolism that prevent the formation of reactive oxygen and allow efficient use of energy during drought are of equal or greater importance in plant performance under moderate drought (Potters et al., 2010; De Block and Van Lijsebettens, 2011; Sharma et al., 2011). Moderate drought can affect redox status even in the absence of cellular damage that leads to uncontrolled ROS production. Stomatal closure to conserve water can change the amount of carbon dioxide in the leaf. Because of this, as well as other stress factors, the chloroplast can be prone to overreduction and ROS generation (Dietz and Pfannschmidt, 2011). Plants have a number of ways to

direct excess reducing potential away from ROS production and maintain a stable redox state (Potters et al., 2010). Many factors affecting redox buffering are also likely to affect drought tolerance. Particularly, cyclic electron flow around photosystem I, which is only recently being characterized, may be a way to deal with excess reductant during drought (Bukhov and Carpentier, 2004; Johnson, 2011).

The key acceptor of electrons from photosystem I is NADP and the redox poise of the NADP(H) pool can exert feedback regulation of photosynthesis via poorly understood mechanisms (Hald et al., 2008). We found that low water potential had a particular effect of decreasing the NADP/NADPH ratio while having less effect on NAD/NADH (Figure 2.3A). Thus, there were fewer NADP electron acceptors available per NADPH, a situation that makes ROS production more likely. Interestingly, we found an even larger decrease of NADP/NADPH in mutants blocked in either proline synthesis (*p5cs1-4* mutant of  $\Delta^1$ -pyrroline-5-carboxylate synthetase1) or proline catabolism (*pdh1-2* mutant of *proline dehydrogenase1*) than in wild type plants (Figure 2.3A). Proline synthesis and turnover have a function in regenerating NADP electron acceptors, while proline catabolism in other parts of the plant is important in supplying energy and reductant for continued growth (Figure 2.3B; Sharma et al., 2011). Other experiments have shown a role of NAD<sup>+</sup> metabolism in drought tolerance: inactivation of poly(ADP-ribose) polymerase (PARP) inhibits NAD<sup>+</sup> breakdown and results in enhanced tolerance to several abiotic stresses (De Block et al., 2005; Vanderauwera et al., 2007). This effect was attributed to more efficient energy metabolism in the PARP-inhibited plants. NAD kinases, which phosphorylate NAD to NADP, have also been suggested to have a role in stress responses (Hashida et al., 2009). All these data indicate a drought tolerance role of redox status and energy metabolism independently from ROS scavenging mechanisms that may be involved in controlling damage under more severe stress.

The ability of mitochondria to dissipate excess reducing potential is also a component of drought tolerance. Alternative oxidases, uncoupling proteins, and other proteins involved in mitochondrial electron transport have been shown to impact drought response (Giraud et al., 2008; Zsigmond et al., 2008; Begcy et al., 2011), and expression of genes encoding these proteins is responsive to abiotic stress. Mitochondrial metabolism likely has roles in sustained respiration and energy production in meristematic regions as well as acting as a safety valve to dissipate excess reductant in photosynthetic tissue (Atkin and Macherel, 2009; Skirycz et al., 2010). Overall, there are many indications that both chloroplast and mitochondrial metabolism are important determinants of drought tolerance and are interrelated, as both contribute to the redox poise of the cell, energy usage, and how energy and reductant are directed away from uncontrolled ROS production and used to support continued growth under mild to moderate stress.



**Figure 2.3** NADP/NADPH ratio as an indicator of redox status of plants at low water potential and the role of proline metabolism in buffering NADP/NADPH. (A) Seven-day-old Arabidopsis seedlings of Columbia wild type, *p5cs1-4* (lacking expression of  $\Delta^1$ -pyrroline-carboxylate synthetase1, which encodes a stress-induced enzyme of proline synthesis) and *pdh1-2* (lacking expression of the proline catabolism enzyme *proline dehydrogenase1*), were transferred to control ( $-0.25 \text{ MPa}$ ) or low water potential ( $-1.2 \text{ MPa}$ ) media and pyridine nucleotide levels measured 96 hours later. Low water potential caused a decline in NADP/NADPH, indicating that a reduced supply of NADP in the chloroplast may increase the potential for reactive oxygen production or inhibition of photosynthesis. In contrast, NAD/NADH was less affected by low water potential. *p5cs1-4* and *pdh1-2* both had a greater decline in NADP/NADPH than wild type at low water potential. *pdh1-2* also had decreased NADP/NADPH in the unstressed control treatment. These data indicated a role of proline metabolism in controlling NADP/NADPH in addition to other protective roles of proline as a compatible solute that accumulates during drought. (B) Model of proline metabolism and its roles in regenerating NADP in photosynthetic tissue and supplying energy and reductant to meristematic and growing tissue to support continued growth during low water potential. In photosynthetic shoot tissue, synthesis of proline is relatively high, indicated by induced expression of *P5CS1* and repressed expression of *PDH1*. Because of its probable location in the chloroplast (reviewed in Verslues and Sharma, 2010; Szabados and Savoure, 2010), proline synthesis can serve to regenerate NADP as an electron acceptor to avoid reactive oxygen production and inhibition of photosynthesis. In growing tissue, especially the root apex, *PDH1* expression is induced rather than repressed by low water potential, and proline serves as an alternative respiratory substrate to sustain growth. Additional investigation of the localization and regulation of *P5CS1* and *PDH1* and mechanisms of proline transport are needed for further understanding of how proline metabolism promotes drought tolerance. Both (A) and (B) are modified from Sharma et al., 2011. For color details, please see color plate section.

#### 2.2.4 Senescence and cell death

Both chloroplast and mitochondrial metabolism also have roles in ROS production leading to cell death, and this is closely tied to another new question that has emerged in drought research: Do plant cells essentially kill themselves under stress by activation of senescence pathways? Is preventing such senescence and cell death a way to increase drought tolerance? Overexpression of a cytokinin synthesis gene (isopentyl transferase [IPT]) under control of a stress-inducible promoter enhanced cytokinin production at the onset of water limitation and led to dramatic differences in drought response (Rivero et al., 2007). In some ways this is similar to the “stay green” phenotype that has been studied in several plants; however, the stay green phenotype has several origins, not all of which involve cytokinin (Thomas and Howarth, 2000). Plants expressing the IPT construct remained green and had reduced leaf senescence under severe stress and enhanced growth under controlled water limitation. The mechanisms by which enhanced cytokinin increases drought tolerance seem to be related to changes in photorespiratory metabolism in the chloroplast, mitochondria, and peroxisome (Rivero et al., 2009), changes in hormone levels that lead to an overall protection of photosynthesis (Rivero et al., 2010), or other changes in drought-associated metabolites (Merewitz et al., 2012). There are still many questions about how manipulation of cytokinin has such dramatic effect on drought phenotypes. Leaf senescence during drought can be a conservative strategy whereby the plant prepares for more severe drought stress by decreasing the transpiring leaf area to conserve water. As described above for shoot growth, it may be advantageous for crop yield to make the plant less conservative during moderate drought.

There is also increasing interest in other cell death/senescence-associated signaling mechanisms and their effect on drought. In mammalian and yeast systems, ER stress and the unfolded protein response (UPR) is a major pathway of response to cellular stresses that lead to increases in unfolded proteins. This system is also present in plants (Urade, 2007; Cacas, 2010; Moreno and Orellana, 2011). Many UPR components are conserved in plants, including homologs of the sensor protein inositol requiring enzyme1 (IRE1), ER resident molecular chaperone binding protein (BiP), and bZIP60, which is alternatively spliced in response to ER stress (Martinez and Chrispeels, 2003; Iwata and Koizumi, 2005; Iwata et al., 2008; Deng et al., 2011; Humbert et al., 2012). Overexpression of BiP has been reported to increase drought tolerance (Alvim et al., 2001; Valente et al., 2009; Reis et al., 2011). This may also be related to delayed senescence or inhibited cell death. Studies of the UPR have involved very severe stress treatments; it will be of interest to determine whether UPR modulation affects plant growth in response to more moderate and longer-term drought stress.

### 2.2.5 *Metabolism*

Most of the above-mentioned drought tolerance mechanisms involve a metabolic component, and better understanding of metabolic regulation is an important part of understanding drought tolerance. One example is the TOR (Target of Rapamycin) pathway and its associated phosphatases and kinases, which are known as metabolic regulators in vertebrates and yeast and are now becoming better studied in plants (Harris et al., 1999; Menand et al., 2002; Mahfouz et al., 2006; Ahn et al., 2011; Moreau et al., 2012; Robaglia et al., 2012). Such metabolic signalling systems are likely to adjust cellular metabolism to the changes in energy and redox status caused by drought (such as the NADP/NADPH changes mentioned above). In the past, research on drought-induced metabolic changes focused on the synthesis of compatible solutes that accumulate to high levels during drought. Now, metabolomics technologies are making it easier to examine many metabolites simultaneously (see, e.g., Keurentjes et al., 2006; Lisec et al., 2008; Lisec et al., 2009; Riedelsheimer et al., 2012). The combination of metabolomics with QTL mapping or genome-wide association mapping has allowed many QTL and candidate genes associated with metabolic variation to be identified. Such approaches have great potential to identify and understand metabolic regulation contributing to drought tolerance but have yet to be widely used for this purpose.

## 2.3 Emerging drought tolerance regulatory mechanisms

Much study of drought-associated signaling and regulation has dealt with changes in gene expression. We still have a lot to learn about transcriptional regulation, as recently highlighted by Des Marais et al. (2012) in their comparison of transcriptional responses of different *Arabidopsis* accessions under slow soil drying. However, it is also clear from the application of omics technologies such as proteomics, metabolomics and next generation sequencing that transcript levels give only a partial picture at best of the many cellular changes that determine drought tolerance. Changes in protein abundances, activity, and interaction are also essential for understanding drought signaling. Here we deliberately omit discussion of transcriptional regulation (see, e.g., Yamaguchi-Shinozaki and Shinozaki, 2006; Mizoi et al., 2012; Nakaminami et al., 2012; Nakashima et al., 2012, for review) and have very little discussion of the rapid advances in ABA signaling (Cutler et al., 2010; Hubbard et al., 2010). Instead, the following sections maintain our focus on drought tolerance traits and some relatively uncharacterized signaling mechanisms that may control them (see Figure 2.1 for summary and illustration of drought tolerance traits and potential signaling mechanism). These traits have relatively less well-characterized signal transduction systems than for ABA signaling and stomatal regulation

(Kim et al., 2010). In our view, the drought tolerance traits described above are likely to be controlled by signaling that is less dependent on ABA or acts to change the plant's response to ABA during drought (i.e., crosstalk of drought and ABA signaling). We start with consideration of mechanisms that may be responsible for the initial sensing of water limitation and lay upstream of both ABA accumulation and ABA-independent drought responses.

### 2.3.1 Drought perception and early signaling

In most reviews of drought signaling, the initial perception of the stress (often referred to as *osmosensing*) is depicted as a black box. We do not open that box in this chapter. However, as this is one of the most fundamental questions in understanding drought tolerance at the molecular genetic level, we do think it worthwhile to exam what is known of osmosensing in other systems and clues this can reveal about plant osmosensing and drought perception. The key parameter that distinguishes drought (which for this discussion also includes osmotic stress imposed by various types of experimental treatments) from other signaling systems is the lack of a simple chemical ligand that can be detected as the initial signal. Drought/osmotic stress are thus likely be perceived by other mechanisms such as changes in turgor pressure, membrane strain, cell wall-plasma membrane connections, or molecular crowding (Bray, 1997; Wood, 1999; Wood, 2011).

Information from prokaryotes and yeast form the basis of many hypotheses of osmosensing mechanisms in plants. Two component systems are involved in many types of bacterial signaling and consist of a membrane-bound histidine kinase and cytoplasmic response regulator. An external stimulus induces autophosphorylation of the histidine kinase followed by transfer of the phosphoryl group to an aspartate residue in the response regulator, leading to activation of downstream genes. Examples of bacterial osmosensing two component systems include MtrB histidine kinase/MtrA response regulator of *Corynebacterium glutamicum* (Maker et al., 2007a; Maker et al., 2007b), and EnvZ/OmpR of *Escherichia coli* (Wang et al., 2012). Yeast also have a two component osmo-sensing system composed of two independent osmosensors, SLN1 (Synthetic Lethal of N-end rule 1) and SHO1 (SH3-Domain Osmosensor1). However, the downstream signaling is more complex: osmosensing by SLN1 transmits the signal to the response regulators Ypd1-Ssk1, which in turn leads to activation of the High Osmolarity Glycerol (HOG) MAP kinase pathway ending with the HOG1 MAP kinase for which the pathway is named (Reiser et al., 2000; Saito and Tatebayashi, 2004; Muzzey et al., 2009; Pelet et al., 2011). The yeast HOG pathway is an exceptionally well-characterized signal transduction system and similar pathways involving the MAPK p38 are present in other eukaryotes (Hilder et al., 2007; Gatidis et al., 2011).

Five of the 11 histidine kinase genes in *Arabidopsis* encode ethylene receptors. Of the others, AHK2, AHK3, and CRE1 are cytokinin receptors (Schaller et al., 2008; To and Kieber, 2008; Perilli et al., 2010). AHK1 is not a cytokinin receptor, and the signal it perceives is not understood. AHK1 has been proposed to be a plant osmosensor (Urao et al., 1999; Tran et al., 2007; Wohlbach et al., 2008) based on its ability to complement yeast *sln1/sho1* mutants. Interestingly though, AHK1, AHK2, AHK3, and CRE1 can all complement yeast *sln1/sho1* mutants despite their different functions *in planta* (Reiser et al., 2003; Tran et al., 2007). It has been proposed that AHK1 positively regulates drought and ABA signaling in *Arabidopsis* (Tran et al., 2007) and that *ahk1* mutants are deficient in ABA and proline accumulation (Wohlbach et al., 2008). Studies in our laboratory (Kumar et al., 2013) indicate that AHK1 regulates ABA sensitivity and stomatal density; however, we found that *ahk1* mutants were not inhibited in proline accumulation, osmotic adjustment, or ABA accumulation induced by low water potential. Thus the apparent drought phenotypes of *ahk1* mutants previously reported (Tran et al., 2007; Wohlbach et al., 2008) are likely caused by differences in leaf water loss and dehydration avoidance rather than any difference in drought tolerance. In our view, this indicates that AHK1 is not a main sensor of drought/osmotic stress analogous to yeast SLN1, as it does not affect core drought tolerance responses (such as osmotic adjustment and ABA accumulation) that should be under control of an osmosensing mechanism. The AHK2, AHK3, AHK4 cytokinin receptors have effects on low water potential-induced proline accumulation and growth under low water potential or salt stress (M.N. Kumar and P.E. Verslues, unpublished), although these effects differ from previous analysis (Tran et al., 2007). Plant AHKs have interesting roles that deserve further investigation; however, the search for plant osmosensing mechanisms is still open.

In bacteria, mechanosensitive channels (MSCs) can also respond directly to hypo-osmolarity (sudden increase in water potential) to release intracellular solutes and avoid cell lysis (Booth and Louis, 1999; Booth et al., 2007). Also, transporters such as ProP of *E. coli* (Wood, 2011) can directly sense osmotic shifts and mediate uptake of compatible solutes (proline in the case of ProP). Mechanosensitive channels and possibly transporters also exist in plants (Kung, 2005; Nakagawa et al., 2007; Jammes et al., 2011; Maathuis, 2011), but how they function in drought response is not known. It has been shown that several mechanosensitive channels of *Arabidopsis* are localized to plasma membrane, plastid, or mitochondrial membranes (Haswell et al., 2008; Veley et al., 2012). Knockout of plastid MSCs alters plastid shape (Haswell and Meyerowitz, 2006) and this phenotype is alleviated by addition of solutes, indicating that plastids normally have a higher solute content than the cytoplasm and need mechanosensitive channel activity to release solutes from the plastid (Veley et al., 2012). Thus, *Arabidopsis* MSCs are a plastid localized system capable of responding to osmotic changes. It will be of interest to see if this system, as

well as mechanosensitive channels on the tonoplast (Maathuis, 2011) or plasma membrane (Nakagawa et al., 2007), has roles in signaling of tissue dehydration during drought.

Other models for perception of drought/osmotic stress are based on sensing of changes in the connection between the plasma membrane and cell wall or in the shape of the cell. The analogous system that may be most relevant in this case is mammalian integrins, which span the plasma membrane and interact with the extracellular matrix and with signaling proteins and the cytoskeleton on the interior of the cell (Campbell and Humphries, 2011; Jean et al., 2011). In plants, the cell wall-plasma membrane-cytoskeleton continuum may also be important for plants to respond to the different environmental cues (Baluska et al., 2003). There are no clear integrin homologs in plants, although there are some clues of integrin-like functions in plants. The *Arabidopsis Non-Race-Specific Disease Resistance Protein1* (NDR1) was found to have similarity to integrins through structural modeling (Knepper et al., 2011). Mutants of *ndr1* had altered cell wall-plasma membrane connections after plasmolysis and were affected in pathogen response. However, it is not known whether NDR1 is involved in drought sensing or signaling. In another example, immunoscreening of an *Arabidopsis* cDNA library using an antibody against mammalian  $\beta 1$ -integrin identified a protein named At14a (Nagpal and Quatrano, 1999). At14a has similarity to integrins in its N-terminal cytosolic portion. There is some evidence that At14a, or other proteins having integrin-related function may be involved in osmotic stress responses and cytoskeleton rearrangements (Lu et al., 2007a, 2007b, 2012). There is also strong evidence for involvement of cytoskeleton rearrangements in salinity stress response (Wang et al., 2011) and in sensing mechanical forces associated with differential growth of adjacent cells during meristem development (Uyttewaal et al., 2012).

There are certainly other possibilities for osmosensing mechanisms in addition to the membrane-based sensors described here and it is likely that plants have multiple osmosensing systems. For example, plants have a plethora of receptor-like kinases that have no known function, and it is plausible that some of these could sense signals other than small molecule ligands. Other cell wall proteins could also be involved. Assay of some of the drought tolerance phenotypes described above coupled with genetic manipulation of osmosensing candidates is a promising approach to open the black box of osmosensing and upstream drought signaling.

### 2.3.2 Alternative splicing

Alternative splicing has recently drawn more attention in plant science in part because next generation sequencing of cDNA is generating much information about splicing variation. Plants can produce alternative spliced transcripts

having intron retention, exon skipping, or alternate acceptor-donor splice sites. These splicing differences can create an alternative start site for translation and creation/deletion of some important domain(s), as well as creation of premature stop codon, which can produce a truncated or non-functional protein. Frequently, these alternative splicing events are coupled with nonsense mediated decay (NMD) or regulated unproductive splicing and translation (RUST) as an extra layer of controlling gene expression and protein levels (Filichkin et al., 2010). It has been estimated that in *Arabidopsis* and rice, approximately 42% and 48%, respectively, of intron containing genes undergo alternative splicing (Filichkin et al., 2010).

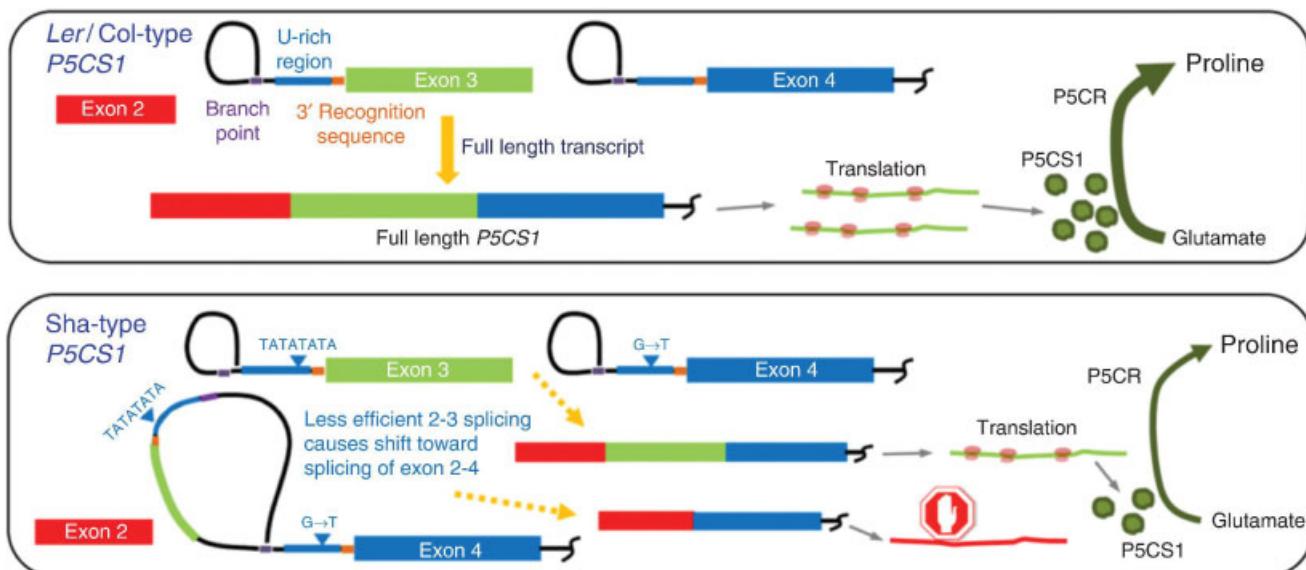
Some regulatory genes are already known to produce alternative spliced transcripts under abiotic stress treatment. Heat, drought, and cold have been shown to change the expression and splicing pattern of genes encoding splicing factor proteins such as the serine/arginine-rich (SR) splicing factors or polypyrimidine tract binding protein homologs (PTBs), which in turn govern the splicing of other genes (Rühl et al., 2013; Iida and Go, 2006; Isshiki et al., 2006). Other types of splicing factors may also be affected by drought and abiotic stress. One example is *Suppressor of ABI3* (SUA), which reduces splicing of a cryptic intron of ABI3 in *Arabidopsis* (Sugliani et al., 2010). More generally, there is also evidence that RNA binding/stabilizing proteins can enhance drought tolerance (see, e.g., Castiglioni et al., 2008). Thus, alternative splicing and RNA processing are a level of post-transcriptional regulation that can affect mRNA stability or change the amount, activity, or stability of the encoded proteins. The effect of alternative splicing on plant transcriptomes and proteomes, and how they change under drought and other stresses, is only beginning to be understood.

Besides splicing factors, changes in cis-acting elements can alter recognition of introns and splice acceptor/donor sites and give rise to alternatively spliced transcripts. For example, a conserved sequence upstream of the splice acceptor site in intron 12 of chloroplast *ascorbate peroxidase* (chlAPX) was found to be critical for efficient splicing (Yoshimura et al., 2002). Studies on maize ADH1 intron 3 led to the proposal that plant 5' and 3' splice sites are recognized based on their position relative to AU-rich elements in the intron (Lou et al., 1993; Lou et al., 1993; McCullough and Schuler, 1993; Merritt et al., 1997). Thus specific U and UA-rich sequences, as well as correct spacing between the 3' splice site, AU-rich region, and the splice acceptor site, are needed for intron recognition and splicing.

We recently found that intron sequence differences have a dramatic effect on alternative splicing and protein production of the drought-associated gene  $\Delta^1$ -pyrroline-5-carboxylate synthetase1 (*P5CS1*) of *Arabidopsis*. *P5CS1* catalyzes the first step of proline synthesis. Many studies have shown that *P5CS1* mRNA level increases under drought and other abiotic stresses (reviewed in

Szabados and Savoure, 2010; Verslues and Sharma, 2010). We found that insertion of extra TA repeats into the sequence coding for the UA-rich region of intron 2 coupled with a specific G to T transversion, which increased the length of a poly-T tract in intron 3, led to a high frequency of alternative splicing where exon 3 was deleted from the mature mRNA (see model in Figure 2.4; Kesari et al., 2012). Disruption of spacing between the 3' splice junction and branch point in intron 2 or altered binding of splicing factors are likely reasons for the dramatic effect of these polymorphisms on *P5CS1* splicing. The *P5CS1* mRNA lacking exon 3 was stable and accumulated to similar level as the full-length transcript; however, it did not produce protein. Thus, increased level of the alternatively spliced *P5CS1* transcript in some accessions led to decreased level of *P5CS1* protein and decreased ability to accumulate proline at low water potential (Kesari et al., 2012). We also observed that the *P5CS1* promoter does not express well when taken out of its genomic context and inserted into transgenic plants (Kesari et al., 2012; S. Sharma, R. Kesari, and P.E. Verslues, unpublished observations). Given this, and the fact that the total *P5CS1* transcript level varied little among most accessions, it seems that *P5CS1* alternative splicing is a way for the level of *P5CS1* protein to be altered by selection even though the promoter activity of *P5CS1* is dependent on its genomic context and not readily changed. The amount of alternatively spliced *P5CS1* was correlated with precipitation and temperature patterns from the accession sites of origin, indicating that alternative splicing of *P5CS1* was a factor in adaptation to differing climates. Such intron-mediated differences in splicing had not been observed before in plants and it will be of interest to look for additional examples.

Alternative splicing has also been observed for other drought-related genes, and certainly there are many more such cases yet to be found. For example, three transcript forms were observed for wheat *Dehydration Responsive Element Binding2* (*DREB2*), and two of these forms were differentially regulated by drought or salt stress (Egawa et al., 2006). In rice, *OsDREB* formed two splice variants, of which only one was functional and showed increased expression during stress conditions (Matsukura et al., 2010). Barley HvDRF1 and maize ZmDREB2A also show splicing variants (Xue and Loveridge, 2004; Qin et al., 2007). MAP kinases also have drought signaling roles, and 5 out of 20 Arabidopsis MAPK genes have been found to have multiple splice variants (Lin et al., 2010). These examples, as well as others (see, e.g., Kong et al., 2003 and Lin et al., 2009) differ from *P5CS1* in that it is presumed (but not known with certainty) that the stress-affected alternative splicing is controlled by differential expression of splicing factor genes. Thus, a more systematic investigation of splicing factors, their expression under drought, and how modulation of their expression changes mRNA profiles and drought phenotypes will be of interest.



**Figure 2.4** Intron sequence polymorphisms lead to varying rates of *P5CS1* alternative splicing and varying capacity for proline accumulation among *Arabidopsis* accessions. The *Arabidopsis P5CS1* transcript can be alternatively spliced into two transcripts: a full-length transcript that encodes *P5CS1* protein and a transcript missing exon 3 (exon 3-skip *P5CS1*) that cannot be translated to *P5CS1* protein. Most accessions produce only a low level of exon 3-skip *P5CS1* and can accumulate high levels of proline in response to low water potential stress. However, in some accessions up to half of the *P5CS1* transcript is the non-functional exon 3-skip *P5CS1*. These accessions have reduced levels of proline accumulation. Accessions having high levels of exon 3-skip *P5CS1* have extra TA repeats in intron 2 and a specific G to T transversion in intron 3; these are sufficient to drive high levels of alternative splicing. The percentage of exon 3-skip *P5CS1* is correlated with temperature and rainfall conditions from the accessions sites of origin, indicating the *P5CS1* and proline synthesis are under selection as part of local adaptation of *Arabidopsis* accession to climates differing in rainfall and temperature patterns. Figure is modified from Kesari et al., 2012. For color details, please see color plate section.

### 2.3.3 Post-translational modification: ubiquitination and sumoylation

Conjugation of protein with ubiquitin or small ubiquitin-related modifier (SUMO) is a common post-translational modification. Ubiquitination, similar to sumoylation, involves activating enzymes (E1s), conjugating enzymes (E2s), and ubiquitin ligases (E3s; Hua and Vierstra, 2011). The RING type E3 ligase KEEP ON GOING (KEG) is a negative regulator of ABA sensitivity based on its role in ubiquitination of the ABA-Insensitive5 (ABI5) transcription factor when ABA levels are low. When ABA levels are higher, phosphorylation changes promote KEG itself to become ubiquitinated and degraded, thus allowing ABI5 to accumulate (Liu and Stone, 2010). In contrast, AtAIRP1, a cytosolic E3 protein with a single C3H2C3 type RING motif, had a positive effect on ABA sensitivity (Ryu et al., 2010). Such differing effects of ubiquitination may be caused by the sheer number of ubiquitination enzymes and target proteins involved. E3 ligases are critical for recognition of the target protein, and Arabidopsis has up to 1,500 proteins that can act as E3 ligases (Hua and Vierstra, 2011).

Sumoylation can have diverse effects on protein properties including stabilization (e.g., by preventing ubiquitination), localization, or conformation/activity changes (Miura and Hasegawa, 2010). Study of the SUMO E3 ligase SIZ1 indicates that sumoylation is important for stress response (Catala et al., 2007; Miura and Hasegawa, 2010; Zheng et al., 2012). The *siz1* mutant had altered expression of a number of drought-associated genes, including *P5CS1* (Catala et al., 2007). Interestingly, we found that *P5CS1* protein is consistently observed to be 8–10 kD heavier than its expected size on western blots (Kesari et al., 2012). Such a size shift would be consistent with Sumoylation. Whether or not this actually indicates sumoylation of *P5CS1* is not known. In another example, *nine-cis-epoxycarotenoid dioxygenase3* (NCED3), an ABA biosynthesis enzyme required for drought-induced ABA accumulation, has been observed to generate two distinct protein bands of either the native or epitope tagged protein in Arabidopsis (Endo et al., 2008; Xu et al., 2012). In this case it is unclear whether one of the bands is a cleavage product or if there are other post-translational modifications of NCED3. Study of these and other drought tolerance proteins will certainly reveal more about the role of post-translational modification. Conversely, interaction screens (Elrouby and Coupland, 2010) or similar approaches can allow more general search for sumoylated or ubiquitinated proteins but have yet to be conducted in a manner designed to find proteins only expressed during drought or specifically modified during drought.

### 2.3.4 Kinase/phosphatase signaling

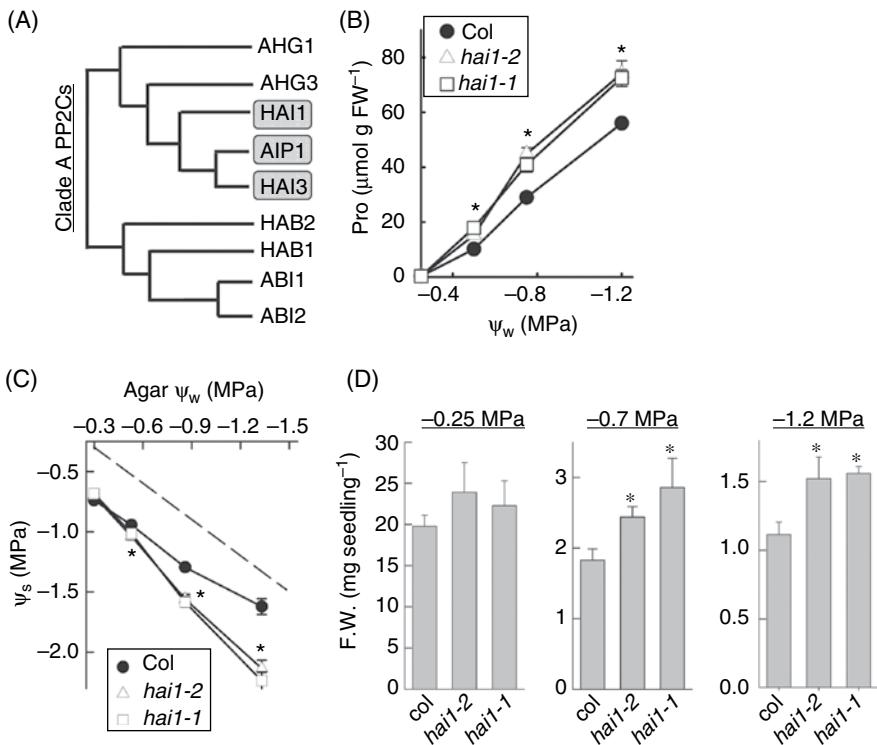
Plants have greatly expanded numbers of protein kinases compared to vertebrates, and most of these kinases are of unknown function (Schweighofer et al., 2004;

Ding et al., 2009). Phosphatases are fewer in number in plants (Schweighofer et al., 2004), but most are still of unknown function. The protein phosphatase 2Cs (PP2Cs) and Sucrose non-fermenting Related Kinase2 (SnRK2) kinases have been known for some time to have key roles in ABA signaling. Clade A PP2Cs and SnRK2s have recently come to the forefront of signaling research based on the discovery of the PYL family of ABA receptors and reconstitution of complete pathways from ABA perception to downstream targets in gene expression or channel regulation (Fujii and Zhu, 2009; Ma et al., 2009; Park et al., 2009; Cutler et al., 2010; Nishimura et al., 2010; Brandt et al., 2012). So far these reconstituted pathways consist of PYL/RCAR ABA receptors, Clade A PP2Cs, SnRK2 or calcium-dependent kinases (CDKs) and a target protein regulated by phosphorylation.

Just six of the nine Clade A PP2Cs have clearly established ABA signaling roles based on mutant phenotypes and PYL interaction. Likewise, of the ten SnRK2 kinases in *Arabidopsis* it is mainly SnRK2.2, 2.3, and 2.7 that have been well studied as part of the PYL-PP2C-SnRK2 signaling pathway (Fujii and Zhu, 2009; Fujii et al., 2011). Mutant analysis has shown that other SnRK2s had contrasting effects on proline accumulation with some promoting and some inhibiting proline accumulation induced by ABA or mannitol (Fujii et al., 2011). It is also known that some SnRK2s can be activated by osmotic stress but not ABA, while other SnRK2s can be activated by both ABA and osmotic stress (Boudsocq et al., 2004, 2007). Thus, even within the Clade A PP2Cs and SnRK2s, there is the strong possibility that some of the genes may be involved in other distinct stress signaling pathways.

Conversely, there are many drought tolerance traits (see above) for which regulatory mechanisms have yet to be established. With this thought in mind, we used a reverse genetic strategy for functional analysis of the Clade A PP2Cs and found that the three *Highly ABA-Induced (HAI)* PP2Cs (Figure 2.5A) have a role in proline accumulation and osmotic adjustment distinct from the ABA signaling roles of the other Clade A PP2Cs. Mutants of *HAI1* in particular had greatly enhanced proline accumulation (Figure 2.5B), osmotic adjustment (Figure 2.5C), and fresh weight (Figure 2.5D) across a range of low water potential treatments. The interesting question for further analysis is whether *HAI1* may work with SnRK2s (or other kinases) of unknown function in a distinct signaling pathway controlling osmotic adjustment and proline accumulation.

One can also apply such logic to other families of signaling proteins for which only a few members have known function while other members remain to be characterized. For example, many mitogen-activated protein kinases (MPKs) have no established function (Andreasson and Ellis, 2010). Interestingly, overexpression of a cotton group C MAP kinase led to improved osmotic adjustment capacity, elevated proline accumulation and



**Figure 2.5** The Highly ABA-Induced Clade A PP2Cs have a role in controlling proline accumulation and osmotic adjustment distinct from that of other Clade A PP2Cs. (A) Phylogenetic tree of the Clade A protein phosphatase 2Cs (PP2Cs) adapted from Schweighofer et al. (2004) showing the relationship of the Highly ABA-Induced PP2Cs (HAI1, AIP1/HAI2, and HAI3) to other Clade A PP2Cs. The other Clade A PP2Cs have well-established roles in ABA signaling, while the roles of the HAI PP2Cs have remained unclear. (B) Proline (Pro) accumulation of Columbia wild type *Arabidopsis* seedlings and *hai1* mutants. Seedlings were transferred to PEG-agar plates of a range of water potentials ( $\psi_w$ ) and proline measured 96 hours after transfer. Asterisks (\*) indicate significant differences between the *hai1* mutants and wild type. (C) Osmotic potential of Columbia wild type and *hai1* mutants 96 hours after transfer to a range of water potentials (Agar  $\psi_w$ ). The *hai1* mutants have reduced osmotic potential ( $\psi_s$ ), indicating increased solute accumulation and osmotic adjustment. The dashed line in the figure indicates where osmotic potential = water potential. Points below this line are consistent with a positive turgor pressure. Asterisks indicate significant differences between the *hai1* mutants and wild type. Both proline and osmotic potential also differed in *aip1* and *hai3* mutants but were less affected or unchanged in mutants of the other Clade A PP2Cs (data not shown). This indicated a specific function of the HAI PP2Cs in regulating osmotic adjustment associated with drought tolerance rather than ABA sensitivity and avoidance of leaf water loss, which are more affected by the other Clade A PP2Cs. (D) Fresh weight of Columbia wild type and *hai1* mutant seedlings 96 hours after transfer to control ( $-0.25$  MPa), moderate low water potential ( $-0.7$  MPa), or more severe low water potential ( $-1.2$  MPa). The *hai1* mutants maintained a higher fresh weight, indicating that their increased solute accumulation promoted greater water uptake and retention at low water potential. Figure is modified from Bhaskara et al., 2012.

upregulated expression of dehydrin, osmotin, and late embryogenesis associated (LEA) genes associated with dehydration tolerance (Zhang et al., 2011). Group C MAP kinases are of relatively unknown function (Andreasson and Ellis, 2010). As the experiments of Zhang et al. (2011) indicated an opposite effect of MPK on proline and osmotic adjustment compared to the *HAI* PP2Cs and MPK-PP2C interaction has been observed in other cases (Leung et al., 2006; Brock et al., 2010), it will be interesting to test whether MPKs and PP2Cs work together in a novel drought tolerance signal pathway(s). This would also be consistent with the model of the HOG pathway in yeast where protein phosphatases antagonize the activation of the pathway by the SLN1 osmosensor.

#### 2.4 Conclusion

Clearly distinguishing drought tolerance traits from drought avoidance, stomatal signaling, and ABA sensitivity illustrates how much remains to be done before we know even the broad outline and most important genes that make up the molecular basis of drought tolerance. Such knowledge of the molecular basis of drought tolerance will allow us to generate new variation in drought tolerance traits and use that variation for plant improvement. It will also be important in understanding how changing climates will affect plants in natural environments. From the basic research perspective, using a wider range of drought-related traits (including those summarized in Figure 2.1) as the basis for genetic studies has a great potential to identify new signaling mechanisms controlling drought tolerance traits. From the applied biology perspective, there is a need to use the molecular genetic tools of model organisms (mostly *Arabidopsis*) to analyze traits for which physiologists, breeders, and ecologists have long been interested. Ultimately, the goal is to match genes and molecular mechanisms with phenotypes, and often it is the phenotype side and execution of well-designed drought experiments that is limiting in closing this gap. We hope that this chapter can make a small contribution to the study of drought tolerance at the molecular level and ultimately to translational research in drought tolerance.

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### 3 Stomatal regulation of plant water status

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#### 3.1 Stomatal transpiration and cuticular transpiration

The gametophytes of mosses and liverworts do not possess true stomata. Instead of stomata, liverwort gametophytes possess air pores. In contrast, most of the sporophyte generations of the bryophytes possess stomata (Ziegler, 1987). Lichens that have wider ecological realms than the bryophytes are completely astomatous. The Isoetaceae have a number of astomatous species, particularly in the genus *Isoetes*. Most astomatous species of *Isoetes* are aquatic, and some phanerogam species become astomatous when submerged, for example, *Lobelia dortmanna* (Pedersen and Sand-Jensen, 1992) and species of *Ranunculus* (Bruni et al., 1996). However, some species of *Isoetes* and *Stylites* are terrestrial, occurring around the edges of oligotrophic bogs, and are astomatous (Keeley et al., 1984, 1994). It has been argued that stomata are either absent or non-functional in species with plastic responses to submergence where gaseous exchange through stomatal pores does not occur; instead gaseous movement through aerenchyma is the norm (Sculthorpe, 1967; Raven, 1984). Stomata are completely afunctional in the parasitic orchid, *Neottia nidus-avis* (Ziegler, 1987). Stomata are incapable of opening in *Neottia*, since the guard cells are fused to each other. In contrast, the stomata in some aquatic species, such as *Nymphaea alba*, *Nuphar lutea*, and *Lemna minor*, remain permanently open (Ziegler, 1987). In *L. minor*, the stomata do not close because the guard cells are mortal. In *Nymphaea* and *Nuphar*, guard cells appear to be cytologically intact, but the absence of substomatal cavities physically prevents the movement of the guard cells (Ziegler, 1987).

Thus, many plants in many natural habitats are either astomatous or lack functional stomata. Astomatous plants are categorized into two groups; a group that never possessed stomata and another group that once had functional stomata but later became completely or functionally astomatous. The former

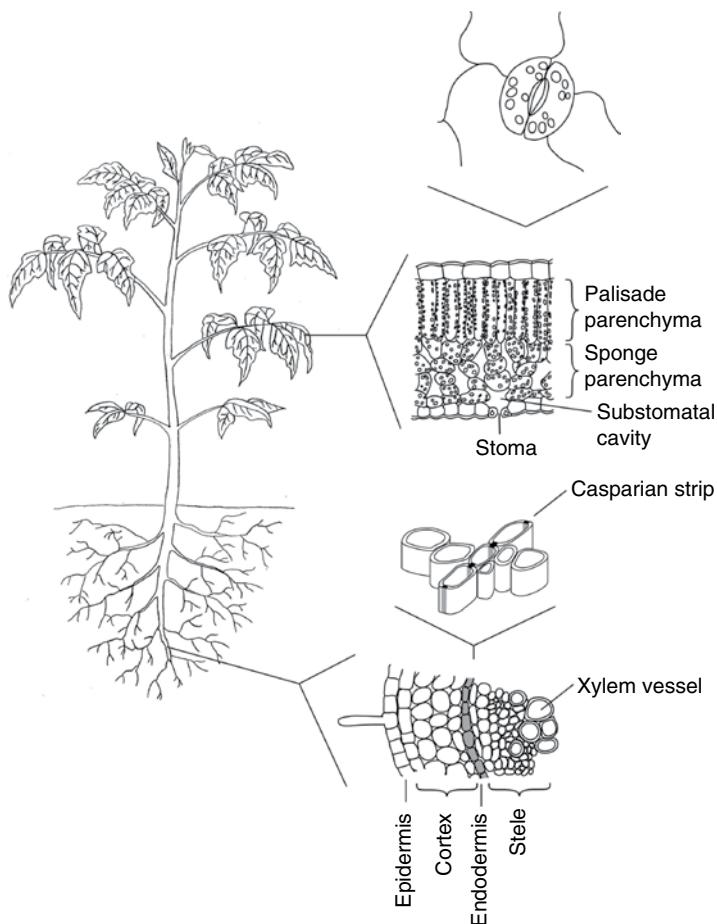
includes bryophytes and lichens and the latter includes the isoetid life forms and the parasitic orchids. In the case of moss gametophytes, the leaves are typically one cell thick and so CO<sub>2</sub> can diffuse into the leaves at a reasonable rate. However, this life form is small, with the tallest moss, *Dawsonia superba* reaching a maximum height of about 50 cm (Parihar, 1965). Water is transported in *Dawsonia* by hydroids and leptoids (Parihar, 1965), which add little strengthening to the stem. Niklas (1992) suggests that 50 cm is close to the maximum height that a stem with an essentially parenchymatous cell elastic modulus can attain.

Terrestrial isoetid plants are also very small, reaching heights of only about 10 cm. Keeley et al. (1994) found a number of species of *Isoetes* at high altitude (ca. 3,500 m) in the Andes. In all cases, the plants were found in bogs around the edges of nutrient-poor lakes. The species of *Isoetes* are astomatous and their CO<sub>2</sub> for photosynthesis is nearly all derived from sediments, entering the plants through their roots (Keeley et al., 1994). The leaves of *Isoetes andicola* have thick cuticles with low CO<sub>2</sub> conductance, at least three orders of magnitude less than that of open stomata (Kluge and Ting, 1978). The leaves, stems, and roots possess large air spaces or lacunae, which are more than sufficient to allow the movement of CO<sub>2</sub> to the photosynthetic cells of the leaf. In contrast, the vascular cylinder is very small.

Plants adapted to the land where the acquisition of water is restricted develop sophisticated water uptake and delivery systems. This water acquiring process is essentially similar to that with which a human drinks water.

Water ingested from the mouth migrates through the gastrointestinal tract and is finally selectively uptaken to the inside of the tissue across the epithelia of the large intestine. The acquired water is transferred to blood vessels and delivered to every part of a body.

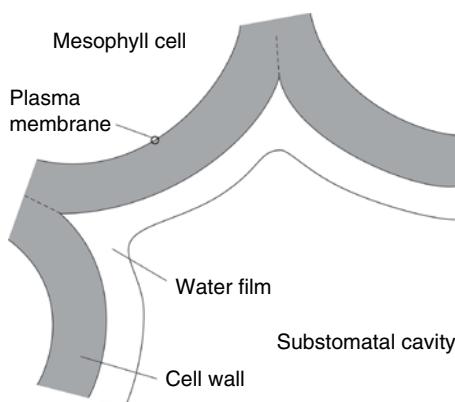
Plants acquire water from the soil by root hairs. A root hair consists of a single epidermal cell that is conceptually equivalent to epithelial cells of a large intestine. Water directly enters the cells from the surrounding environment in root epidermis and is turned over to cortical cells in the root. Some roots possess an exodermal layer beneath the epidermal layer. Exodermal cells form a single cell layer and are surrounded by suberin deposited in cell walls between exodermal cells, called *Casparian strip*. Casparian strip is virtually impermeant to water. Hence, exodermal cells serve the second gate to secure specificity of water uptake in roots. Water moves on toward the stele in cortical cells, which are connected to each other with plasmodesmata (symplastic pathway) or in apoplastic space around the cortical cells (apoplastic pathway). Before entering the stele, water faces the third gate, endodermis. Endodermal cells are girdled with Casparian strip, allowing selective uptake of minerals and water into the cells and successively loading them to the stele. Xylem parenchyma cells surrounding xylem vessels in the vascular bundle introduce water to the xylem.



**Figure 3.1** Plant structure involving acquisition and distribution of water.

Entering a leaf, vascular bundles form veins that are surrounded by bundle sheath. Bundle sheath consists of a single layer of parenchyma cells called *bundle sheath cells*. Water is unloaded from the xylem tissue through bundle sheath cells to parenchyma cells in leaves, mesophyll cells. Water channels (also known as aquaporin) that are membrane-integrated proteins facilitating water molecules to go across lipid bilayers function for water to pass through plasma membrane or tonoplast. This process is illustrated in Figure 3.1.

Many plants have a greater number of stomata on the abaxial surface of a leaf than the adaxial side, while some do solely on either surface or the same number on both sides. Behind the stomatal complex in the epidermis, a space called *substomatal cavity* exists (Figure 3.2). Around the substomatal cavity,



**Figure 3.2** Water film surrounding cell wall of mesophyll around substomatal cavity.

the surface of cell wall is wrapped with water film that evaporated to the air space of the cavity. Water vapor in the cavity diffuses away to the atmosphere through the stomatal aperture.

Early in the evolution of land plants (~420 million years ago, Silurian period), atmospheric CO<sub>2</sub> concentrations were 10–12 times higher than that of the present day (0.35–0.4%[v/v]), estimated from carbon isotope composition in calcium carbonate in fossil soils. A recent report argued against this estimation to be overestimated and predicted the atmospheric CO<sub>2</sub> concentrations in late Silurian to early Devonian to be approximately 0.2% (Breecker et al., 2010). Edwards et al. (1996) discussed the physiological problems confronting astomatous land plants at that time. Graham et al. (1995) suggested that small plants had moderate but presumably adequate peak photosynthetic rates, on the order of 6 µmol m<sup>-2</sup> s<sup>-1</sup>, but that the absence of stomata may have eliminated the water potential gradient, which would have limited the supply of water and nutrients to the plants. The small amount of xylem likely to occur in these early plants is thought to be sufficient to supply the volume and rate of water for transpiration, even for early stomatous species (Raven, 1993). However, the rate of nutrient supply from the soil to the plant, which occurred in the transpirational flux, would have been very low and potentially limiting to plant growth (Edwards et al., 1996).

According to Taiz and Zeiger (2002), for every gram of new plant material, approximately 500 g of water is absorbed by the roots, transported to the leaves, and then lost to the atmosphere. In fact, a leaf may exchange up to 100% of its internal water every hour. This continuous cycling of water through the plant is called *transpiration*, a process that can be divided into two main components: (1) stomatal transpiration, which is a gas-phase water diffusion through open stomata, and (2) cuticular transpiration, which is the diffusion of solid-phase water across the cuticle membrane itself.

Under water-sufficient conditions, the majority of water loss occurs through open stomata. Under environmental conditions that cause stomata to close, such as during drought, water loss is mainly executed via solid-phase cuticular transpiration.

Körner et al. (1994) demonstrated the effects of stomatal closure on water loss. The minimum conductance ( $g_{\min}$ ) was found to range from <1% of the maximum conductance ( $g_{\max}$ ) in succulents to 5.6% of  $g_{\max}$  in herbaceous shade plants. Here,  $g_{\min}$  and  $g_{\max}$  correspond to the water loss when the stomata are presumably closed and fully open, respectively. Thus, in well-watered environments, ~95–99% of all water loss occurs through the pores of stomata. By comparison, in conditions where plants close their stomata, such as during times of water insufficiency, water loss rates are determined primarily by the permeability of the plant cuticle.

The diffusional resistance of the transpiration pathway consists of two varying components. One is the resistance associated with diffusion through the stomatal pore, the leaf stomatal resistance ( $r_s$ ), and another is the resistance due to the layer of unstirred air next to the leaf surface through which water vapor must diffuse to reach the turbulent air of the atmosphere, the leaf boundary layer resistance. Because the boundary layer is smaller and less rate limiting in moving air than in still air, the stomatal aperture has more control over transpiration in moving air (Farquhar and Sharkey, 1982).

Because small stomata can open and close more rapidly than large stomata (Aasamaa et al., 2001) and tend to occur at high densities (Hetherington and Woodward, 2003), they have the capacity to rapidly vary the stomatal conductance of a leaf to water ( $g_s$ ).

### 3.2 Abiotic stress

Abiotic stress is a broad term that includes multiple stresses such as heat, chilling, excessive light, drought, waterlogging, wounding, ozone exposure, UV-B irradiation, osmotic shock, and salinity. It has been estimated that only 10% of arable land can be classified under the non-stress category, which implies that crops grown on the other 90% of arable lands experience one or more environmental stresses. Some of these stresses, like drought, extreme temperature, and high salinity, dramatically limit crop productivity. The prediction is that water deficits will continue to be the major abiotic factor likely to affect crop yields globally (Sharma and Lavanya, 2002).

#### 3.2.1 Drought

When plants are turgid, cells expand and press against the cell wall. During drought, the water content in a plant decreases, cells shrink, and the turgor pressure decreases. The decrease in cell volume concentrates solutes in cells.

Due to the decrease of cell surface area, the lipid bilayer of the plasma membrane bows inward or outward, even becomes physically more compressed and thicker. This process may be one of the earliest events in which a plant cell recognizes the decrease of water status. Plant processes that are most sensitive to a loss of turgor are leaf expansion and root elongation.

The total leaf area of a plant decreases during periods of water stress as a result of leaf senescence and abscission. Such a leaf area adjustment is an important long-term change that improves the plant's fitness in a water-limited environment. Indeed, many drought deciduous, desert plants drop all their leaves during drought and emerge new ones after a rain. This cycle can occur multiple times in a single season. Abscission during water stress results largely from enhanced synthesis of and responsiveness to the endogenous plant hormone ethylene (Sexton and Roberts, 1982).

A shoot will grow until its water requirements are greater than can be provided by the roots. Conversely, roots will grow until their demand for photosynthate is greater than can be provided by the shoot. This functional balance is shifted if the water supply decreases.

As stated above, leaf expansion is affected very early when water uptake is curtailed, but photosynthetic activity is much less affected. Inhibition of leaf expansion reduces the consumption of carbon and energy, and a greater proportion of the plant's assimilates can be distributed to the root system, where they can support further root growth. At the same time, the root apices in dry soil lose turgor.

All these factors lead to a preferential root growth into the soil zones that hold sufficient moisture. Plants generally develop shallow roots when all soil layers are wet. As the water deficit increases, the upper layers of the soil usually dry first, so that roots proliferate to the deeper layers. Deeper root growth into wet soil can be considered as a second line of defense against drought (Henckel, 1964).

Enhanced root growth into moist soil zones during stress requires allocation of assimilates to the growing root tips. During water deficit, assimilates are directed to the fruits and away from the roots. For this reason, the enhanced water uptake resulting from root growth is less pronounced in reproductive plants than in vegetative plants. Competition for assimilates between roots and fruits is one reason why plants are generally more sensitive to water stress during the reproduction stage.

A common development response to water stress is the production of a thicker cuticle that reduces water loss from the epidermis (cuticular transpiration; Kerstiens, 2006). Although waxes are deposited in response to water deficit both on the surface and within the cuticle inner layer, the inner layer may be more important than the surface because the inner layer controls the rate of water loss in complex ways rather than increasing the amount of wax present (Jenks et al., 2002).

A thicker cuticle also decreases CO<sub>2</sub> permeability, but leaf photosynthesis remains unaffected because the epidermal cells underneath the cuticle are non-photosynthetic. Cuticular transpiration, however, accounts for only 5–10% of the total leaf transpiration, so it becomes significant only when water stress is severe or when the cuticle has been damaged (e.g., by wind-driven sand).

The preceding section described the changes in plant development/architecture in response to short- and long-term challenges. Not only is whole plant development plastic (e.g., leaf size, root mass, and cuticle thickness) but also the number of stomata per unit area is plastic. Abscisic acid (ABA) reduces the stomatal index, which is defined as the number of stomata divided by the number of epidermal cells in a unit area. Atmospheric CO<sub>2</sub> concentration affects the stomatal index, so that the stomatal index of a fossil leaf can be used to estimate atmospheric CO<sub>2</sub> levels in earlier ages. When plants are fully developed, the strategy to change the morphology is not effective any more. The onset of drought/desiccation stress can be far too rapid for the plant to respond morphologically. However, under rapid dehydrating conditions, plants can rapidly respond by closing stomata to reduce water loss. Thus, stomatal closure can be considered a third line of defense against drought.

The influx and efflux of water through plasma membrane of guard cells modulates the turgor of the cells, causing the movement of stomata. Guard cells, which are localized in the epidermis of aerial parts of plants, lose turgor as a result of a direct loss of water by evaporation to the atmosphere. Hydropassive stomatal closure results from such evaporative water loss. This closing mechanism is likely to operate in air of low humidity, when direct water loss from the guard cells is too rapid to be balanced by water movement into the guard cells from adjacent epidermal cells (Raschke, 1975).

A second mechanism, referred to as hydroactive stomatal closure, occurs when the whole leaf and/or the roots are dehydrated and is affected by diurnal metabolite fluctuation in the guard cells. A reduction in the solute content of the guard cells (increase in osmotic potential) results in water loss and decreased turgor, causing the stomata to close; thus the hydraulic mechanism of hydroactive closure is almost a reversal of the mechanism of stomatal opening. However, it should be noted that the control of hydroactive closure is not entirely a reverse process of opening but subtly differs in important ways (Raschke, 1975).

Solute loss from guard cells can be indirectly triggered by a decrease in the water content of the leaf, and ABA plays an important role in this process. The transcripts encoding ABA synthesis-relating enzymes are localized in vascular tissue. It is conceivable that a decrease of water status in the plant body is recognized rapidly in vascular tissue, which is simply connected to other tissues with the xylem elements. Upon recognition of a water shortage by the vascular parenchyma cells, ABA may be released into xylem sap so that it readily reaches to the guard cells through the transpirational stream. Although ABA import transporter in guard cells and export transporter in xylem parenchyma

cells have been identified, it is unclear whether stored free ABA or conjugated ABA in other tissue, such as xylem sap, contributes to the pool of the physiologically active ABA (Nambara and Marion-Poll, 2005).

Although mild water stress can decrease photosynthesis by decreasing stomatal conductance, it more severely restricts leaf expansion. As stomata close during the early stages of water stress, water-use efficiency (WUE: the ratio of photosynthetic assimilation to transpiration) may increase (i.e., more CO<sub>2</sub> may be taken up per unit of water transpired) because stomatal closure inhibits transpiration more than it decreases intercellular CO<sub>2</sub> concentrations. The increase of WUE is attributed to the simple physical characteristics of the diffusions of CO<sub>2</sub> and H<sub>2</sub>O in the gas phase.

However, as stress becomes severe, the dehydration of mesophyll cells inhibits photosynthesis, which impairs mesophyll metabolism, which in turn decreases WUE. Many studies have shown that severe water stress decreases stomatal conductance much more than it decreases photosynthesis. The effects of water stress on photosynthesis and stomatal conductance can be separated by exposure of stressed leaves to air containing high concentrations of CO<sub>2</sub>. Any effect of water stress on stomatal conductance is eliminated by the high CO<sub>2</sub> supply, and differences between photosynthetic rates of stressed and unstressed plants can be directly attributed to damage from the water stress to photosynthesis (Farquhar and Sharkey, 1982).

Water stress decreases both photosynthesis and the consumption of assimilates in the expanding leaves. As a consequence, water stress indirectly decreases the amount of photosynthate exported from leaves. Because phloem transport depends on turgor, decreased water potential in the phloem during stress may inhibit the movement of assimilates. However, experiments have shown that translocation is unaffected until late in the stress period, when other processes, such as photosynthesis, have already been strongly inhibited.

This relative insensitivity of translocation to stress allows plants to mobilize and use reserves where they are needed (e.g., in seed growth), even when stress is extremely severe. The ability to continue translocation assimilates is a key factor in almost all aspects of plant resistance to drought.

### 3.2.2 *Light and heat*

If the net radiant balance is positive, as occurs in full sunlight, the leaf temperature rises. Then the boundary layer conductance must increase to allow a concomitant increase in sensible heat loss, in order to avoid lethal temperatures. The conductance would increase in areas with a tall canopy or a lot of wind. However, only plants having cuticular transpiration can occupy high irradiance habitats. The presence of stomata markedly increases energy dissipation by latent heat transfer, increasing the thermal tolerance of high irradiance sites.

The reduction of leaf temperature by vaporization heat through transpiration is most effective in the presence of infrared irradiation. The problem when stomata are open is achieving the balance between necessary water loss and water gain into the plant.

The depletion of ozone layer in the stratosphere has resulted in increased UV-B (280–315 nm) radiation at the earth's surface since the 1980s (UNEP, 2002). The stomata of plants exhibit complex responses to UV-B radiation. UV-B was reported to provoke stomatal closure and reduction in stomatal conductance (Musil and Wand, 1993; Nogués et al., 1999; Jansen and Noort, 2000). However, high fluences of UV-B stimulate either stomatal opening or stomatal closure, depending on the metabolic state of the guard cells, and neither of these responses is readily reversed. That is, once stomata have been exposed to UV-B, they are unable to readjust their aperture in response to environmental stimuli like changes in light, humidity, or ABA (Jansen and Noort, 2000). This lack of responsiveness is unlikely to cause widespread cellular damage, because UV-induced stomatal closure is largely reverted in response to the H<sup>+</sup>-ATPase activator fusicoccin (Jansen and Noort, 2000).

Although the response of stomata to UV-B radiation is well known, the underlying mechanism remains to be clarified. In *V. faba*, UV-B-induced stomatal closure is accompanied by hydrogen peroxide production, which is mediated by SHAM-sensitive peroxidase but not by NADPH oxidase (He et al., 2011). Moreover, the UV-B-induced stomatal closure involves ethylene synthesis (He et al., 2011).

Climate modeling predicts future increases in global temperature (Battisti and Naylor, 2009). High temperature increases the risk of both heat damage and water shortage to plants. High atmospheric temperature promotes evapotranspiration (sum of evaporation from Earth's ground and plant transpiration) from the soil as well as plant body. The risk of heat damage can be minimized by leaf cooling through the evaporation of water from stomata (i.e., transpiration; Hetherington and Woodward, 2003; Grill and Ziegler, 1998; Radin et al., 1994). Under well-watered conditions, plants consume considerably more water than is necessary for optimum yield, where the majority is lost via transpiration (Grill and Ziegler, 1998). Here, leaf cooling capacity has been shown to positively correlate with fruiting prolificacy and plant fitness (Radin et al., 1994). In water-limited environments, there is a trade-off between leaf cooling and the potentially injurious effects of excessive water loss.

Leaf temperature depends on stomatal conductance to water vapor, absorbed net radiation, air humidity, air temperature, and boundary layer conductance, which determine the leaf energy balance and also stomatal conductance (Jones, 1999; Nobel, 1999). Transpirational water loss is associated with leaf cooling. The water potential of the air in the substomatal cavity is equilibrated with that of apoplast fluid facing to the gas phase. As water vapor goes through a stomatal pore to the atmosphere, water is vaporized from the surface area of the substomatal

cavity to reach a new equilibrium between water vapor pressure in cells and water vapor pressure in cavity, where vaporization heat was taken from the surface area to the atmosphere.

Stomatal opening is crucial for maintaining leaf temperature. For example, the leaves of *open stomata* mutants have lower leaf surface temperature than wild-type plants, as shown by infrared thermography (Merlot et al., 2002).

On the other hand, in *Arabidopsis* plants developed at high temperature (28 °C), water loss is increased and leaf cooling capacity is enhanced, even though the leaves have fewer stomata, an elongated architecture, and reduced size. Because the rate of transpiration in plants is proportional to the number of stomata, plant architectural adaptions to high temperature may also enhance evaporative leaf cooling in well-watered environments (Crawford et al., 2012).

### 3.2.3 Carbon dioxide

The ability of plants to moderate water loss while allowing sufficient CO<sub>2</sub> uptake for photosynthesis can be expressed by the transpiration ratio, which is the number of water molecules transpired by the plant divided by the number of carbon dioxide molecules assimilated by photosynthesis. The inverse of the transpiration ratio is called the *water-use efficiency*. For plants in which the first stable product of carbon fixation is a three-carbon compound (such plants are called C3 plants), about 500 molecules of water are lost for every molecule of CO<sub>2</sub> fixed by photosynthesis, giving a transpiration ratio of 500 and a WUE of 1/500, or 0.002.

The large ratio of H<sub>2</sub>O efflux to CO<sub>2</sub> influx results from three factors:

1. The concentration gradient driving water loss is about 50 times larger than that driving the influx of CO<sub>2</sub>. In large part, this difference is due to the low concentration of CO<sub>2</sub> in air (about 0.038%) and the relatively high concentration of water vapor within the leaf.
2. CO<sub>2</sub> diffuses about 1.6 times more slowly through air than does water because CO<sub>2</sub> molecules are larger than H<sub>2</sub>O molecules.
3. Before CO<sub>2</sub> is assimilated in the chloroplast, it must cross the plasma membrane, the cytoplasm, and the chloroplast envelope, which increase the resistance to CO<sub>2</sub> diffusion.

Solubilized, CO<sub>2</sub> is converted to carbonic acid, bicarbonate, and protons. Thus the sensing mechanism could either rely on measuring CO<sub>2</sub>, protons, and bicarbonate or monitor the interconversion of a protein via CO<sub>2</sub> binding. Experiments using either the pH-sensitive dye 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) or fluorescence microphotometry found no evidence for a change in cytosolic pH after elevation of CO<sub>2</sub> up to 1,000 ppm (Bearley et al., 1997). These data showed that the sensing of CO<sub>2</sub> by plants is not mediated through changes of cytosolic pH.

Carbonic anhydrase (CA) is a metalloenzyme that catalyzes the decomposition of carbonic acid into carbon dioxide and water. In taste buds of animals, CAs are involved in detection of CO<sub>2</sub> (Chandrashekhar et al., 2009). A recent study revealed that carbonic anhydrases,  $\beta$ CA1 and  $\beta$ CA4, mediate CO<sub>2</sub> response directly in guard cells, suggesting that CAs are also involved in detection of CO<sub>2</sub> in plant cells. On the other hand, in *Drosophila*, CO<sub>2</sub> is sensed as an olfactory stimulus by a novel G protein-coupled receptor (Jones et al., 2007).

The HT1 (High Leaf Temperature 1) protein kinase is the first identified molecular component that functions as a major negative regulator in the high CO<sub>2</sub>-induced stomatal closure pathway (Hashimoto et al., 2006). The *ht1* mutants retain responsiveness to ABA, suggesting that HT1 functions upstream of the convergence of the CO<sub>2</sub>- and ABA-induced stomatal closure pathways (Hashimoto et al., 2006). *ca1 ca4 ht1-2* triple mutants exhibit the same constitutive high-CO<sub>2</sub> response as *ht1-2* mutants, suggesting that *HT1* masks the phenotypes of  $\beta$ CA1 and  $\beta$ CA4.

In a genetic screen, Negi et al. (2008) isolated a mutant with an impaired stomatal response to elevated CO<sub>2</sub>. The mutant was found to be deficient in slow anion channel-associated 1 (SLAC1), which is responsible for slow-type anion channel activity of guard cell plasma membrane.

CO<sub>2</sub> signaling is not a stand-alone pathway because the CO<sub>2</sub> signal pathway commonly shares downstream signal components with other pathways and because a core component of ABA signaling, OST1 kinase, is required for guard cell CO<sub>2</sub> signaling (Xue et al., 2012). Hence, acclimation to elevated CO<sub>2</sub> can affect the response of guard cells to other signals. For example, elevated CO<sub>2</sub> can sensitize stomatal response to osmotic stress (Leymarie et al., 1998; Leymarie et al., 1999).

### 3.2.4 Ozone

A recent analysis of 30 years of satellite data (Salby et al., 2011) detected an upward trend in total ozone over Antarctica since the late 1990s that is presumably due to reductions in the use of chlorofluorocarbons (CFCs). However, circulation in the upper atmosphere, which can change temperatures and affect the formation of polar stratospheric clouds, may also affect ozone levels, so the recent increase in ozone levels does not preclude the formation of ozone holes in the future.

Exposure of *Arabidopsis* to acute ozone results in a rapid transient decrease in stomatal conductance (within 3–6 minutes of exposure) accompanied by a burst of reactive oxygen species (ROS) in the guard cells, followed by a slower recovery to initial rates of stomatal conductance (Kollist et al., 2007; Vahisalu et al., 2008). A minimum concentration of ozone of 80 ppb is required to trigger the rapid transient decrease in stomatal conductance (Vahisalu et al., 2008).

Chronic ozone exposure is not as important as leaf age and plant developmental stage in determining stomatal conductance (Bernacchi et al., 2006; Uddling et al., 2009). Ozone does not affect the existing wax but severely reduces the biosynthesis of cuticle wax *de novo* (Percy et al., 1994). Moreover, chronic ozone exposure prevents stomata from closing rapidly in response to environmental stimuli (Mills et al., 2009).

Additionally, stomatal sensitivity to ABA may be compromised in ozone-stressed plants (Mills et al., 2009; Wilkinson and Davies, 2009; Wilkinson and Davies, 2010). *slac1* mutants were genetically isolated by three independent mutant screens: one for CO<sub>2</sub>-insensitive stomatal closure mutants and the others for ozone-sensitive mutants (Negi et al., 2008; Vahisalu et al., 2008; Saji et al., 2008). SLAC1 is a plant ion channel that controls turgor pressure in the guard cells of stomata. *slac1* mutants display impaired stomatal closure in response to ABA.

### Ozone distribution in the atmosphere

The ozone layer in the stratosphere began to form 2.3 billion years ago. The ozone layer that absorbs UV-B was established around 400 million years ago. The absorption of UV-B by the ozone layer is crucial for the survival of terrestrial organisms, since UV-B critically damages DNA.

The dense ozone layer is formed in the stratosphere at an altitude of approximately 20–40 km, where the ozone production rate exceeds the decomposition rate. Very low concentrations of ozone are found in the upper to middle levels of the troposphere. The highest ozone concentration in the troposphere is found at ground level, although it is not as high as the concentration in the stratosphere.

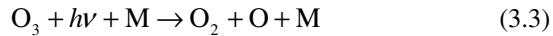
In Canada, the ozone concentration in the troposphere remained stable or declined during 1980–1990, while it strongly increased in 1990–2000. Similarly, the ground level ozone had decreased in the 1970–1980s in Saitama, Japan, then turned upward in 1990–2000. The ozone production in troposphere (ground level) is the latest issue that is currently affecting plants. Saitama prefecture estimated that the rice yield decreased by 10% due to ambient ozone in 2000 and expected 30–40% loss in 2050 in the north Kanto area surrounding Tokyo (Yonekura, 2008). Why did the ozone concentration increase at the ground level? Let's look at the mechanism of ozone production.

High-energy photons ( $\lambda < 240 \text{ nm}$ ) photolyze the molecular oxygen (O<sub>2</sub>), producing two ground level oxygen atoms (<sup>3</sup>O, 1s<sup>2</sup> 2s<sup>2</sup> 2p<sub>x</sub><sup>2</sup> 2p<sub>y</sub><sup>2</sup>).

The  ${}^3\text{O}$  readily combine with molecular oxygen ( $\text{O}_2$ ) to form ozone ( $\text{O}_3$ ) in the presence of a third party (M), as follows:



$\text{O}_3$  decays with lower-energy photons ( $\lambda < 320\text{nm}$ ) to form  $\text{O}_2$  and O. At the same time, another reaction for  $\text{O}_3$  decrease occurs in the stratosphere.



Ozone in the troposphere is produced by a different mechanism. Photochemical oxidants mainly consisting of  $\text{O}_3$  are formed with photochemical reactions of nitrogen oxides (NOx) and volatile organic compounds (VOC). The major sources of NOx and VOC are smoke dusts from fossil fuel. Use of coal resulted in the London smog in 1952. Modern photochemical smog caused by automobile emission was first observed in Los Angeles in 1943. The mechanism underlying the production of Los Angeles-type smog is clear. The heavy smog in Beijing is much in the news today. In areas with heavy smog all forms of life suffer from the ozone stress in the biosphere.

### 3.3 Abiotic stress and biotic stress

Plants have evolved to live in environments where they are exposed to multiple stresses. Multiple environmental stresses can have additive or interactive effects on plants.

#### 3.3.1 Interaction between ABA signaling and MeJA signaling

Jasmonates regulate various physiological processes in plants such as pollen maturation, tendril coiling, senescence, and responses to wounding and pathogen attacks (Turner et al. 2002). Like ABA, jasmonates also trigger stomatal closure in many plants, including *Arabidopsis thaliana* (Suhita et al., 2004), *Hordeum vulgare* (Tsonev et al., 1998), *Commelina benghalensis* (Raghavendra and Reddy, 1987), *Vicia faba* (Xin et al., 2005), *Nicotiana glauca* (Suhita et al., 2003), *Paphiopedilum supersuk* (Gehring et al., 1997), and *Paphiopedilum tonsum* (Gehring et al., 1997). To date, pharmacological and reverse genetic

approaches have revealed many important signal components involved in MeJA-induced stomatal closure and suggest a signal crosstalk between MeJA and ABA in guard cells.

*Arabidopsis coronatine insensitive 1* encodes an F-box protein that forms high affinity jasmonate co-receptors with transcriptional repressor JAZ proteins (Sheard et al., 2010). The disruption of *COI1* gene hampers MeJA-induced stomatal closure (Munemasa et al., 2007), suggesting that MeJA-induced stomatal closure is one of the plant physiological responses.

ROS and nitric oxide (NO) have been shown to function as second messengers in guard cell ABA signaling. In guard cells ABA triggers ROS production, which is mediated by homologs of the neutrophil NADPH oxidase gp91<sup>phox</sup>, AtrobohD, and AtrobohF (Pei et al., 2000; Kwak et al., 2003). In the *Arabidopsis atrbohD* and *atrbohF* mutant, MeJA as well as ABA fails to induce stomatal closure (Suhita et al., 2004), and MeJA-induced stomatal closure is inhibited by an NADPH oxidase inhibitor, dephenylene iodonium (Suhita et al., 2004; Munemasa et al., 2007), suggesting that ROS produced by NADPH oxidase functions in both ABA signaling and MeJA signaling. The radical signal gas NO was shown to play a crucial role in both MeJA and ABA signaling. However, the detailed mechanism of how NO is produced during MeJA- and ABA-induced stomatal closure is still unclear. A regulatory A subunit of protein phosphatase type 2A (PP2A), RCN1, is involved in both MeJA and ABA signaling upstream of ROS and NO production. Myrosinases TGG1 and TGG2 function downstream of ROS production in guard cell MeJA and ABA signaling (Islam et al., 2009). A calcium-dependent kinase, CPK6, is required for activation of  $I_{Ca}$  channels and S-type anion channels in both MeJA and ABA signaling (Munemasa et al., 2007).

An ABA-insensitive mutant, *abi2-1*, is insensitive to MeJA (Munemasa et al., 2007) and another ABA-insensitive mutant, *ost1*, is less sensitive to MeJA (Suhita et al., 2004). PP2Cs and SnRK2 participate in ABA core components together with the ABA receptor PYR/PYL/RCAR (Park et al., 2009), suggesting that MeJA affects regulation of the ABA receptor complexes.

On the other hand, MeJA signal transduction leading to stomatal closure in *Arabidopsis* guard cells requires endogenous ABA (Hossain et al., 2011), suggesting that ABA priming is involved in the MeJA signal transduction.

### 3.3.2 Interaction with other signaling

Salicylic acid (SA) is a critical signaling molecule that mediates plant defense responses against numerous biotrophic/hemibiotrophic pathogens as well as induction of systemic acquired resistance that confers a long-lasting, broad-spectrum resistance against pathogen infection (Durrant and Dong, 2004). Recently, NPR3 and NPR4 were identified as SA receptors (Fu et al., 2012), and NPR1 was also shown to function as an SA receptor (Wu et al., 2012).

Several studies have demonstrated antagonistic interactions between the ABA and SA responses (Mosher et al., 2010; Moeder et al., 2010).

Interestingly, SA and ABA both rapidly induce stomatal closure. SA requires ROS as a second messenger to trigger stomatal closure, but SA-induced ROS production is mediated by SHAM-sensitive cell wall bound peroxidases rather than NADPH oxidases (Mori et al., 2001; Khokon et al., 2011).

Elicitors are chemical or biological molecules from various sources that mimic pathogen attack and induce marked physiological changes of the target living organism (Zhao et al., 2005). Cell wall fragments of plants or pathogens can serve as elicitors in many species. Exposure of plants to either elicitors or pathogens triggers an array of defense reactions, including the accumulation of defensive secondary metabolites such as phytoalexins (Zhao et al., 2005). The early responses of plant tissues to elicitors are typical of signal transduction from elicitor perception to defense reactions. For example, elevation in cytosolic  $\text{Ca}^{2+}$  (Mithöfer et al., 1999; Blume et al., 2000) and production of ROS and NO (Garcia-Brunner et al., 2006; Mur et al., 2006) are common in plant tissues exposed to elicitors during plant pathogen interactions.

Like SA, yeast elicitor (YEL) and chitosan (CHT) induce stomatal closure, which is accompanied by ROS production mediated by SHAM-sensitive cell wall peroxidases (Khokon et al., 2010a; Khokon et al., 2010b). Bacteria such as *Pseudomonas syringae* pv *tomato* (*Pst*) strain DC3000 and bacterial flagellin (flg22) induced stomatal closure, which is mediated by NADPH oxidases (Mersmann et al., 2010; Desclos-Theveniau et al., 2012). YEL evokes  $\text{Ca}^{2+}$  oscillations in guard cells, which is like ABA-induced  $\text{Ca}^{2+}$  oscillation (Klüsener et al., 2002). In future, single-cell ROS imaging analysis at high spatio-temporal resolution would allow us to understand how the produced ROS integrate abiotic and biotic signal crosstalk in guard cells, along with the interaction between ROS productions and  $\text{Ca}^{2+}$  oscillation patterns during the signal crosstalk.

### 3.4 C4 plants and crassulacean acid metabolism

Dehydration avoidance mechanisms involve the maintenance of a high (favorable) plant water status during stress. Such strategies include minimized water loss (e.g., stomatal closure, reduced leaf area, and senescence of older leaves) or maximal water loss afforded by increased root proliferation at depths where the water is available. However, in determining transpiration rates, there is a trade-off between biomass accumulation and stress avoidance because the acquisition of photoassimilates is dependent on stomatal aperture and leaf area (Araus et al., 2008; Blum, 2009, 2011). Tolerance to low water potentials requires maintaining plant functions under limited water availability and/or the rapid recovery of plant water status and plant function after stress. Stomatal

closure is the best means to promptly reduce water loss because stomata can quickly close in response to stress and because stomata can promptly reopen if the stress has gone.

However, the responses of plants to drought observed under field conditions are generally much more complex than those measured under controlled environmental conditions because other factors accompanying water deficits influence the stress response. Water deficits lead not only to low tissue water status and cell dehydration but also to nutrient deprivation and osmotic stress and often additionally heat stress linked to decreased transpiration.

Biomass production is tightly linked to transpiration, WUE, and nitrogen accumulation. Blum (2011) has argued that breeding for high WUE under drought conditions will ultimately result in low-yielding genotypes with reduced transpiration and water use. Therefore, biomass production under most drought conditions can only be enhanced by maximizing soil moisture capture for transpiration, which also involves minimizing water loss by soil evaporation. Breeding for maximal soil moisture capture for transpiration is perhaps the most important target for yield improvement under drought stress conditions.

C<sub>4</sub> plants are considered to be better adapted to water-limiting environments because they are able to maintain leaf photosynthesis with closed stomata. C<sub>4</sub> plants have high WUEs, and the presence of the CO<sub>2</sub>-concentrating mechanisms makes C<sub>4</sub> photosynthesis more competitive in conditions that promote carbon loss through photorespiration, such as high temperatures, high light intensities, and decreased water availability (Edwards et al., 2004). C<sub>4</sub> photosynthesis is characterized by the presence of a metabolic CO<sub>2</sub> pump that concentrates CO<sub>2</sub> in the vicinity of the main enzyme of carbon dioxide fixation, ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco; Edwards et al., 2001, 2004). This confers a number of important advantages in terms of WUE because it allows high rates of photosynthesis to occur even when stomata are closed, while limiting flux through the photorespiratory pathway.

Many cacti, orchids, bromeliads, and other succulent plants with crassulacean acid metabolism (CAM) have stomatal activity patterns that contrast with those found in C<sub>3</sub> and C<sub>4</sub> plants. CAM plants open their stomata at night and close them during the day, exactly the opposite of the pattern observed in guard cells in leaves of C<sub>3</sub> and C<sub>4</sub> plants. At night, atmospheric CO<sub>2</sub> diffuses into CAM plants, where it is combined with phosphoenolpyruvate (PEP) and fixed into malate.

The ratio of water loss to CO<sub>2</sub> uptake is much lower in CAM plants than it is in either C<sub>3</sub> or C<sub>4</sub> plants. This is because stomata are open only at night when temperatures are lower and humidity is higher than during daytime conditions. Both lower temperatures and higher humidity contribute to a lower transpiration rate.

The main photosynthetic constraint to CAM metabolism is that the capacity to store malic acid is limited, and this limitation restricts the total amount of

$\text{CO}_2$  uptake. However, some CAM plants are able to enhance total photosynthesis during wet conditions by fixing  $\text{CO}_2$  via the Calvin cycle at the end of the day, when temperature gradients are less extreme. Under water-limited conditions, stomata only open at night.

Cladodes (flattened stems) of cacti can survive after detachment from the plant for several months without water. Their stomata are closed all the time, and the  $\text{CO}_2$  released by respiration is refixed into malate. This process, which has been called *CAM idling*, also allows the intact plant to survive for prolonged drought periods while losing remarkably little water.

The leaf-to-air vapor pressure difference that drives transpiration is much reduced at night, when both the leaves and air are cool. As a result, the WUEs of CAM plants are among the highest measured. A CAM plant may gain 1 g of dry matter for only 125 g of water used—a ratio that is 3–5 times greater than the ratio for a typical C3 plant.

CAM is very prevalent in succulent plants such as cacti. Some succulent species display facultative CAM, switching to CAM when subjected to water deficits or saline conditions. This switch in metabolism is a remarkable adaptation to stress, involving accumulation of the enzymes PEP carboxylase, pyruvate-orthophosphate dikinase, and NADP-malic enzyme, among others.

CAM metabolism involves many structural, physiological, and biochemical features, including changes in carboxylation and decarboxylation patterns, transport of large quantities of malate into and out of the vacuoles, and reversal of the periodicity of stomatal movements. Thus, CAM induction is a remarkable adaptation to water deficit that occurs at various levels in plants.

### 3.5 Conclusion

Stomata rapidly and slowly respond to a range of abiotic stress, regulating water status. Stomatal regulation of water status is prompt, low cost, reversible, and safe for plants under multiple environmental stresses. However, we can find gaps in knowledge about short-term and long-term responses and about responses to one stress and another stress. We need to fully understand the molecular mechanism of stomatal regulation of water status in order to breed plants tolerant to multiple environmental stresses.

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## 4 Root-associated stress response networks

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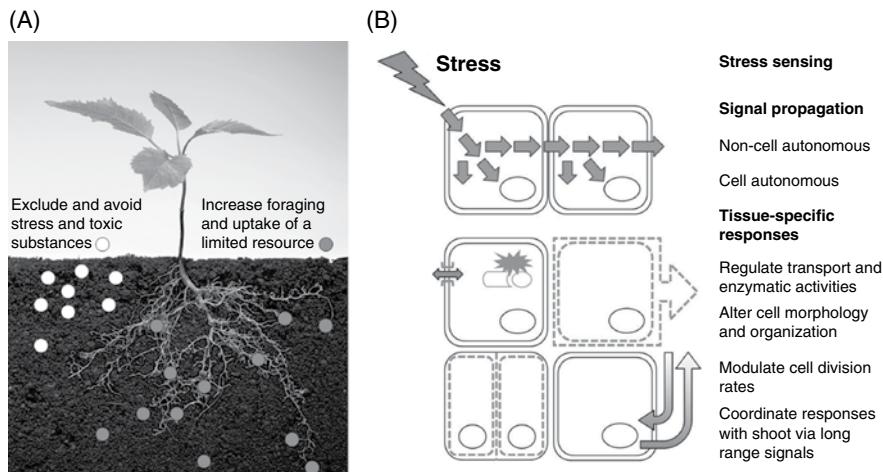
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### 4.1 Introduction

The root system is the major point of physical interaction between the plant and its growth substrate. In land plants, roots are required for physical anchorage and for uptake of water and nutrients from the soil. Variable edaphic conditions and weather patterns require that root systems sense and respond to deficiency in water and nutrients, as well as unfavorable conditions such as high content of toxic solutes (Lynch, 1995). Although roots generally do not directly participate in carbon fixation and reproductive processes, they contribute significantly to overall plant fitness and success. Roots must evaluate soil conditions to forage and secure resources for plant growth while excluding and avoiding accumulation of toxic substances (Figure 4.1A). A significant portion of photosynthetic energy is invested in root growth and establishment in order to secure soil resources. The total fine root surface area of terrestrial plants has been estimated to be matching, if not exceeding, the total photosynthetic leaf surface area across a variety of ecosystems (Jackson et al., 1997). In order to adapt to growth environments, root system architecture exhibits high developmental plasticity and genotype-specific variation (de Dorlodot et al., 2007). Root traits that enhance plant tolerance to abiotic stresses have been termed *traits of the second green revolution* and are a growing focus for crop improvement (Lynch, 2007; Den Herder et al., 2010).

Recent advances in studying root stress responses have been made possible by the use of (1) model genetic systems with tools to dissect root biology and stress responses at the molecular level (Benfey et al., 2010; Hirayama and Shinozaki, 2010), (2) new technologies to resolve these molecular changes at the cell-type-specific level (Lee et al., 2005), (3) high-content “omics” characterizations paired with advanced bioinformatics to integrate these molecular changes into response networks (Long et al., 2008; Lee et al., 2010; Urano et al., 2010), and (4) improved root phenotyping and modeling methods to



**Figure 4.1** (A) Schematic of root responses to abiotic stresses at the whole plant level. Overall root architecture changes to increase root proliferation toward limited nutrients and avoid stress and toxicity. Cellular and tissue-specific responses contribute to promote accumulation of water and key nutrients, and to exclude toxic substances. Gray circles represent nutrients in limited supply. White circles represent substances that incur stress or toxicity. (B) Schematic of root responses to abiotic stress at the cellular level. The initial sensing of the stress signal stimulates a network of cell autonomous and non-autonomous pathways to activate tissue-specific responses. Tissue-specific responses include regulating transport and enzymatic activities, altering cell morphology and organization in tissue layers, modulating the rates of cell division and expansion, and coordinating responses with other tissues in the root and shoot via a variety of long-range signals.

evaluate these responses in the context of the whole plant (Tardieu and Tuberosa, 2010; Zhu et al., 2011). These advances have built on physiological studies to make functional predictions and construct a systems view of root stress responses. The emerging picture of root stress responses is an aggregate of a highly coordinated set of stress and tissue-specific networks that give rise to root system adaptation to the environment.

A generalized view of the response to abiotic stress in the root can be outlined as follows (Figure 4.1B): the initial stress-sensing mechanism in the root triggers rapid cellular responses to regulate gene expression and function (Monshausen and Gilroy, 2009). The initial stress signal is propagated through a network of overlapping cell autonomous and non-autonomous signals, such as calcium, reactive oxygen species (ROS), phospholipids, amino acids, and phytohormones such as auxin (De Tullio et al., 2010; Van Norman et al., 2011). Tissue-specific responses are activated in order to promote accumulation of water and key nutrients and to exclude toxic substances. These tissue-specific responses include regulating transport and enzymatic activities, altering cell morphology and organization in tissue layers, and modulating the rates of cell division and expansion. Changes in cell division and expansion modify root

organization and growth to give rise to adaptive changes in root architecture (Lynch, 2011). Stress responses in the root are also coordinated with stress and nutritional cues from the shoot via a variety of long-range signals (Schachtman and Goodger, 2008), including a simple hydraulic signal (Christmann et al., 2007), and phytohormones such as abscisic acid (ABA) and cytokinin (Christmann et al., 2006; Ha et al., 2012).

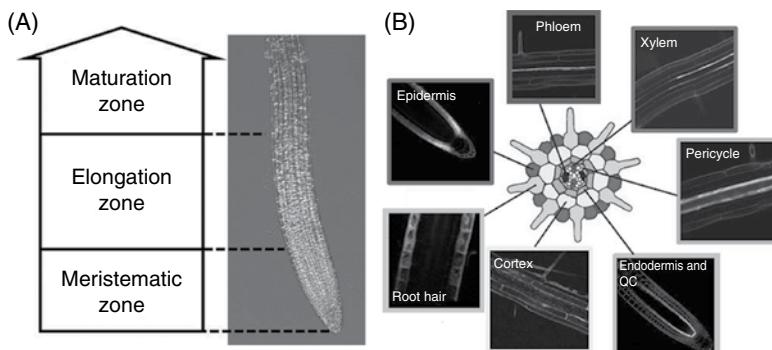
Root responses to abiotic stresses are dynamically regulated at the molecular level and coordinated across cell types and organs to modulate root physiology and growth (Galvan-Ampudia and Testerink, 2011; Peret et al., 2011; Gutierrez, 2012; Jones and Ljung, 2012). Ultimately a stress response can be reduced to a change in gene activity. This regulation can impact gene activity at various stages, such as activation or silencing of transcription, transcript stability, translation initiation or inhibition, protein turnover, and/or protein modifications. However, the functional significance of a gene must be considered in the context of its interacting partners, the gene network within the tissue-of-interest, and the role of the tissue in the context of the physiology of the plant. In this chapter, we will (1) review the organization and function of the plant root in relation to a variety of abiotic stress responses, (2) summarize recent progress in resolving root-associated stress response networks, (3) discuss how these networks give rise to phenotypic plasticity of the root system, and finally (4) speculate on strategies to manipulate these stress response networks for crop improvement.

## 4.2 Root organization

In order to dissect stress response networks in the root, an understanding of the basic organization and function of the root system is necessary. We will begin by briefly defining the tissue organization of the root and highlighting tissue-specific functions, signaling, and developmental pathways that are relevant to root stress responses. Readers may wish to refer to recent reviews for more extensive descriptions of root growth and development (de Dorlodot et al., 2007; Osmont et al., 2007; Jones and Ljung, 2012; Perilli et al., 2012; Petricka et al., 2012).

### 4.2.1 Root developmental zones

The root can be broadly classified into three developmental zones based on cellular division, elongation, and differentiation activities. In the model plant *Arabidopsis*, the meristematic, elongation, and maturation zones are readily distinguishable along the longitudinal axis of the root (Figure 4.2A; Beemster and Baskin, 1998; Perilli et al., 2012). In other plant species such as maize, these regions are overlapping, with activities of cell division, elongation, and



**Figure 4.2** (A) Developmental zones in the *Arabidopsis* root are arranged along the longitudinal axis in the following order from the root tip: meristematic, elongation, and maturation zone. (B) Tissue types in the *Arabidopsis* root are arranged radially. Longitudinal images of tissue-specific marker lines were captured by confocal microscopy. Note that images for the epidermis and endodermis marker lines were captured in the meristematic and elongation zones, images for the root hair marker line were captured in the elongation zone prior to root hair maturation, and images for cortex, phloem, xylem, and pericycle marker lines were captured in the maturation zone. Cellular boundaries were visualized by light propidium iodide staining. Strong fluorescence is observed in GFP-marked cells.

maturation peaking in order from the root tip toward the root-shoot junction (Ishikawa and Evans, 1995).

Root growth responses occur primarily at the tip. New cells are produced from a group of actively dividing initials in the root apical meristem (Scheres, 2007). These stem cells surround an organizing center of slowly dividing cells called the *quiescent center*. The stem cells divide to give rise to distinct radial tissue layers of the root in the proximal meristem (see next section). Cells in the proximal meristem divide several times before transiting to the elongation and differentiation zones (Perilli et al., 2012). The size of the meristem is a major determinant of the rate of root growth (Beemster and Baskin, 1998) and is controlled by the balance between auxin and cytokinin (Dello Ioio et al., 2008). This hormonal balance is also modulated by nutritional cues. The PLETHORA (PLT) subfamily of the APETALA2 (AP2) family of transcription factors is required for root apical meristem maintenance and patterning (Galinha et al., 2007). Auxin coordinates both PLT and redox pathways to regulate root apical meristem activity (De Tullio et al., 2010). Ethylene signaling also regulates cell division patterns in the meristem (Ortega-Martinez et al., 2007) and interacts with redox pathways (De Tullio et al., 2010). The transitional boundary between the meristematic and elongation zones, also termed the *transitional zone*, has been shown to be specified by a gradient of ROS (Tsukagoshi et al., 2010), which is regulated by hormonal, developmental, and stress response pathways (Mittler et al., 2011). Under stress, the root meristem can accumulate ROS and terminate (De Tullio et al., 2010); the root apical meristem can reorganize

to cease root growth at the tip, and lateral roots can be initiated to redirect growth (Shishkova et al., 2008; De Tullio et al., 2010; Galvan-Ampudia and Testerink, 2011).

In the elongation zone, cells undergo non-isodiametric cell expansion along the longitudinal axis of the root. This rapid elongation is driven by vacuolar expansion due to water uptake and is radially restricted by cortical microtubules and cellulose microfibrils (Burk and Ye, 2002; Sedbrook and Kaloriti, 2008). Root elongation is coordinated by auxin and ethylene (Muday et al., 2012). Maintenance of root elongation under dehydration and osmotic stress has been observed in many plant species including maize (Sharp et al., 1988). This maintenance occurs in the zone of rapidly elongating cells in maize root tips and is mediated by osmotic adjustments and cell wall loosening (Sharp et al., 2004). The elongation zone also controls root bending by differential cell expansion. Root gravitropic bending is regulated by auxin transport, whereas hydrotropic bending is in part mediated by ABA and interferes with gravitropism (Takahashi et al., 2009). Stress avoidance by agravitropic root bending has been observed under high salinity and is mediated through interfering with auxin transport (Sun et al., 2008; Dinneny, 2010).

Root tissue differentiation begins in the elongation zone and peaks in the differentiation zone. In the differentiation zone, cells in each radial tissue acquire distinct cell fates (see below). In *Arabidopsis*, the differentiation zone is defined as the region where cell elongation ceases and trichoblast differentiation into hair cells is detected. Except for pericycle cells, all cell types eventually become terminally differentiated in maturity. Pericycle cells maintain the ability to initiate lateral roots (see below). Generally, water and mineral uptake activities are concentrated in younger regions of the root and these transport activities diminish with increasing maturity (Volder et al., 2005). In mature regions of the root, overall permeability to water and solutes is reduced by secondary thickening in multiple tissue layers including exodermal, endodermal, and vascular tissues (Wasson et al., 2012).

#### 4.2.2 Root tissue types

Root tissues are organized in radially concentric cylinders along the length of the root (Figure 4.2B). At the center of the root is the stele, which comprises the vascular cylinder encircled by the pericycle. The vascular cylinder includes xylem and phloem tissues and their cellular components. Differentiation of the vascular tissues is controlled by a complex network of signals and receptors from a variety of pathways, including radial and polarity patterning, plant hormones (cytokinin, auxin, and brassinosteroids), small signaling factors, and downstream transcriptional networks (Lehesranta et al., 2010). Xylem vessels transport water, minerals, and metabolites from the roots to the shoots by transpiration stream. Root-to-shoot signals that communicate root status are also transported by the xylem.

For example, under osmotic stress, ABA is produced in the roots and transported to the shoots to regulate stomatal conductance in leaves (Thompson et al., 2007; Schachtman and Goodger, 2008). Phloem transports sucrose and other metabolites from source to sink tissues. Shoot-to-root signals transported by the phloem can communicate the nutritional and stress status of the plant. For example, glutamate has been proposed to be the systemic signal that communicates nitrogen status from the shoot to regulate root architecture (Forde and Walch-Liu, 2009). Some long-range signals are produced in both the root and the shoot and can be transported both shootward and rootward by xylem and phloem, respectively, to coordinate plant growth and metabolism. For example, cytokinins are detected in both xylem and phloem exudates but carry distinct side chain structures that distinguish the direction of transport and signaling (Sakakibara et al., 2006). The size of the vascular cylinder has been shown to be responsive to drought, salinity, and ABA signals (Burssens et al., 2000; Chen et al., 2006). An overall increase in vascular tissue and root diameter to lower axial resistance has been correlated with improved water uptake and drought resistance in rice (Nguyen et al., 1997; Wasson et al., 2012).

The pericycle encircles the vascular cylinder and is the key tissue for regulating root architecture. In the model dicot *Arabidopsis*, lateral roots initiate from the xylem pole pericycle, whereas in monocots such as maize and rice, lateral roots initiate from the phloem pole. Sites of lateral root initiation, termed *prebranch sites*, are specified by a periodic oscillation network (Moreno-Risueno et al., 2010). Lateral root development initiates in an asymmetric cell division in the pericycle and is followed by a series of ordered cell divisions to produce a new root meristem (Malamy and Benfey, 1997). Auxin can activate cell cycle progression in founder cells in the pericycle to initiate lateral root primordia (Himanen et al., 2002). Lateral root development is modulated to evade stress and to forage for limited resources such as water, low nitrogen, and low phosphorus (Osmont et al., 2007). For example, lateral root primordia emergence in *Arabidopsis* is repressed by drought stress (Deak and Malamy, 2005) and induced by local nitrate-rich patches in an auxin dependent manner (Walch-Liu et al., 2006). Split root experiments in many plant species, including *Arabidopsis* and rice, have demonstrated that local and systemic signals coordinate lateral root growth (Remans et al., 2006b; Ruffel et al., 2010; Mirzaei et al., 2011).

The endodermis is the boundary cell layer between the stele and outer root cell layers. The endodermis regulates water and nutrient transport between the ground tissue and the vasculature and also plays an integral role in regulating root growth and patterning (Miyashima and Nakajima, 2011). The cortex and endodermis both arise from a common progenitor of the ground tissue, the cortex/endodermal initial cell, and are specified by the GRAS family transcription factors *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*; Di Laurenzio et al., 1996; Helariutta et al., 2000), which are required for meristem maintenance and root growth. The endodermis blocks apoplastic movement of water and

solutes by developing a Casparyan strip, which has been associated with lignin and suberin deposits on the anticlinal cell walls (Enstone et al., 2002; Chen et al., 2011). Under salt stress, enhanced development of the Casparyan strip has been observed in maize roots (Karahara et al., 2004). Recently, the primary component of the Casparyan strip in Arabidopsis has been identified as lignin, whereas suberin is produced later in root development (Naseer et al., 2012). Nonetheless, other evidence still demonstrates that suberin plays a role in controlling root permeability. Increased suberin in the root endodermis has been shown to decrease water uptake and transport from the root to shoot and to increase wilting resistance by reducing water leakage from the root (Baxter et al., 2009). The endodermis cell body also controls transport of water and selected solutes into and out of the vascular cylinder by selective channels and transporters (Baxter et al., 2009). An increase in the number of endodermal cell layers has been correlated with adaptation to high salt environments (Inan et al., 2004).

The cortex forms the bulk of the ground tissue in most plant species. Cortex cells exhibit high plasticity in morphology and function during stress responses. For example, under high salinity, microtubules in Arabidopsis cortex cells disassemble to disrupt anisotropic expansion, resulting in radial swelling (Wang et al., 2011). Salt-stressed Arabidopsis roots exhibit chloroplast differentiation in the cortex, which has been hypothesized to be a response to ROS accumulation (Dinneny et al., 2008). In rice, the number of cortical cell layers varies between root types and growth conditions as a developmental adaptation to cultivation under submergence (Pauluzzi et al., 2012). Under hypoxic conditions, rice cortex cells form aerenchyma, which are air-filled cells, to limit water uptake and improve oxygenation (Coudert et al., 2010). Formation of cortical aerenchyma is also induced by low availability of water and nutrients and has been correlated with drought tolerance in maize (Zhu et al., 2010; Lynch, 2011). This drought tolerance has been attributed to a metabolic advantage in substituting living cortical tissue for air space, which can reduce energy costs for soil exploration under limited resources (Lynch, 2011). In plants such as maize and rice, older roots and roots under stress form a distinctive cortex-derived cell layer beneath the epidermis called the *exodermis* (Enstone et al., 2002). This structure is notably absent in the model plant Arabidopsis. Exodermal cells accumulate secondary cell wall deposits between cells that form a Casparyan strip, which creates a physical barrier to limit water and solute permeability between the root and the soil. Similar to the endodermis, the exodermis Casparyan strip is associated with salt response and has also been correlated with salt stress tolerance among rice varieties (Cai et al., 2011).

The epidermis forms the outer layer of most roots and consists of both root hair and non-hair cells (Dolan, 2006). Root hair cells extend the root surface area and enhance contact with the soil for water and nutrient uptake, as well as for the release of exudates (Wasson et al., 2012). Many transporters for mineral

nutrients are expressed in the epidermis to regulate intake, exclusion, and excretion in response to nutritional cues (Gilroy and Jones, 2000). Differentiation of root hair cells is partially specified by positional cues from the cortex (Dolan, 2006). Root hair growth is regulated by positive feedback between calcium and ROS signals (Monshausen et al., 2007; Takeda et al., 2008). Both root hair initiation and elongation are regulated by abiotic stress via auxin and ethylene signals (Muday et al., 2012). Root hair length and density are adaptive traits that are responsive to environmental conditions. For example, in maize and Arabidopsis, root hair length increases under phosphorus deprivation and has been associated with phosphorus use efficiency (Bates and Lynch, 2000; Zhu et al., 2005; Lynch, 2011). A transient reduction in root hairs is also observed under salt stress (Dinneny et al., 2008).

The root cap protects the growing root tip, and its cells are continuously shed and regenerated as the tip grows (Iijima et al., 2008). The root cap reduces resistance to root penetration during soil exploration. In addition, statoliths in the columella root cap are involved in sensing the gravity vector (Morita and Tasaka, 2004). The gravity signal is transmitted via auxin transport through the lateral root cap to elongating cells in the epidermis to control root angle and bending (Swarup et al., 2005). The lateral root cap has also been implicated in moisture sensing in hydrotropic root bending (Taniguchi et al., 2010). Under salt stress, an ion dependent mechanism has been shown to mediate statolith degradation in the columella root cap, as well as redistribution of auxin, which result in agravitropic root bending linked to stress avoidance (Sun et al., 2008; Dinneny, 2010).

### 4.3 Systems analysis of root-associated stress responses

A systems approach has been used to analyze stress response networks across levels of root organization. Systems biology uses systematic genome-scale datasets to construct and test hypotheses (Chuang et al., 2010). Large-scale datasets can be collected for the genome, transcriptome, proteome, and/or metabolome to describe temporal dynamics, developmental patterns, and tissue-specific functions in response to abiotic stresses.

In the model plant *Arabidopsis*, tissue-specific promoters have been exploited to enable high throughput separation of specific cell types and their contents by a variety of methods. These include fluorescent activated cell sorting (FACS) of protoplasted tissue-specific fluorescent reporter lines (Birnbaum et al., 2005), isolation of nuclei tagged in specific cell types (INTACT; Deal and Henikoff, 2011), and immunopurification of cell-type-specific mRNA-ribosome complexes (Mustroph et al., 2009). The development of laser capture microdissection (LCM) has also facilitated the collection of cell-type-specific datasets in other plant species, including maize and rice, without requiring the production

of transgenic marker lines (Nakazono et al., 2003). Currently, transcript profiling methods are applied to materials collected by all the above methods and provide the highest amount of interpretable data that can be associated with genome-wide regulatory mechanisms. In FACS and LCM, isolated tissues can also be analyzed by high content methods for RNA transcripts, protein, and metabolites. Recent advances in next generation sequencing technologies have further increased the sensitivity of transcript profiling assays to detect transcripts and alternative splice forms from low levels of starting material (Ramskold et al., 2012a, 2012b). Furthermore, next generation sequencing methods can accommodate *de novo* transcript analysis in plant species without a reference genome sequence or extensive expressed sequence tag (EST) information (Schmid et al., 2012), which can facilitate the analysis of crops and stress-tolerant extremophiles.

Although these “omics” datasets are a rich resource of information, interpreting large-scale datasets to extract meaningful information can be challenging. In order to detect overall trends in stress responses and reduce the complexity in large datasets, a clustering analysis is often used to group genes and their responses on the basis of co-expression patterns and functional categories (Orlando et al., 2009). Dynamic modeling methods can also be used to characterize the kinetics of time-dependent responses. Co-regulated gene sets are often used to infer interacting genetic pathways. Gene ontology (GO) terms are a set of standardized functional classifiers that aid in interpreting biological significance of responses.

The extraction of value from systems datasets depends on bioinformatic methods to integrate and construct response networks for further hypothesis generation and testing (Lee et al., 2010; Hwang et al., 2011; Petricka and Benfey, 2011). In gene networks, nodes represent genes and edges can represent known genetic interactions and gene regulatory pathways. Edges can also be predicted based on co-expression, biochemical interactions, phenotypic classification, or orthologous networks. Highly connected genes, termed *hubs*, are often predicted to be critical points of control in these networks. Using a “guilt by association” approach, edges indicate a shared biological feature between connected genes that can be used to predict a functional relationship. These relationships can then be continually refined by iterative hypothesis generation and experimental testing. While the “guilt by association” approach has been widely used for predicting gene function, recent work has cautioned that the robustness of biological networks depends on a small population of hubs, and only a subset of the edges from these hubs encode functional information (Gillis and Pavlidis, 2012). Identification of these functional edges is dependent on the specificity and relevance of the incorporated datasets (Bhat et al., 2012). Thus, high-resolution data, at relevant stress conditions and tissue types, are important to understanding gene function in stress responses.

#### 4.4 Root-tissue to system-level changes in response to stress

The complexity of root stress responses and the diversity of tissue functions require resolution of specific stress responses at the cell-type-specific level in order to reconstruct a systems view of the stress response networks. A variety of methods have been used to elucidate root stress response networks, including genetic screens, large-scale gene expression profiling, analysis of cis-elements and transcription factors, and genome-wide association studies with root phenotyping approaches.

The detailed mechanisms of various abiotic stress responses in the whole plant and strategies for enhancing tolerance to these stressors are covered in other chapters of this book. Here we will focus on tissue-specific responses to nitrogen and salinity as two examples that illustrate root stress responses involved in (1) increasing foraging and uptake of a limited resource, and (2) excluding and avoiding a substance that is toxic at high levels. For each stress condition, we will first review root-associated responses and highlight recent advances in constructing root-associated response networks.

##### 4.4.1 Nitrogen

Nitrogen (N) is required for key biological building blocks, including amino acids, nucleic acids, lipids, and many important metabolites, and is the mineral nutrient in highest demand in the plant. Although organic N constitutes the bulk of the total N in soil (> 98%), it does not constitute the bulk source of plant N. Instead, plant N is typically obtained through root uptake of nitrate and ammonium (Crawford and Forde, 2002; Kraiser et al., 2011), although some organic N can be taken up by roots as amino acids or peptides (Nasholm et al., 2009). A small subset of plant species, such as legumes, can form symbiotic relationships with N-fixing microbes to directly utilize atmospheric N. Nitrate is the most abundant inorganic N source in aerobic soils, and its concentrations range between a few hundred micromolar to 70 mM, with ammonium concentrations averaging at about one-eighth of the nitrate concentration (Garnett et al., 2009; Dechorganat et al., 2011). In this section, we will focus our discussion on soil inorganic N, with a proportional emphasis on nitrate.

Most soil inorganic N is fixed from atmospheric N through microbial, atmospheric, and industrial processes, or converted from soil organic matter by microorganisms. Because inorganic N is highly mobile in solution, its concentration is also affected by local variations in water content and physical properties of the soil. The spatially and temporally variable distribution of soil N requires the plant to sense N conditions and make adaptive changes to enhance N use under N deficiency and concentrate N uptake in localized N-rich patches.

N deficiency results in stunted growth and a decrease in plant fitness and reproductive success. In particular, an N deficit can be easily observed as chlorosis due

to a decline in photosynthetic machinery. In agriculture, N deficiency results in a dramatic reduction in biomass and seed yield. N fertilization is widely practiced in order to increase and stabilize soil N content. Worldwide consumption of N fertilizers has been steadily rising over the last 50 years and has reportedly reached 105 million tons/year in 2009 (FAOSTAT, 2012). However, crop utilization of N fertilizers has been reported to be as low as one-third of the input in cereals, with the remaining two-thirds being lost to the environment (Raun and Johnson, 1999). Inorganic N fertilizers are highly diffusible in solution, and the mobility of nitrate in soil is particularly high due to its weak propensity to form surface complexes with soil minerals (Dechorganat et al., 2011). Excess N fertilizers are primarily lost by nitrate leaching into the soil, ammonia volatilization, or microbial denitrification (Vitousek et al., 1997; Ju et al., 2009). These processes result in economic losses to farmers and serious environmental impacts. Nitrate leaching through the soil can deplete minerals such as calcium and potassium, which can lead to crop nutrient deficiencies. Excessive nitrate accumulation in fresh or coastal waters has led to eutrophication and acidification of aquatic systems. Volatilization of nitrogenous gases can lead to production of greenhouse gases and has been linked to overall alteration of the global N and C cycles. Efforts to minimize N loss to the environment and maintain yield have involved improving N fertilizer management (Ju et al., 2009) as well as increasing N acquisition and assimilation by crop plants (Good et al., 2004). Although N deficiency is not generally encountered in fertilized agricultural soils, low N conditions are increasing in occurrence with progressive depletion of topsoils, particularly in low-input farms where soil nutrients are not replenished fully after each harvest (Vitousek et al., 2009). With rising energy costs, fertilizer costs are becoming less affordable to farmers. In addition, the increasing demands of a growing world population for food and biofuel crops are driving the expansion of agricultural practices into marginal lands. Thus a better understanding of the plant response to N deficiency is required to improve plant N use efficiency (NUE) for sustainable agriculture (Kant et al., 2011). Strategies for improving NUE can be designed based on knowledge gained from N-response networks (Kant et al., 2011; Gutierrez, 2012). Root-specific strategies to improve NUE have also been proposed (Garnett et al., 2009).

#### *Root responses to N*

The root response to N conditions involves modulation of cellular N transport and assimilation to enhance N use under systemic N deficiency, as well as modification of root system architecture to continue soil exploration for local N-rich patches, which are the coordinated results of sensing and responding to low and high N conditions.

Roots take up N from the soil primarily as nitrate and ammonium (Crawford and Forde, 2002; Kraiser et al., 2011). Both low-affinity and high-affinity

transport systems (LATS and HATS) have been described for nitrate and ammonium (Crawford and Forde, 2002; Ludewig et al., 2007). Inducible HATS for both nitrate and ammonium respond to systemic signals, are induced by N starvation signals, and are repressed by feedback inhibition. For example, the high-affinity nitrate transporter *NRT2.1* is transcriptionally upregulated under N deficiency and downregulated in response to high N supply. Specifically, upregulation of the high-affinity nitrate transporters *NRT2.1* and *NRT2.4* under low N has been reported to occur primarily in the root epidermis, particularly in root hairs (Wirth et al., 2007; Kiba et al., 2012). The low-affinity ammonium transporter AMT1;3 and high-affinity ammonium transporter AMT1;5 are also transcriptionally induced by N deficiency (Yuan et al., 2007). In addition, an inducible HATS for nitrate responds to local stimulation and concentrates nitrate uptake in local nitrate-rich patches (see below; Crawford and Forde, 2002). Consistent with the expression of transporters, root hair length has been observed to be negatively correlated to N supply.

After nitrate is taken up by the root, much of it is loaded into the xylem vessels and translocated to different parts of the plant for metabolism, storage, or as a long-range signal (Dechorganat et al., 2011). In the root, nitrate can be stored to high levels in vacuoles or reduced to nitrite and further to ammonium by nitrate reductase and nitrite reductase, respectively (Crawford and Forde, 2002). Ammonium is toxic at high levels and is preferentially assimilated upon uptake (Bloom et al., 2012). Ammonium that is taken up by transporters or reduced from nitrate is further assimilated through the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. The amount of N assimilation that takes place in the root varies significantly between plant species and growth environments (Garnett et al., 2009). Nevertheless, these storage and assimilation processes are tightly regulated by nitrate concentrations, plant N status, and carbon-nitrogen (CN) balance (Kant et al., 2011).

In addition to cellular changes in nutrient uptake and metabolism, many plant species have demonstrated the ability to increase root proliferation to explore local nutrient-rich patches (Hodge, 2004). Similarly, a localized high nitrate supply stimulates lateral root initiation and growth in many plant species, including *Arabidopsis* and barley (Walch-Liu et al., 2006). Interestingly, the increase in lateral root growth in response to nitrate supply has been attributed to increased rates of cell division rather than cell elongation (Zhang et al., 1999). In addition, lateral root proliferation is regulated by systemic signals from the shoot: N deficiency increases lateral rooting, whereas N sufficiency suppresses lateral rooting. In split root experiments, lateral root proliferation in an N-rich zone is further enhanced by partial exposure of the root system to low N conditions, suggesting that long-distance signals are involved in communicating N status between portions of the root system (Remans et al., 2006a; Ruffel et al., 2011). These long-range signals can be mediated by nitrate itself (Zhang et al., 1999), or by the N assimilation intermediate glutamate (Forde and

Walch-Liu, 2009). Changes in root growth and architecture are also in part coordinated with shoot metabolic and developmental processes through auxin (Zhang et al., 1999), cytokinin (Sakakibara et al., 2006), and ABA (Signora et al., 2001).

#### *Molecular dissection of N responses*

A number of genetic screens have been conducted in Arabidopsis to elucidate components of the N sensing and signaling pathway. A forward genetics screen for mutants resistant to a chlorine analog of nitrate, chlorate (*chl*), identified a number of alleles of the nitrate transporter *NRT1.1* as well as mutants affected in nitrate reductase activity (Oostindier-Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1982; Cheng et al., 1988; Tsay et al., 1993). *NRT1.1* was subsequently implicated as a nitrate sensor based on its requirement for a number responses to nitrate supply, including local lateral rooting (Remans et al., 2006a) and induction of the high-affinity nitrate transporter *NRT2.1* (Wang et al., 2003; Muños et al., 2004; Krouk et al., 2006). Upregulation of *NRT2.1* under N limitation is also in part regulated by *NRT1.1*. Consistent with a role in local nitrate sensing and uptake, *NRT1.1* is expressed in the root cap and in the epidermis across different root developmental zones (Huang et al., 1996). In addition, *NRT1.1* is also expressed in the endodermis in mature tissues, which may be involved in sensing systemic N status from nitrate content in the stele. *NRT1.1* was found to be a dual affinity transporter as it switches from low- and high-affinity states based on phosphorylation of the amino acid T101 (Liu and Tsay, 2003). Recently the dual transporter/receptor (transceptor) role of *NRT1.1* was uncovered by the characterization of a novel allele, *chl1-9*, which disrupts its role in nitrate transport but retains its role in nitrate sensing, thus indicating that these two roles are separable (Ho et al., 2009). Under low nitrate conditions, *NRT1.1* is activated as a high-affinity nitrate transporter by T101 phosphorylation by a CBL-INTERACTING PROTEIN KINASE (CIPK23) and *NRT2.1* is expressed. Under high nitrate conditions, *NRT1.1* is not phosphorylated at T101 and it functions at a low-affinity state and further induction of *NRT2.1* expression above the *NRT1.1* high-affinity state occurs. Nitrate transport function in the *chl1-9* mutant protein was found to be strongly reduced, but the primary response of *NRT2.1* induction was not affected, indicating that *NRT1.1* functions as a transceptor. Another CBL-INTERACTING PROTEIN KINASE, CIPK8, has been shown to activate low-affinity nitrate transport in a similar manner, but no target has yet been identified (Hu et al., 2009). Interestingly, the epidermal high-affinity ammonium transporter AMT1;1 also exhibits phosphorylation-dependent switching between high- and low-affinity states (Lanquar et al., 2009). It would be interesting to find out if the transceptor model may extend to other N substrates and transporters. In addition, other N transporters have also been implicated in N sensing or signaling. The ammonium transporter

AMT1;3 has been shown to mediate ammonium-induced lateral root branching in a manner independent of its role in ammonium transport function (Lima et al., 2010). *NRT2.1* has been implicated in the N signaling pathway regulating lateral root initiation in response to low N conditions, although contrasting evidence from loss-of-function mutants indicates both stimulating and repressive roles (Little et al., 2005; Remans et al., 2006b), possibly due to assay conditions that may reflect distinct responses to local and systemic cues. The precise roles of these N transporters in N sensing remain to be uncovered.

Downstream of N perception, molecular studies have uncovered a number of players in the N signal transduction pathway. Recently, one member of an Arabidopsis gene family homologous to nodule initiation genes in legumes, *ODULE INCEPTION-LIKE PROTEIN 7 (NLP7)*, was found to be a positive regulator of nitrate sensing and assimilation genes (Castaings et al., 2009). NIN proteins contain BASIC REGION/LEUCINE ZIPPER TRANSCRIPTION FACTOR (bZIP) domains that can dimerize with other bZIP transcription factors. Similar to *NRT1.1*, *NLP7* was also found to be expressed in the root cap and epidermis, as well as near the vasculature toward the distal elongation and maturation zones. A forward genetic screen for mutants defective in the primary nitrate response of *NRT2.1* induction has also identified alleles of both *NRT1.1* and *NLP7* (Wang et al., 2009). Further molecular evidence will ascertain if *NLP7* functions downstream of *NRT1.1* in the primary N response of transcriptional activation of *NRT2.1*.

Several N-responsive genes that regulate N assimilation have been identified in Arabidopsis. These are generally broadly expressed across root and shoot tissues and have been implicated in modulating plant nutritional status. For example, The RING-type ubiquitin ligase gene *NITROGEN LIMITATION ADAPTATION (NLA)* was isolated in a genetic screen for mutants that failed to accumulate anthocyanins and senesced early under N deficiency, in part due to compromised N remobilization and metabolism (Peng et al., 2007b). Mutants in the bZIP transcription factors, *elongated hypocotyls 5 (hy5)* and *hy5-homology (hyh)*, were found to be impaired in light-mediated enhancement of the expression of the nitrate reductase gene, *NIA2*, and repression of the nitrate transceptor *NRT1.1* (Jonassen et al., 2009). *HY5* and *HYH* are likely to mediate interaction between light, carbon, and N nutritional cues. Three members of the *LATERAL ORGAN BOUNDARY DOMAIN* family of transcription factors, *LBD37*, *LBD38*, and *LBD39*, are negative regulators of anthocyanin biosynthesis, as well as N uptake and metabolism (Rubin et al., 2009). In Arabidopsis, overexpressing any of these three LBD transcription factors resulted in reduced amino acid levels and overall stunted growth, whereas loss of function mutations resulted in increased N uptake and assimilation. Currently, most characterization of these genes has been conducted at the whole plant level. Further tissue-specific dissection of the function of these genes may illuminate N-responsive assimilation responses in roots and allow the development of enhanced strategies for increasing NUE.

The first N responsive transcription factor, *ARABIDOPSIS NITRATE REGULATED 1 (ANR1)*, was identified in a screen for nitrate regulated genes in roots (Zhang and Forde, 1998). *ANR1* is required for enhanced lateral root growth in response to local nitrate-rich patches, and its expression has been shown to be induced by both nitrate resupply to nitrate-starved plants (Zhang and Forde, 1998) and N starvation of N sufficient plants (Gan et al., 2005). *ANR1* expression also overlaps with *NRT1.1* and is responsive to nitrate signals in an *NRT1.1* dependent manner, indicating that *ANR1* is likely to function downstream of *NRT1.1* (Remans et al., 2006a). However, root-specific overexpression of *ANR1* induced lateral root growth, but this effect was still responsive to nitrate stimulation, indicating that nitrate regulates other functions downstream and/or in parallel with the *NRT1.1/ANR1* pathway to regulate lateral root growth (Gan et al., 2012). *ANR1* belongs to the type-II MADS box transcription factor family, which includes a well-characterized subset of family members that heterodimerize to activate transcription that results in floral organ identity specification. Other root-expressed MADS-box transcription factors that are N responsive have been identified (Gan et al., 2005), and an auxin-regulated root-expressed MADS-box transcription factor has been found to regulate root apical meristem activity (Tapia-Lopez et al., 2008), but thus far no direct evidence has been found for genetic or biochemical interaction with *ANR1*.

Phytohormone signals such as auxin, cytokinin, and ABA are modulated via altering hormone levels and/or signaling pathway components. Reduced auxin content in maize roots has been correlated with inhibition of root growth under high N supply (Tian et al., 2008). In Arabidopsis, nitrate can directly regulate auxin levels by inhibiting *NRT1.1*-mediated auxin transport (Krouk et al., 2010a). Local induction of lateral roots by nitrate is mediated in part by AUXIN RESISTANT 4 (AXR4; Zhang et al., 1999), which is an accessory protein for auxin transport (Dharmasiri et al., 2006). Nitrate induces cytokinin biosynthesis by transcriptional upregulation of *ISOPENTENYL SYNTHASE 3 (IPT3)*, which controls the rate limiting step in cytokinin biosynthesis (Takei et al., 2004). In both Arabidopsis and maize, N supply has been found to induce expression of a subset of cytokinin response regulators through upregulation of cytokinin biosynthesis (Sakakibara et al., 2006). In addition, ABA biosynthesis and signaling are required for high nitrate repression of lateral root growth (Signora et al., 2001). While cytokinin and auxin levels and signaling pathways have been shown to coordinately regulate meristematic activity in the root (Dello Ioio et al., 2008), how all of these nitrate-regulated long-range signals interact to control both primary root elongation and lateral root growth, as well as coordinate shoot responses, remains largely unknown.

#### *Systems analysis of N response*

Evidence from the molecular studies builds a model of nitrate sensing by one or more receptors, including *NRT1.1*, which activates downstream responses that

alter N uptake, metabolism, and lateral root growth. However, the identification of the components in these pathways remains incomplete (see above), and how the signal is transduced from the receptor to transcription factors to downstream cellular processes is largely unknown. In order to build a more comprehensive view of the N signaling networks, several groups have conducted systematic queries of the N response. The most widely used approach has been transcriptional profiling microarrays (Wang et al., 2003; Munos et al., 2004; Palenchar et al., 2004; Scheible et al., 2004; Wang et al., 2004; Lian et al., 2006; Bi et al., 2007; Gutierrez et al., 2007; Peng et al., 2007a; Gifford et al., 2008; Gutierrez et al., 2008; Vidal and Gutierrez, 2008; Krouk et al., 2009). More recently, these global queries have also been expanded to profiling miRNAs, proteins, and metabolites (Tschoep et al., 2009; Vidal et al., 2010; Kusano et al., 2011; Wang et al., 2012). These approaches have also been applied to a variety of plant tissues using a variety of N treatments, including N depletion and/or N addition. In general, N addition and starvation treatments have found enriched GO categories such as altered N transport, C and N metabolism, and stress responses, as well as auxin and cytokinin signaling.

Characterization of *Arabidopsis* root transcriptional responses to C/N interactions has found metabolic, protein degradation, and auxin signaling components overrepresented among the differentially regulated genes (Gutierrez et al., 2007). In addition, a network model has predicted that the master regulator of the circadian clock, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, is also a central regulator of N response, in particular to the N assimilation intermediate glutamate (Gutierrez et al., 2008). *CCA1* directly regulates the expression of a transcription factor, *bZIP1*, to control expression of key N assimilation enzymes *ASPARAGINE SYNTHETASE 1 (ASN1)*, *GLUTAMINE SYNTHASE 1.3*, and *GLUTAMATE DEHYDROGENASE 1*. *bZIP1* regulates nutritional status and modulates C and N metabolism (Obertello et al., 2010). The *CCA1/bZIP1* pathway provides a point of integration between day/night cycles and C and N status in regulating N metabolism (Gutierrez et al., 2008).

A time series dataset has further uncovered a sequence of plant responses to N signaling: initial induction of ribosomal genes to increase protein synthesis, followed by upregulation of N transport and metabolism, and finally interactions with hormonal signals (Krouk et al., 2010b). Dynamic predictive modeling of the time-resolved dataset identified the transcription factor *SPOROCYTELESS 9 (SPL9)* as a gene hub controlling core N response genes that affect N transport and assimilation.

A root cell-type-specific transcriptional profiling experiment was conducted to analyze the effect of N resupply to N-starved *Arabidopsis* seedlings at tissue-specific resolution (Gifford et al., 2008). GFP marker lines that were specific to five distinct root tissues were N starved and then resupplied with nitrate. These specific cell types were released by protoplasting and isolated by FACS for expression profiling. This high-resolution dataset increased the overall sensitivity

in detecting N responses. Most (> 87%) of the responsive genes were found to be specific to a subset of tissues. Different subsets of auxin and cytokinin signaling components showed up or down regulation among the five cell types, indicating that the responses in individual tissues were also distinct. In particular, the identification of low N induction of *AUXIN RESPONSE FACTOR 8 (ARF8)* in the pericycle uncovered a novel pericycle-specific network for regulation of lateral root initiation: nitrate (via organic N assimilation) repression of the micro-RNA, miR167, induces *ARF8* expression in the pericycle, which increases lateral root emergence. Thus, these results provide a mechanism for auxin-mediated changes in root architecture in response to N supply.

Recently, another miRNA network that modulates root architecture response to N supply was detected by large-scale miRNA sequencing (Vidal et al., 2010). *miR393* was found to be upregulated by N supply and specifically targeted the auxin receptor *AUXIN SIGNALING F-BOX PROTEIN 3 (AFB3)* in the root. *AFB3* expression is induced rapidly by nitrate supply in the pericycle and enhances lateral root initiation. *miR393* upregulation can be induced by nitrate, ammonium or glutamate, indicating that it is likely to be induced by N metabolites downstream of nitrate assimilation. Overexpression of *miR393* represses *AFB3* expression and nitrate-responsive lateral root growth. This finding implicates an additional mechanism for integration of external nitrate and internal N metabolite signals to modulate auxin sensitivity in regulating root architecture.

While molecular and systems approaches have identified a number of key players in N response, how these players are activated by N sensors and how they interact to coordinate physiological outcomes are still largely unknown. For example, the transcriptional networks involved in the primary N response for *NRT2.1* induction are still unclear. Some network models have been generated for responses to N supply, but the responses to low N have not been characterized in as much detail. Further resolution of the low N response in cell type and time dimensions will allow identification of additional signaling components to connect these networks and build a systems model of the root N response. These models will enable the generation of new hypotheses that can be rigorously tested with targeted molecular approaches.

#### 4.4.2 Salinity

Salinity affects ~6% of the world's total land area. High salt conditions are prevalent in arid and semi-arid zones as well as along coastal regions. Salt, mainly sodium ( $\text{Na}^+$ ), can be deposited by wind and rain from weathering rocks and the ocean. In addition, salinity is becoming increasingly problematic in farmland, as rising water tables due to land clearing and irrigation practices concentrate salts in soils at root depth (Munns and Tester, 2008). Currently, approximately 20% of irrigated farmland has been estimated to be salinized.

The physiological response to salt stress has been widely characterized in a variety of plant species and thoroughly reviewed in other recent articles (Munns, 2005; Tran et al., 2007b; Flowers and Colmer, 2008; Munns and Tester, 2008). Salt stress manifests itself at two general stages: an early phase manifests itself as osmotic stress due to high salt concentrations outside the root cells and largely overlaps with drought stress, whereas the later phase is a specific ionic stress caused by accumulation of sodium and chloride ions to toxic levels that inhibit key enzymatic reactions in plant cells, most notably affecting photosynthesis and shoot growth. Salt stress responses span a broad spectrum across species: glycophytes have limited mechanisms to cope with salt stress, whereas halophytes are adapted to salt conditions and have enhanced abilities to compartmentalize and tolerate salinity. Salinity tolerance is covered in depth in another chapter of this book. Here, we will mainly highlight salt stress responses specific to root tissues and aspects of root physiology and architecture that may enhance salinity tolerance. We will emphasize current studies on *Arabidopsis*, rice, and maize, which are all salt-sensitive glycophytes.

#### *Root response to high salt*

Adaptive salt stress responses in the root include regulation of transporters and metabolites to control cellular water potential; cell-type-specific regulation of transporters and cellular permeability to exclude salt and maintain ion homeostasis in shoot tissues; maintenance of root growth to facilitate water and nutrient uptake; redirection of root growth to avoid salt stress; and activation of signaling pathways for root to shoot communication (Munns and Tester, 2008; Dinneny, 2010; Galvan-Ampudia and Testerink, 2011). Many of these root responses are mediated in part by ABA, which is often referred to as the stress hormone. ABA synthesis in roots is induced by osmotic stress incurred by salinity. ABA has been implicated in osmotic adjustment (Sharp et al., 2004), cell wall loosening (Sharp et al., 2004), hydrotropic root bending (Takahashi et al., 2009), and lateral root outgrowth (Galvan-Ampudia and Testerink, 2011). ABA has also been proposed to act as a root to shoot signal that can regulate stomatal conductance in the leaf (Thompson et al., 2007; Schachtman and Goodger, 2008).

Recently, cytokinin function has been implicated in the salt stress response in part through interactions with ABA signaling. Cytokinin can function as a long-range signal to coordinate root and shoot stress responses. Cytokinin biosynthesis is reduced under stress and ABA treatment, and mutants with reduced cytokinin content or sensitivity exhibit enhanced salt tolerance. These results indicate antagonistic interactions between cytokinin and ABA (Nishiyama et al., 2011).

In the early osmotic phase of salt stress, a number of cellular activities are altered in order to control water potential (Munns and Tester, 2008). The activities of

Na<sup>+</sup>/H<sup>+</sup> transporters are increased to exclude sodium from cells and/or sequester sodium in vacuoles. The production and import of organic solutes such as proline and hexoses are also increased in order to balance the osmotic pressure in the cell with the external environment (Sharp et al., 2004). Lowering the water potential in the cell is necessary for cell expansion for root elongation.

Maintenance of root elongation near the tip has been observed across a broad range of plant species and is achieved as a consequence of both osmotic adjustment and enhanced cell wall loosening (Sharp et al., 2004). ABA increases the expression of proline transporters such as *LATE EMBRYOGENESIS ABUNDANT (LEA)* proteins for osmotic adjustment, and *XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)* for cell wall loosening (Sharp et al., 2004). Root elongation under osmotic stress has been observed to be restricted to the apical regions of the root, where the roots also appear thinner. The altered morphology in root tips indicates that cell expansion is restricted to the longitudinal direction and suggests an adaptive mechanism for conserving resources for root growth. *Arabidopsis* cell wall biogenesis mutants, such as *cobra*, are defective in this restriction and exhibit radial root swelling with reduced root and shoot growth (Roudier et al., 2005). The ability to maintain growth under osmotic stress has been observed to be stronger in roots than shoots, indicating that this property is also adaptive for continued soil exploration (Sharp et al., 2004).

Recent observations of agravitropic root bending under high salt further implicate a stress avoidance response that is mediated by a sensing mechanism in the root cap, and that activates differential cell expansion in the root elongation zone (Dinneny et al., 2008; Sun et al., 2008). Interference of auxin transport has also been linked to this agravitropic root bending through redistribution of the expression of auxin transporter *PIN2* (Sun et al., 2008).

Under extreme and prolonged stress, the root meristem undergoes programmed cell death, and growth ceases at the root tip (De Tullio et al., 2010) but can be reinitiated and redirected in lateral root outgrowth from the maturation zone (Galvan-Ampudia and Testerink, 2011). ABA has been shown to play a role in regulating lateral root growth, in a manner involving the transcription factors *ABSCISIC ACID INSENSITIVE 4* and *ABSCISIC ACID INSENSITIVE 5* (*ABI4* AND *ABI5*; Signora et al., 2001). Regulation of lateral root growth is also mediated by auxin and cytokinin. In *Arabidopsis*, prolonged exposure to high salt decreases the vascular diameter, induces radial swelling in the cortical cells (Burssens et al., 2000), and reduces root hair length (Halperin et al., 2003). Root meristem organization is disrupted while an increase in lateral root emergence has also been observed (Burssens et al., 2000).

#### *Molecular dissection of the salinity response*

Many genetic screens have been conducted to uncover genes involved in salt stress response pathways (Saleki et al., 1993; Werner and Finkelstein, 1995;

Wu et al., 1996; Ishitani et al., 1997). One of the most extensively characterized pathways was identified from a forward genetic screen for *Arabidopsis* mutants that exhibited salt overly sensitive (SOS) phenotypes in the root (Zhu, 2000). In the SOS pathway, salt stress induced calcium oscillations are sensed by the EF hand calcium binding protein SOS3 (Liu and Zhu, 1998; Ishitani et al., 2000). Upon calcium activation, SOS3 dimerizes with SOS2 and activates the kinase activity of SOS2, an Snf1-like kinase (Halfter et al., 2000). The activated SOS2/SOS3 complex phosphorylates and activates the Na<sup>+</sup>/H<sup>+</sup> antiporter SOS1 to facilitate sodium transport out of the cell (Wu et al., 1996; Shi et al., 2000; Qiu et al., 2004). While this model clearly links a physiological salt stress response to calcium oscillations, the salt sensing mechanism that triggers the calcium oscillations has remained elusive. Overexpression of *SOS1*, *SOS2* and *SOS3* has been reported to enhance salt tolerance in *Arabidopsis* (Shi et al., 2003; Yang et al., 2009).

A salt exclusion strategy is common among many plant species. The *HIGH AFFINITY K<sup>+</sup> TRANSPORTERs (HKTs)* have been shown to mediate sodium tolerance among many plant species, including wheat, rice and *Arabidopsis* (Horie et al., 2009). The *Arabidopsis HKT (AtHKT1)* localizes to the plasma membrane of xylem parenchyma cells and mediates sodium removal from xylem sap during salt stress (Sunarpi et al., 2005). Constitutive overexpression of *AtHKT1* in *Arabidopsis* increased salt content in the shoot and incurred sodium toxicity, whereas pericycle-specific and vascular bundle-specific overexpression of *AtHKT1* increased salt content in the root but decreased salt content in shoots, leading to overall enhanced salt tolerance (Møller et al., 2009). These results suggest that this salt exclusion must be directional and tissue-specific in order to be beneficial. Similarly, root cortex-specific expression of *AtHKT1* in rice has also been found to enhance salt tolerance (Plett et al., 2010), suggesting that generating a net sodium sink in root tissues can exclude sodium from the shoot. In durham wheat, the introgressed *TmHKT1;5A* gene from the wheat relative, *Triticum monococcum*, improved grain yield on saline soils (Munns et al., 2012). Interestingly, ionic profiling studies using genetically diverse *Arabidopsis* populations have associated a weak-expressing *AtHKT1* allele with high leaf sodium accumulation and adaptation to high salt environments, (Rus et al., 2006; Baxter et al., 2010), suggesting that natural selection for this weak allele occurs concurrently with mechanisms to reduce cellular sodium toxicity. One potential mechanism for reducing cellular sodium toxicity is vacuolar compartmentalization by NHX Na<sup>+</sup>/cation-H<sup>+</sup> antiporters, which has been shown to enhance salt tolerance when overexpressed (Apse et al., 1999). Further enhancement of these salt exclusion modules can be a potential strategy for crop improvement.

While activation of salt exclusion mechanisms is specific to salt stress, additional salt stress response pathways overlap with ABA-mediated abiotic stress responses such as those of drought, osmotic, and cold stress. Three transcriptional

regulatory pathways have been characterized by first isolating salt stress responsive genes, identifying cis-acting elements that control stress-inducible gene expression, and isolating transcription factors that bind and activate these cis-elements (Nakashima et al., 2009).

The DEHYDRATION-RESPONSIVE ELEMENT BINDING (DREB) transcription factors were isolated in a yeast one-hybrid screen using the dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element. The DRE/CRT element is common to genes induced by drought, salt, and cold stress (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998). Additional members of the DREB transcription factor family were found among a super family of *APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF)* transcription factors. Some specificity in function among these stress response factors was found: members of the DREB2 subfamily of transcription factors were generally responsive to salt, osmotic, dehydration and heat stress, whereas members of the DREB1 subfamily were responsive to cold stress (Nakashima et al., 2009). Both DREB2A and DREB2B are induced by salt stress in Arabidopsis, rice and maize roots (Nakashima et al., 2000; Qin et al., 2007) and expression of DREB2s in grasses has been shown to be regulated in part by alternative splicing (Egawa et al., 2006; Qin et al., 2007), although the mechanisms are unclear. Targets of DREB2s include other transcription factors and stress response genes associated with osmotic balance and detoxification pathways, which largely overlap with ABA-dependent pathways. For example, overexpression of maize ZmDREB2A in Arabidopsis induces genes encoding LEA proteins, which are involved in cellular proline accumulation for balancing water potential and preventing protein aggregation under osmotic stress (Qin et al., 2007). *ABI4* is an orphan member of the DREB transcription factor family that regulates lateral root emergence and may integrate ABA and DREB pathways in this response (Signora et al., 2001).

The stress-responsive *NAC (NO APICAL MERISTEM, ATAF1-2, AND CUP SHAPED COTYLEDON 2)* domain transcription factors were isolated by their ability to bind a stress responsive MYC-like cis-element using the yeast one-hybrid assay (Tran et al., 2004). This MYC-like sequence was identified in the promoter of the gene *EARLY RESPONSIVE TO DEYDRATION STRESS 1 (ERD1)*, a Clp ATP-dependent protease, which is induced by drought and salinity in an ABA-independent manner. Further yeast one-hybrid screening identified an additional *ZINC FINGER HOMEODOMAIN (ZFHD1)* transcription factor, which was also required for induction of *ERD1* (Tran et al., 2007a). Expression of three stress responsive Arabidopsis *NAC* transcription factors, *ANAC019*, *ANAC072* and *ANAC055*, is induced by high salinity and drought. Overexpression of these three *ANACs* altered the expression of glyoxylase enzymes that detoxify aldehydes and increased plant survival under prolonged drought treatment (Tran et al., 2004). A rice *NAC*, *ONAC063* has recently been proposed to enhance stress tolerance in a similar manner (Yokotani et al., 2009).

Other rice salt stress inducible *NAC* transcription factors have been identified. *OsNAC6* overexpression in rice activated a peroxidase gene and improved tolerance to high salt stress and dehydration, as well as disease resistance, which suggests a general role in redox regulation (Nakashima et al., 2007). *OsNAC9* and *OsNAC10* are root-expressed *NACs* that are induced by salt and other stresses. Recent studies have shown that overexpression of both genes alters root development and enhances drought tolerance (Jeong et al., 2010; Redillas et al., 2012). Interestingly, *OsNAC9* overexpression upregulated genes involved in ABA biosynthesis, cortical aerenchyma formation and cell wall biosynthesis (Redillas et al., 2012). Overexpression of *ONAC9* produced larger stele diameter and cortical aerenchyma, indicating that the ABA and *NAC* pathways may interact to regulate root architecture in adaptive responses to stress.

As discussed above, many salt stress response genes are also ABA-responsive. In Arabidopsis and rice, an ABA-responsive element (ABRE) was found to be enriched among ABA-responsive genes and was also found to function independently with DRE/CRT in regulating stress responses (Narusaka et al., 2003). A yeast one-hybrid screen using an ABRE bait isolated *ABRE-BINDING FACTORS (AREBs/ABFs)*, which comprised a subfamily of 13 bZIP transcription factors in Arabidopsis (Choi et al., 2000; Uno et al., 2000). *AREB1*, *AREB2*, and *ABF3* are all expressed in roots (Yoshida et al., 2010) and are induced by high salinity, dehydration, and ABA treatment (Fujita et al., 2005). Their transcriptional activation requires phosphorylation by three Snrk2 kinases in an ABA-dependent manner (Fujita et al., 2009; Yoshida et al., 2010). Overexpression of an activated form of *AREB1* resulted in ABA hypersensitivity and enhanced drought tolerance, whereas loss-of-function combinations of *areb1*, *areb2*, and *abf3* resulted in loss of ABA sensitivity, impaired stress-induced gene expression, and reduced drought tolerance. One member of the *AREB* family, *AB15*, has been shown to be a negative regulator of lateral root growth through modulating ABA and auxin responses (Signora et al., 2001; Yang et al., 2011), and may mediate salt responsive changes in root architecture.

In order to identify additional players in salt stress responses and identify general regulatory patterns, multiple groups have queried the salt stress response using transcriptional profiling with microarrays (Kawasaki et al., 2001; Kreps et al., 2002; Ozturk et al., 2002; Seki et al., 2002; Rabbani et al., 2003; Ueda et al., 2004; Matsui et al., 2008). Additional genome-wide studies including miRNA, proteome, and metabolome profiling have also been conducted in several crop plants and halophytes (Ding et al., 2009; Lugan et al., 2010; Zhang et al., 2011). The root transcriptional response to salt stress has been profiled in a number of plants including Arabidopsis (Kreps et al., 2002; Maathuis et al., 2003), rice (Kawasaki et al., 2001) and barley (Ueda et al., 2004). In general, these studies found many overlapping stress responsive genes that were common to salt, cold, and drought stress. This overlap has been attributed to common short-term effects on cellular osmotic potential imposed by the respective

treatments (Munns, 2005). For example, expression of aquaporins, which are water channel proteins, was commonly found to be upregulated in response to salt, drought, and cold stress (Kawasaki et al., 2001; Maathuis et al., 2003). This upregulation is a consequence of overlapping functions of DREB, *NAC*, and ABRE regulons in salt, cold and drought stress. Consistent with this, systematic analysis of salt-responsive seedling and root transcriptomes has identified additional DREB and *NAC* transcription factor family members. However, expression profiles of *Arabidopsis* seedlings exposed to a longer 27-hour salt stress treatment uncovered a set of genes that were distinct from the short-term stress responses, consistent with a later phase ion-specific response (Kreps et al., 2002).

#### *Tissue-specific analysis of salt stress response*

A first indication of the tissue-specificity of the root salt stress response came from studies using a set of tissue-specific calcium reporters in *Arabidopsis* roots (Kiegle et al., 2000). Calcium oscillations exhibited distinct patterns between salt, cold and osmotic stress. Salt and osmotic stress induced similar initial responses, consistent with a short-term osmotic phase in salt stress, whereas cold stress elicited a distinct pattern of calcium oscillations. In the short term, salt and osmotic stress induced a large initial calcium spike in all cell types tested, including epidermis, endodermis and pericycle in the maturation zone, as well as epidermis and cortex in the elongation zone. These results indicate that the osmotic response is fairly widespread among root cell types. In addition, an extended wave of calcium oscillations persisted in the endodermis and pericycle under salt stress, suggesting that the calcium signal may activate later salt stress-specific signaling pathways. Interestingly, in addition to modulating cellular transport, EF hand calcium binding protein SOS3 has recently been linked to auxin mediated regulation of lateral root emergence in response to salt stress (Zhao et al., 2011). These results implicate a role for the extended wave of calcium oscillations in the pericycle.

A correlative approach was first used to investigate tissue specificity of stress responses (Ma and Bohnert, 2007) by comparing *Arabidopsis* microarray expression datasets collected in bulk tissues across a variety of abiotic stress, biotic stress, light and hormone responses (Zeller et al., 2009), with cell-type-specific datasets in roots (Birnbaum et al., 2003). The data were analyzed using fuzzy k-means clustering methods to identify general patterns of gene regulation (Ma and Bohnert, 2007). A set of genes that were significantly regulated across multiple treatments was found to include evolutionarily conserved modules such as mitogen-activated protein kinase (MAPK), Snf1/SnRK, phospholipid, ROS, and calcium signaling pathways. Overall, by comparing stress-responsive expression in whole seedlings with tissue-specific expression in non-stressed roots, the authors predicted that ABA-dependent stress response

networks occurred among genes that were expressed in many cell types, whereas ABA independent networks tended to have more specific expression patterns.

Recently, salt-stressed *Arabidopsis* roots were transcriptionally profiled at tissue-specific resolution (Dinneny et al., 2008). Tissue-specific GFP marker lines were treated with high salinity or control treatments and then specific cell types were released by protoplasting and isolated by FACS for expression profiling. Under the assay conditions, salt stress responses were found to be predominantly constrained by tissue or developmental stage. Specifically, >87% of the differentially regulated genes were found at the developmental and tissue-specific level and were undetectable in a bulk root dataset. Gene ontology analysis also found that most enriched gene functions were constrained by tissue or developmental context. For example, salt stress responsive and epidermal patterning-dependent genes were found to be specifically salt-regulated in hair cells. Regulators of root hair development, such as *COBRA-LIKE 9 (COBL9)* and *ROOT HAIR DEFECTIVE 2 (RHD2)*, exhibited transient downregulation and recovery of expression under salt stress, which correlated with the dynamics of root hair development. Analysis of cis-elements among differentially regulated genes revealed that DRE responses were generally ubiquitous, but a subset of ABRE responses was found to be tissue specific. Comparisons of the cell-type-specific salt expression profiling dataset with similar low iron, low sulfur, and low pH datasets also found that although ABA responses were commonly found among analyzed stress responses, the specific subsets of ABA responses found in each stress, tissue, and developmental context were different (Iyer-Pascuzzi et al., 2011). Generally, no common stress response was found under high-resolution analysis. Furthermore, the cell identity regulator *SCARECROW (SCR)* was found to bind promoters of ABA response genes, and the expression of *SCR* itself was found to be salt regulated in different tissue types, indicating that cell identity regulators can mediate interactions between stress and developmental signals. These cell-type-specific studies highlight the functional importance of tissue-specificity in strategies for understanding and improving stress tolerance.

#### 4.4.3 Root system architecture in stress responses

The ultimate goal for a systems level study of root stress responses is to integrate these gene networks into the phenotypic plasticity that gives rise to root system adaptation. Root system architecture (RSA) describes the overall spatial configuration of all roots of a plant (Hochholdinger and Tuberrosa, 2009; Peret et al., 2009; Coudert et al., 2010; Jones and Ljung, 2012), and is the net result of the ordered growth and branching of different root types. In *Arabidopsis*, the RSA consists of a single primary root that gives rise to lateral roots and higher order branches. Monocots such as maize and rice generate a fibrous root system that

originates from embryonic primary and seminal roots, as well as postembryonic nodal roots, which all give rise to lateral roots and higher order branches. Hence, RSA is regulated by cell division and expansion activities near the root tips of all the root types, cell divisions that initiate axile and lateral roots from the pericycle, and root tropisms that regulate the direction of root growth. Collectively, a plant's RSA responses to different environmental conditions is referred to as the root phenome (Lynch, 2011). As discussed in the previous sections, environmental conditions influence division, expansion and differentiation of different cell types within the root that contribute to RSA. These changes in RSA are the ultimate result of the coordination of local and systemic signals with cellular activities to allow soil exploration for nutrients and evasion of toxic substances. Understanding adaptive RSA responses to stress can also predict constitutive root architecture features that are predisposed to stress tolerance.

Recent years have seen a surge in development of methods to characterize root system architecture, ranging from controlled, reductionist and high throughput lab-based methods to open field conditions that are exposed to complex variable conditions (Tardieu and Tuberrosa, 2010; Zhu et al., 2011; De Smet et al., 2012). These analyses can be conducted across genetic varieties and the genetic basis underlying these RSA traits can be identified using genomic methods such as quantitative trait locus (QTL) analysis and genome-wide association studies (GWAS). Variation in RSA has been observed between individuals of a genetic population that have been subjected to different stress conditions, between genetic varieties within a species isolated from different environments, and between species that occupy distinct ecological niches (Lynch, 1995; de Dorlodot et al., 2007). These variations in RSA between genetic varieties selected for different environments have informed root ideotypes for enhanced stress tolerance. For example, faster growing and deeper rooting systems have been associated with the ability to capture deeper soil moisture for drought tolerance. Additionally, in well-drained soils, deeper roots have also been associated with more efficient uptake of highly mobile nutrients such as inorganic N. In contrast, broader and shallower roots have been associated with better foraging for nutrients with lower mobility in soil such as phosphorus (Lynch, 2011; Wasson et al., 2012). The effect of RSA traits on overall plant fitness is also dependent on soil quality, weather patterns, and interactions with plant neighbors and the rhizosphere. For example, in environments where deeper soil moisture is salinized, deeper roots can incur toxicity, whereas shallower root systems may result in better performance; in agricultural systems that depend on stored water moisture, efficient capture of deeper soil moisture during early developmental stages may incur drought during flowering, which will damage yield (Wasson et al., 2012). The current challenge is to associate RSA features and ideotypes with genes that coordinate cell-type-specific activities for these complex traits in order to develop crop improvement strategies for specific target environments.

#### 4.5 Conclusion

Over recent years, a comprehensive approach has been adopted to uncover complex root response networks. This includes high-resolution characterization of stress responses by transcriptomic profiling, genomic identification of primary sensors and key response regulators, physiological characterization of root traits and their effect on plant fitness in the lab and field. The genetic models and “omics” tools have accelerated the identification of stress response genes to allow the construction of stress response networks. Fully sequenced genomes have accelerated research to identify components in root response networks (Benfey et al., 2010), albeit the coverage of the *Arabidopsis* genome in current network models is relatively low (Chae et al., 2012). The outstanding question remains: how is a stress signal transduced to alter a cell-type-specific gene expression response? Additional datasets, particularly with increased specificity and resolution in stress and tissue-specific contexts, can improve the coverage and robustness of these networks.

At present, our understanding of adaptive stress responses in the root may still be limited by the physiological limitations of the model systems employed for these studies. For example, within the spectrum of salt sensitivity of glyco-phytes, *Arabidopsis* ranks as a relatively salt-sensitive plant and may be a limited resource for salt tolerance mechanisms (Munns and Tester, 2008). The experimental conditions that are currently used to interrogate this model system are often outside its tolerance range. Application of physiologically relevant conditions may be necessary to uncover additional signal transduction components in relevant stress response and adaptation pathways. Genome association studies in crop varieties bred for different environments or in natural populations with higher genetic diversity can be used to dissect the genetic effect on root stress responses and adaptive RSA. The development of appropriate model systems to study specific stress responses, such as the halophyte *Thellungiella* (Wu et al., 2012) for salt tolerance (Wu et al., 2012, or the C4 monocot *Setaria* for drought tolerance (Bennetzen et al., 2012), may further shed light on adaptive mechanisms that can be used to engineer stress tolerance into crops.

Current strategies for improving low nutrient and stress tolerance have predominantly focused on the shoots. For example, attempts to improve NUE have largely focused on N assimilation pathways, which are primarily carried out by plastids in shoot tissues (Good et al., 2004). Root-focused strategies can be directed at the site of interaction with the soil to increase nutrient uptake efficiency, or toxicity exclusion and avoidance. This can be achieved by three main strategies: (1) enhancing tissue-specific nutrient uptake capacity and toxicity exclusion/compartmentalization, (2) modulating source-sink flux of nutrients and toxic solutes, and (3) improving root architecture to increase foraging for limited nutrients and stress avoidance.

One obvious target group of genes for crop improvement are transporters, including NRT nitrate transporters for N uptake and *HKT* for sodium export. The directional nature of transport requires that expression strategies for engineered traits should be specific to targeted source or sink tissues. For example, constitutive overexpression of *AtHKT1* failed to increase salt tolerance, whereas root tissue-specific expression strategies conferred tolerance by enhancing the sink function of root cells, which excludes sodium toxicity from the shoot (Møller et al., 2009; Plett et al., 2010). Similarly, constitutive overexpression of the tobacco high-affinity nitrate transporter (*NpNRT2.1*) resulted in slightly enhanced nitrate uptake in roots under low N, but no significant difference in nitrate content in shoots and no noticeable effect on overall NUE (Fraisier et al., 2000). So far, no attempt to engineer enhanced N uptake in specific cell types has been reported. The inability to enhance NUE by NRT overexpression may also be in part due to the post-transcriptional regulation and complex metabolic networks that are involved in regulating N use (Vidal and Gutierrez, 2008; Laugier et al., 2012). Successful engineering of increased NUE will require a systems approach to coordinate transport and metabolic pathways in relevant tissue types.

An alternative target group of genes for crop improvement are transcription factors that control coordinated pathways. For example, overexpression of N-regulated *LBD* transcription factors in Arabidopsis can alter the expression of N transport and assimilation genes (Rubin et al., 2009). However, overexpression of rice *LBDs* (*OsLBDs*) has been reported to enhance N metabolism in Arabidopsis but repress N metabolism in rice (Albinsky et al., 2010), which cautions against overgeneralization when transferring orthologous gene sets between plant species. In addition, overexpression of DREB2A resulted in increased drought tolerance as well as some undesired growth retardation (Qin et al., 2007). Thus, not unsurprisingly, constitutively upregulating stress responses may incur fitness costs. Recently, overexpression of *NACs* in rice has improved plant tolerance to high salt and drought with little change in basal plant morphology or yield (Tran et al., 2010), suggesting that some classes of transcription factors may confer less of a yield drag. A systems approach may be used to predict transcription factors with selective targets in relevant tissue types. Furthermore, the implementation of stress and/or tissue-specific expression strategies may reduce the fitness costs. These specific expression strategies will require the development of advanced and targeted expression tools.

Root architecture traits are generally multigenic and will require advanced genomic methods to identify contributing genetic loci and predict stress-tolerant genetic combinations. Identifying these multigenic complex traits will require high throughput root phenotyping technologies to characterize diverse germplasms, as well as advanced computational models to associate phenotyping and genotyping data. Predictions of gene functions from systems analysis will aid in understanding the contributions of identified genetic loci.

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## **5 Plant low-temperature tolerance and its cellular mechanisms**

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### **5.1 Introduction**

Many plants can tolerate “low-temperature” stresses, a capacity that is essential for their survival. In particular, plants in temperate regions can enhance this capacity when air temperatures decrease in mid-fall and early winter. This phenomenon, known as cold acclimation, is associated with dynamic physiological changes within plant cells (Levitt, 1980) caused by altered gene expression with multiple effects, including modifications in membrane composition and the accumulation of compatible solutes, as described in this chapter.

How do some plants tolerate “low-temperature” stress? To answer this question, we must know how “low-temperature” stress injures plants. Many studies of both cold tolerance and injury mechanisms have been done over the past hundred years, but we still have an incomplete understanding of these processes. “Low-temperature” stress is complex. For example, low temperatures, in some cases even above 0 °C, directly affect the physicochemical nature of lipids and proteins and, consequently, the fluidity of the lipid bilayer decreases, some proteins are denatured, and enzyme activities decrease as described below. The combination of these events may lead to complex injuries if plants do not have tolerance mechanisms. In addition, the chilling tolerance mechanism is difficult to identify directly, because, in general, the distinct physiological changes during chilling treatment cannot be observed in chilling-resistant plants. Thus, only studies comparing chilling-sensitive with chilling-resistant plants have yielded progress in understanding chilling tolerance mechanisms.

On the other hand, because the melting point of water is at 0 °C, when temperatures decrease to below that and the supercooling of water is broken, the water in the plant is frozen. During freezing, plant cells must not only avoid intracellular freezing but also tolerate the dehydration and mechanical stresses that are induced by extracellular freezing. Thus, temperatures below 0 °C cause complex stresses, including the low-temperature effects described above as

well as freezing stresses. While freezing tolerance has been studied via freezing injury and cold acclimation, low-temperature tolerance has been studied via chilling injury at temperatures above 0°C. Although studies of low-temperature tolerance have been performed at non-freezing temperatures, these results are thought to apply to the cells of freezing-tolerant plants as well. In general, the following hypothesis is widely accepted: plants that can survive freezing temperatures possess tolerance mechanisms against low temperatures, and their irreversible injuries are probably directly caused by freezing itself, rather than low temperatures.

In this chapter, we mainly focus on the mechanisms of chilling injury, freezing injury, cold acclimation, and freezing tolerance at the cellular level.

## 5.2 Chilling injury

Generally, chilling injuries are observed in tropical and subtropical plants, which exhibit marked physiological dysfunction when exposed to non-freezing temperatures below about 12°C (Lyons, 1973). Studies on the mechanisms of chilling injury have elucidated how plants have evolved chilling tolerance mechanisms. For a better understanding of the mechanisms by which chilling injures plants, the primary sites that sense low temperatures and the effects of environmental conditions on chilling sensitivity must be revealed. Chilling injuries that are described below are summarized in Figure 5.1.

### 5.2.1 Cold inactivation of vacuolar H<sup>+</sup>-ATPase

Vacuolar H<sup>+</sup>-ATPase (V-ATPase) is extremely sensitive to cold and was preferentially inactivated early during chilling of mung bean hypocotyls (Yoshida et al., 1989), in suspension cultures of mung bean (Yoshida, 1991), in cotyledons of cucumber (Yoshida et al., 1999), and in chilling-sensitive suspension-cultured cells of rice (Kasamo, 1988). Cold-induced inactivation of the vacuolar V-ATPase occurs long before the appearance of cell injury and the general decrease in the activities of enzymes that are associated with the plasma membrane, endoplasmic reticulum (ER), and mitochondria (Yoshida et al., 1989). Therefore, damage to vacuolar V-ATPase is a primary cellular event that results directly from exposure to low temperatures. Chilling injury may be closely related to the cold inactivation of V-ATPase, because loss-of-function mutants of V-ATPase subunits show defects in development and Golgi body organization, and furthermore, knockout mutants of V-ATPase subunits are lethal (Schumacher et al., 1999; Dettmer et al., 2005; Strompen et al., 2005).

The mechanism of cold inactivation of V-ATPase may be related to structural changes in the enzyme during ATP hydrolysis (Moriyama and Nelson, 1989). V-ATPase with a molecular mass of 700–800 kDa is composed of 11–13 subunits.

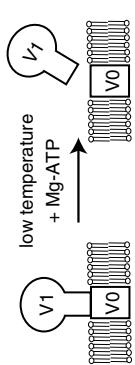
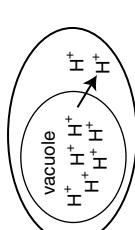
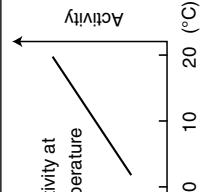
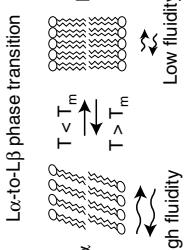
Stress	Primary site	Sensitive molecule	Molecular mechanism	Observed cellular injury
Low temperature	Vacuolar membrane	V-ATPase	low temperature + Mg-ATP ? 	cold inactivation
Low temperature	Vacuolar membrane	pH regulation (V-PPase + ?)	 H <sup>+</sup> efflux to cytoplasm	Cytoplasmic acidification
Low temperature + low light	Thylakoid membrane	APX	Low activity at low temperature 	Increase in AOS → damage of PSII
Low temperature + high light	Thylakoid membrane	Saturated species of PG	 L <sub>α</sub> → L <sub>β</sub> T < T <sub>m</sub> T > T <sub>m</sub>	Decrease in D1 turnover → damage of PSII

Figure 5.1 Summary of chilling injuries in chilling-sensitive plants.

The enzyme complex is organized into a peripheral  $V_1$  domain, responsible for ATP hydrolysis, and an integral  $V_0$  domain responsible for the  $H^+$  pump (Stevens and Forgac, 1997; Forgac, 1999). In parallel with cold inactivation of V-ATPase, the subunits of the  $V_1$  domain decrease in concentration in the vacuolar membrane fraction (Matsuura-Endo et al., 1992). On the other hand, the proteolipid subunit, which constitutes the  $V_0$  domain, hardly decreases in quantity in the vacuolar membrane, even when the ATP hydrolysis and  $H^+$ -pumping activities of V-ATPase markedly decrease (Matsuura-Endo et al., 1992). These results indicate that the  $V_1$  domain detaches from the  $V_0$  domain during chilling, inactivating V-ATPase. In general, purified V-ATPase is stable at low temperature, but the enzyme is inactivated when the  $V_1$  domain is released from the  $V_0$  domain by *in vitro* cold treatment in the presence of Mg-ATP and chaotropic anions, which weaken hydrophobic bonds. Moriyama and Nelson (1989) have speculated that V-ATPase becomes unstable during ATP hydrolysis, and consequently the  $V_1$  and  $V_0$  domains separate.

In contrast to the behavior of V-ATPases in chilling-sensitive leguminous plants such as mung bean, adzuki bean, and kidney bean, the V-ATPases in chilling-tolerant legumes, such as pea and broad bean, are more stable for long periods of exposure to cold (Yoshida et al., 1999). Furthermore, upon cold incubation of vacuolar membranes isolated from these plants in the presence of Mg-ATP and chaotropic anions, such as  $Cl^-$ ,  $NO_2^-$ , and  $NO_3^-$ , the susceptibility of the enzyme to chilling differs markedly. The enzymes from chilling-sensitive plants are more susceptible to lower concentrations of chaotropic anions than the enzymes from chilling-tolerant plants (Hotsubo et al., 1998), suggesting that plant V-ATPases can be categorized into chilling-sensitive and chilling-tolerant types.

### 5.2.2 Lipid phase transition ( $L_\alpha$ to $L_\beta$ )

Chilling has been believed to cause membrane lipids of chilling-sensitive plants to transition from a fluid lamellar  $L_\alpha$  phase to a gel  $L_\beta$  phase, which impairs membrane functions and leads to irreversible injury (Lyons and Raison, 1970; Raison et al., 1971; Raison, 1973; Martin, 1986; Raison and Lyons, 1986). A decrease in the degree of unsaturation of membrane lipids elevates the temperature at which this phase transition happens and consequently decreases membrane fluidity at low temperatures (Murata et al., 1982; Murata, 1983; Murata et al., 1992; Wada et al., 1990). The sensitivity of higher plants to chilling is closely correlated with the degree of unsaturation of the fatty acids in the thylakoid membranes of their chloroplasts (Murata et al., 1982; Murata, 1983; Roughan, 1985). Thus, this phase-transition hypothesis of chilling injury in plants is widely accepted. However, while the activities of integral membrane enzymes are negatively affected by the transition to  $L_\beta$  phase (Lyons and Raison, 1970; Raison, 1973; Yoshida and Matsuura-Endo, 1991), irreversible

chilling injuries have not been shown to be due only to a decrease in enzyme activity caused by phase transition.

In cyanobacteria, further exposure to chilling induces a phase-separated state in membranes; this state occurs after the phase transition (Ono and Murata, 1982). An increase in ion permeability at low temperatures has been confirmed (Murata et al., 1984; Ono and Murata, 1981). Chilling-sensitive plant cells have also been assumed to enter a phase-separated state in which membranes cannot maintain ionic gradients and in which subsequent metabolic disruptions lead to irreversible cell injuries (Nishida and Murata, 1996). However, in plants, the permeability of protons through the vacuolar membrane has been reported to be lower at lower temperatures, including those below the phase-transition temperature in the presence of  $P_2O_7^{4-}$  (PP<sub>i</sub>; Yoshida and Matsuura-Endo, 1991; Kawamura, 2008). In addition, cytosolic PP<sub>i</sub> affects membrane thermodynamic characteristics and stabilizes the membrane at low temperatures (Kawamura, 2008). Based on these results, the effusion of ions from plant membranes is unlikely to be due to phase separation during chilling treatment.

### 5.2.3 Chill-induced cytoplasmic acidification

When treated with cold, cells of chilling-sensitive mung bean suffer a rapid acidification of the cytoplasm (Yoshida, 1994), and simultaneously, an alkalization of vacuoles before irreversible injury occurs (Yoshida, 1995). Cytoplasmic acidification during chilling treatment has also been observed in leaf mesophyll cells of *Episcia*, *Saintpaulia*, and *Cucumis*, all of which are sensitive to chilling (Yoshida, 1994). Because the proton gradient between the cytoplasm and vacuole provides the energy for secondary active transport and maintains homeostasis of cytoplasmic ion and metabolite concentrations, the effusion of protons from the vacuole into the cytoplasm may perturb the metabolic system and ultimately lead to irreversible cell damage if continued for a long period. Cytoplasmic acidification may affect other organelles. For example, the selective inactivation of the oxygen-evolving system in photosystem II (PSII) may be related to acidosis near the chloroplast (Shen and Inoue, 1991). At present, a possible explanation for chilling-induced cytoplasmic acidification is that the  $\Delta pH$ -stat between the cytosol and vacuole ( $\Delta pH_{vac}$ -stat) is perturbed by chilling, rather than a decrease in membrane semipermeability or dysfunction of the primary active H<sup>+</sup>-transporter (Kawamura, 2008).

What is a  $\Delta pH$ -stat? Plant cells must respond rapidly to sudden changes in environmental temperatures, and they are assumed to possess mechanisms to biochemically adjust cytoplasmic pH to, for example, maintain a relatively stable cytoplasmic pH and a certain  $\Delta pH$  across the vacuolar membrane (i.e.,  $\Delta pH_{vac}$ -stat). Cytoplasmic pH may be regulated, in part, by plasma membrane H<sup>+</sup>-pumping, anion channels, and H<sup>+</sup>-pumping into vacuoles (Xia and Roberts, 1996; Johannes et al., 1998; Oja et al., 1999). In particular, the regulation of

leaf cell cytoplasmic pH, under acid stress, involves H<sup>+</sup>-pumping from the cytosol into the vacuole but not into the apoplast (Oja et al., 1999). In suspension cells of sycamore, cytoplasmic pH couples to vacuolar pH following changes in the external pH, and the vacuole is thought to be able to counteract proton invasion from the extracellular space, thereby contributing to cytoplasmic pH homeostasis (Gout et al., 1992).

In mung bean, PP<sub>i</sub>-dependent H<sup>+</sup>-accumulation, which includes H<sup>+</sup> influx driven by vacuolar H<sup>+</sup>-pyrophosphatase (V-PPase) and PP<sub>i</sub>-stabilized H<sup>+</sup>-efflux, may be essential to maintain a ΔpH<sub>vac</sub>-stat during temperature changes (Kawamura, 2008). For example, over the temperature range from 0 to 20 °C, the H<sup>+</sup>-influx mediated by PPase was balanced by the PP<sub>i</sub>-dependent suppression of H<sup>+</sup> efflux; consequently a constant pH of ca. 5 could be maintained in vesicles (pH<sub>in</sub>) during temperature changes (Kawamura, 2007, 2008). However, the ΔpH<sub>in</sub> driven by ATP decreased as the temperature dropped. In vacuolar vesicles isolated from seedlings chilled at 0 °C for 1 d, the PP<sub>i</sub>-dependent H<sup>+</sup>-accumulation maintained pH 5.6 in vesicles during temperature changes. Thus, cytoplasmic acidification may be caused by the breakdown of ΔpH<sub>vac</sub>-stat, which is generated by PP<sub>i</sub>-dependent H<sup>+</sup>-accumulation (Kawamura, 2008).

#### 5.2.4 Light-dependent chilling injury

The modalities of chilling injury have been reported to be very different in light and dark conditions. For example, chilling injury in greening plants in the light is more substantial than those in darkness and is thought to be caused by the photo-oxidation of chloroplasts at low temperatures (van Hasselt, 1972, 1974; De Kok and Kuiper, 1977; van Hasselt and van Berlo, 1980; Powles, 1984; Wise and Naylor, 1987; Hodgson and Raison, 1989; Sonoike and Terashima, 1994; Terashima et al., 1994; Sonoike, 1995, 1996, 1998). In addition, the manner of photo-oxidation is dependent on the light intensity: in low or moderate light conditions (<200 μmol/m<sup>2</sup>/s), photosystem I (PSI) is mainly damaged, and under high light (>500 μmol/m<sup>2</sup>/s), PSII is mainly damaged. While the two photo-oxidation mechanisms are different, they both result in irreversible damage to chilling-sensitive plants (Sonoike, 1996).

In earlier studies in which high-intensity light was used, photoinhibition at low temperatures was thought to occur mainly in PSII. In this scenario, because PSII is affected first, the flow of electrons to PSI stops, and consequently, PSI is protected (Sonoike, 1996). Thus, selective photoinhibition of PSI was not discovered until the work of Terashima's group was published (Terashima et al., 1994). The photoinhibition at PSI is caused by active oxygen species (AOS), which are produced mainly through the reduction of O<sub>2</sub> by electrons from PSI (Asada, 1999). Generally, in non-stressful conditions, PSI is protected from AOS by the Asada pathway, which includes thylakoid

ascorbate peroxidase (APX), a key enzyme in H<sub>2</sub>O<sub>2</sub>-scavenging. However, the activity of thylakoid APX at 5 °C in cucumber, a chilling-sensitive plant, is about 20% of that measured at 25 °C (Terashima et al., 1998). Thus, a net production of H<sub>2</sub>O<sub>2</sub> occurs when the rate of AOS scavenging decreases at low temperatures. Finally, hydroxyl radicals can be produced by the Fenton reaction via Fe-S centers in PSI and cause damage not only to PSI (Terashima et al., 1998) but also to PSII, especially when thylakoids are stacked (Tjus et al., 2001). Interestingly, even in chilling-tolerant plants, some photoinhibition occurs at chilling temperatures, and the AOS scavenging system protects them from damage (Tjus et al., 1998).

Under high-light and chilling-temperature conditions, the D1 protein in the PSII complex is damaged, and PSII photoinhibition occurs (Aro et al., 1990; Aro et al., 1993). In undamaged and less-damaged plants, the disrupted D1 is degraded, removed from the PSII complex, and replaced by newly synthesized D1 to restore photochemical activity (Aro et al., 1993). Therefore, the extent of PSII photoinhibition corresponds to the relative rates at which D1 is photodamaged and at which the PSII complex is restored with newly synthesized D1 (Greer et al., 1986). Studies have shown that chilling sensitivity in plants is closely correlated with the degree of unsaturation of the fatty acids in their thylakoid membranes (Murata et al., 1982; Murata, 1983; Roughan, 1985). Interestingly, the unsaturation phosphatidylglycerol (PG) fatty acids in thylakoid membranes accelerates the recovery of damaged PSII complexes (Moon et al., 1995). Because PGs in thylakoid membranes are preferentially involved in protein-lipid interactions (Li et al., 1989; Murata et al., 1990), the unsaturation of PG fatty acids may affect the turnover of D1 in the PSII complex (Moon et al., 1995). While at present it is unclear whether the decrease in D1 turnover is related to the lipid phase transition in thylakoid membranes, Hamada et al. (1998) have reported that the injuries introduced by lipid phase transition during chilling treatment is mainly related to chloroplast damage.

### 5.3 Freezing injury

No living cells can survive intracellular freezing (Levitt, 1980). Therefore, plant cells that can survive temperatures below 0 °C must possess mechanisms to prevent intracellular freezing (Yamada et al., 2002). However, even if plant cells avoid intracellular freezing, they are subjected to dehydration stress when extracellular water freezes and are also physically pressured by the solid ice crystals that form outside the cells (mechanical stress). The plasma membrane is thought to be the primary site of injury induced by extracellular freezing (Steponkus et al., 1993). Freezing injuries that are described below are summarized in Figure 5.2.

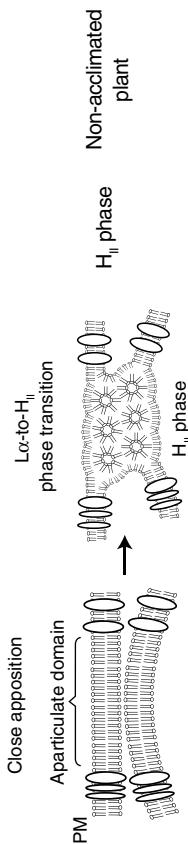
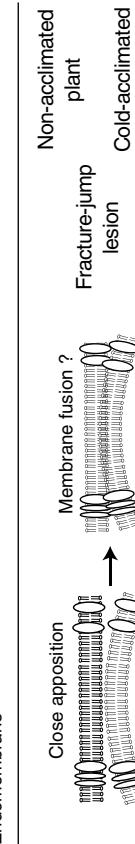
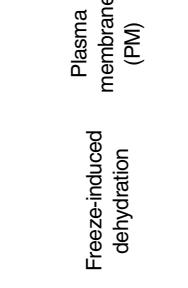
Stress	Primary site	Molecular mechanism	Observed cellular injury	Observed sample
Freeze-induced dehydration	Plasma membrane (PM)	<p>Close apposition</p>  <p>→</p> <p><math>\text{L}_\alpha\text{-to-}\text{H}_\text{II}</math> phase transition</p>  <p><math>\text{H}_\text{II}</math> phase</p>	$\text{H}_\text{II}$ phase	Non-acclimated plant
Freeze-induced dehydration	PM	<p>Close apposition</p>  <p>→</p> <p>Membrane fusion ?</p> 	Fracture-jump lesion	Non-acclimated plant Cold-acclimated plant
Freeze-induced mechanical stress	PM	<p>Close apposition</p>  <p>→</p> <p>Ice growth</p>  <p>Cell wall</p> <p>PM</p>	Mechanically disrupted membrane	Non-acclimated plant Cold-acclimated plant
Freeze-induced dehydration (only in protoplast)	PM	<p>Endocytotic vesiculation</p>  <p>→</p> <p>Freezing</p>	Lysis Thawing EIIL	Non-acclimated protoplast Non-acclimated plant

Figure 5.2 Summary of freezing injuries.

### 5.3.1 Freeze-induced ultrastructures in the plasma membrane

Freezing injury of the plasma membrane is closely related to the fact that the plasma membrane is adjacent to the membranes of intracellular organelles or to itself due to distorted cell shrinkage resulting from freeze-induced dehydration alone (Steponkus et al., 1993) or in combination with mechanical stress (Fujikawa et al., 1999). First, this close apposition causes aparticulate domains, which are intramembranous particle-free areas. All observations using freeze-fracture replica electron microscopy have revealed that aparticulate domains occur in the plasma membranes of freeze-damaged plant cells. Second, ultrastructural changes in the aparticulate domain are thought to lead directly to irreversible cell injury. However, these ultrastructures differ among studies by different research groups. Thus, there is little consensus on the detailed mechanism by which the plasma membrane is injured by distorted cell shrinkage.

One ultrastructure in aparticulate domains is the hexagonal<sub>II</sub> phase ( $H_{II}$ ). Steponkus and his colleagues observed the  $H_{II}$  phase in protoplasts prepared from non-acclimated leaves of winter rye, spring oat, and *Arabidopsis* (Gordon-Kamm and Steponkus, 1984a; Steponkus et al., 1993; Webb et al., 1994; Uemura et al., 1995). They showed that when freeze-induced dehydration causes the plasma membrane and internal endomembranes to come into close apposition, both membranes undergo a transition from lamellar  $L_{\alpha}$  phase to  $H_{II}$  phase. The membranes subsequently fuse, causing irreversible injury (Steponkus et al., 1993). The  $L_{\alpha}$ -to- $H_{II}$  phase transition has also been observed in intact leaf cells of non-acclimated rye and in cortical parenchyma cells of mulberry in summer (Webb and Steponkus, 1993; Fujikawa, 1994), while, for this transition, freeze-induced dehydration requires longer times in leaves than in isolated protoplasts. (Webb and Steponkus, 1993). Thus, Steponkus and his colleagues proposed that the  $H_{II}$ -phase formation is the main cause of irreversible freezing injury in non-acclimated cells.

Another ultrastructure in aparticulate domains is the fracture-jump lesion, which refers to the occurrence of a localized deviation in the fracture plane in the aparticulate domain (Steponkus et al., 1993). The fracture-jump lesion has been interpreted as a site of membrane fusion (Steponkus et al., 1993; Fujikawa, 1995), although it is physicochemically unclear how fracture-jump lesions develop during freezing. When cold-acclimated cells are injured by freezing, only fracture-jump lesions, not  $H_{II}$  phases, occur in their plasma membranes. This phenomenon has been confirmed in a wide variety of plant species (Steponkus et al., 1993; Fujikawa, 1995; Nagao et al., 2008). Thus, the occurrence of fracture-jump lesions in the plasma membranes of cold-acclimated cells has been widely accepted to be the primary cause of irreversible freezing injury.

In contrast, when using intact plant cells with cell walls, Fujikawa and his colleagues observed fracture-jump lesions not only in cold-acclimated cells but also in non-acclimated cells, and they never observed the  $H_{II}$  phase, even in non-acclimated cells, except in mulberry cortical parenchyma cells in summer

(Fujikawa et al., 1999; Nagao et al., 2008). In addition, the frequency of fracture-jump lesions is closely related to the extent of freezing injury in cortical parenchyma cells of mulberry in all seasons, including summer (Fujikawa, 1994). Thus, Fujikawa and his colleagues concluded that the formation of  $L_{\alpha}$ -to- $H_{II}$  phase transitions in plasma membranes was restricted to protoplasts from non-acclimated plants and that the occurrence of fracture-jump lesions in the plasma membrane was the primary cause of freezing injury in both non-acclimated and cold acclimated cells with cell walls.

### 5.3.2 Another freeze-induced injury of the plasma membrane

While aperticulate domains with  $H_{II}$  phases or fracture-jump lesions are caused by the distorted cell shrinkage that results from freeze-induced dehydration, some irreversible damage to the plasma membrane has been suggested to be caused by freeze-induced mechanical stress only, rather than by dehydration (Yamazaki et al., 2008a). Although how the plasma membrane is damaged is unknown, one possibility is the pressure against the cell caused by ice crystal growth. For example, the plasma membranes of plant cells sandwiched between ice crystals may be mechanically pressed as the crystals grow. In electron microscopy studies, plant cells in tissue have been observed to be mechanically deformed by extracellular ice crystals (Pearce, 1988; Pearce and Ashworth, 1992; Fujikawa et al., 1999). Another possibility is that the damage is due to excess adhesion between ice and the plasma membrane during a freeze/thaw cycle. Adhesion energy has been hypothesized to develop between ice and hydrophilic polymers during freezing as they compete for liquid water, and the ice adhesion eventually damages the plasma membrane (Olien, 1974; Olien and Smith, 1977).

In another case, in protoplasts isolated from non-acclimated plants, endocytotic vesicles have been observed to form during freeze-induced dehydration. This phenomenon was visualized using bright-field microscopy with a computational edge-enhancement technique (Dowgert and Steponkus, 1984). During subsequent thawing, the endocytotic vesicles could not be incorporated into the plasma membrane, and consequently, the protoplasts lysed during osmotic expansion after thawing of the suspension buffer, a process referred to as expansion-induced lysis (EIL; Gordon-Kamm and Steponkus, 1984b; Steponkus et al., 1993; Kawamura and Uemura, 2003; Uemura et al., 2006). However, because EIL is restricted to protoplasts, the physiological meaning of EIL in cells with cell walls remains unclear.

## 5.4 Cold acclimation

Cold acclimation is essential for plants to survive the lower temperatures that come with seasonal changes. After perceiving low temperature, plants initiate cold acclimation by producing transcription factors, such as CBF/DREBs and

ICEs (Thomashow, 1998, 1999; Chinnusamy et al., 2007; Lissarre et al., 2010). In addition, cold acclimation is affected by light conditions, such as day length and wavelength (Wanner and Junntila, 1999; Kim et al., 2002; Franklin and Whitelam, 2007; Catala et al., 2011; Lee and Thomashow, 2012). Cold treatment in the dark does not enhance freezing tolerance in *Arabidopsis* (Wanner and Junntila, 1999). During cold acclimation, specific sets of genes are induced (Thomashow, 1998, 1999; Seki et al., 2001; Benedict et al., 2006; Oono et al., 2006) and many physiological, biochemical, and structural changes (Levitt, 1980) progress in cells. These events are necessary for the cells to survive low-temperature and/or freeze-induced stresses. Some of these responses directly increase the cryostability of the plasma membrane. In fact, cold acclimation minimizes the occurrence of freeze-induced plasma membrane lesions (Steponkus et al., 1993).

#### *5.4.1 Lipid composition of the plasma membrane during cold acclimation*

Because lipids are a main component of biomembranes, the lipid composition of the plasma membrane has been studied in relation to membrane cryostability. These studies have revealed that the lipid composition of the plasma membrane is associated with differences in freezing tolerance among plant species and with increases in freezing tolerance induced by cold acclimation (Yoshida, 1984; Uemura and Yoshida, 1984, 1986; Yoshida and Uemura, 1984; Lynch and Steponkus, 1987; Steponkus et al., 1993; Uemura and Steponkus, 1994; Uemura et al., 1995). These differences in lipid composition affect the cryostability of the plasma membranes, accounting for some, but not all, of the freezing tolerance observed (Steponkus et al., 1993).

The most marked change in lipid composition during cold acclimation is an increase in the proportion of phospholipids (Steponkus et al., 1993; Uemura et al., 2006). This phenomenon is conserved across a wide range of species, from monocotyledonous to dicotyledonous plants and from herbaceous to woody plants. In the early stage of cold acclimation, an increase in plasma membrane phospholipids occurs, whereas a decrease in cerebrosides occurs gradually throughout the cold-acclimation process. In many plant species, increases in phospholipids resulted primarily from increases in the proportions of unsaturated molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which are two major phospholipid classes in the plasma membrane. Also, the proportion of cerebrosides decreases in a wide range of plants. In addition, comparative studies of plants with different freezing tolerances revealed that no single lipid species is unique to the plasma membranes of either non-acclimated or cold-acclimated leaves or to a particular plant species (Steponkus et al., 1993). Instead, in the plasma membrane, the relative proportions of almost every lipid species changes during cold acclimation, and these proportions vary widely among plant species.

#### 5.4.2 *Changes in plasma membrane proteins during cold acclimation*

Many studies have demonstrated that gene expression and/or protein profiles, including those of plasma membrane proteins, change during cold acclimation (Uemura and Yoshida, 1984, 1986; Yoshida and Uemura, 1984; Yoshida, 1984; Thomashow, 1999; Seki et al., 2002; Kawamura and Uemura, 2003; Oono et al., 2006). In particular, recent studies have identified many of the plasma membrane proteins that quantitatively change during cold acclimation (Kawamura and Uemura, 2003; Minami et al., 2009; Li et al., 2012), because new proteomics approaches using mass spectrometry and genome sequence databases allow us to identify the sequences of femto- to picomole amounts of protein.

The plasma membrane includes proteins with many different functions, including signal transduction, transport, and stress resistance. In fact, proteomics studies have revealed that many kinds of proteins increase in abundance during cold acclimation, including proteins associated with membrane repair, protection of the membrane against osmotic stress like dehydrins, enhancement of CO<sub>2</sub> fixation, proteolysis, membrane transport, membrane trafficking, and cytoskeleton interaction (Kawamura and Uemura, 2003; Minami et al., 2009; Li et al., 2012). Because some of these proteins may be required during cold acclimation or for low-temperature tolerance, rather than for freezing tolerance, the proteins that directly function in the cryostability of the plasma membrane are difficult to identify from proteomics data alone.

#### 5.4.3 *Compatible solute accumulation during cold acclimation*

Most plants accumulate osmolytes when exposed to abiotic stresses, such as drought, high salinity, and low temperature. The organic osmolytes, the so-called compatible solutes, have low molecular masses and high solubility in water and are non-toxic to the plants, even at high concentrations. Sugars (glucose, fructose, sucrose, and raffinose), amino acid (proline), and glycine betaine are the known compatible solutes. Studies with transgenic plants expressing genes for the biosynthesis of compatible solutes have revealed significant improvements in the tolerance to abiotic stresses, especially water stress (Kishor et al., 1995; Lilius et al., 1996; Hayashi et al., 1997; Romero et al., 1997; Sakamoto et al., 2000).

During cold acclimation, cellular osmotic concentrations quickly increase, primarily owing to the accumulation of various compatible solutes, such as sucrose, raffinose, and proline (Koster and Lynch, 1992; Hurry et al., 1995; Takagi et al., 2003; Kamata and Uemura, 2004). While their functions in freezing tolerance have not been clarified, sugars of the raffinose family have been implicated in plant tolerance to abiotic stresses. In many species, the accumulation of raffinose-family oligosaccharides during cold acclimation appears to correspond to enhanced freezing tolerance (Koster and Lynch, 1992; Bachmann et al., 1994; Castonguay et al., 1995; Gilmour et al., 2000).

### 5.5 Freezing tolerance

Freezing tolerance is essential for plants living in areas with subzero winter temperatures (Levitt, 1980). While precisely how plants survive freezing temperatures is unknown, to survive freezing, plants must increase the cryostability of their plasma membranes, which may be associated with changes in the plasma membrane per se and/or changes in other cellular components surrounding the plasma membrane. At present, changes in the composition of lipids and membrane proteins and increases in highly hydrophilic molecules, such as sugars and dehydrins, are mainly thought to induce plasma membrane cryostability. Freezing tolerance mechanisms described below are summarized, including the information about cold acclimation, in Figure 5.3.

Injury to be reduced	Molecular mechanism of tolerance	Related phenomenon during cold acclimation	Observed sample
H <sub>II</sub> phase	Close apposition PM Endomembrane Unsaturated species of PC	No phase transition Increase in unsaturated species of PC	Cold-acclimated plant
H <sub>II</sub> phase Fracture-jump lesion	PM Endomembrane Hydrophilic proteins • Compatible solutes	Avoiding the close apposition of membranes? Freezing Increase in hydrophilic proteins & compatible solutes	Cold-acclimated plant
Fracture-jump lesion	PM ER vesicles Endomembrane	Avoiding the close apposition of membranes? Freezing Increase in ER	Cold-acclimated woody plant
Mechanically disrupted membrane	Ca <sup>2+</sup> Cell wall PM Calcium-dependent membrane repair	Increase in SYT1 Non-acclimated plant Cold-acclimated plant	
Mechanically disrupted membrane	Cell wall PM Ice growth	?	Cold-acclimated plant
EIL (only in protoplast)	Exocytotic extrusion Freezing Thawing	Increase in unsaturated species of PC	Cold-acclimated protoplast

Figure 5.3 Summary of freezing tolerance mechanisms.

### 5.5.1 Membrane cryostability due to lipid composition

Changes in membrane lipid composition during cold acclimation are responsible for the decreased propensity of the  $H_{II}$  phase to form during freezing. Severe dehydration induced the  $H_{II}$  phase in liposomes formed from a total lipid extract of the plasma membrane of non-acclimated leaves but not in liposomes formed from the total lipids of plasma membranes of cold-acclimated leaves (Cudd and Steponkus, 1988). In addition, artificial enrichment of the plasma membrane with di-unsaturated species of PC precluded the participation of the plasma membrane in the freeze-induced formation of the  $H_{II}$  phase (Steponkus et al., 1988; Sugawara and Steponkus, 1990).

As described above, EIL is an irreversible freezing injury in protoplasts isolated from non-acclimated plants. In contrast, the plasma membrane of protoplasts isolated from cold-acclimated plants forms exocytotic extrusions during freeze-induced osmotic contraction, and the surface area is conserved such that EIL does not occur (Gordon-Kamm and Steponkus, 1984c). This difference in plasma membrane cryobehavior was also observed in liposomes prepared from total lipid extracts of the plasma membranes of non-acclimated and cold-acclimated rye leaves, suggesting that the differential cryobehavior during osmotic contraction was a consequence of alterations in the lipid composition of the plasma membrane (Steponkus and Lynch, 1989; Steponkus et al., 1993).

Direct evidence has been obtained by membrane engineering, in which the plasma membrane of protoplasts isolated from non-acclimated rye leaves was artificially enriched with mono- or di-unsaturated species of PC. In these protoplasts, the endocytotic vesiculation of the plasma membrane did not occur during osmotic contraction; instead, exocytotic extrusions formed (Steponkus et al., 1988). In addition, this lipid transformation increased freezing tolerance, because of a decrease in EIL (Steponkus et al., 1988; Uemura and Steponkus, 1989).

### 5.5.2 Membrane cryostability due to hydrophilic proteins

A number of genes that are regulated by low temperatures have been identified (Thomashow, 1998; Seki et al., 2001; Oono et al., 2006). The *COR* (cold-regulated) genes of *Arabidopsis* are one group of these genes and have been shown to encode various proteins, such as COR6.6, COR15a, COR78, and COR47. Many proteins encoded by these genes are hydrophilic polypeptides, but none have been shown to be membrane proteins with transmembrane domains. Because the amounts of mRNA encoding these proteins, as well as the amounts of the proteins themselves, correlate positively with freezing tolerance, these genes are thought to play roles in increasing freezing tolerance (Thomashow, 1998).

*COR15am*, the final product of the *COR15a* gene, is localized in the chloroplast stroma (Lin and Thomashow, 1992; Nakayama et al., 2007). Overexpression

of *COR15a* in *Arabidopsis* increased freezing tolerance in non-acclimated plants. Interestingly, studies with overexpression mutants have shown not only that the freezing tolerance of chloroplasts was enhanced (Artus et al., 1996) but also that the cryostability of the plasma membrane during freeze-induced dehydration increased as a consequence of a decrease in  $H_{II}$  phase formation (Steponkus et al., 1998). However, how *COR15am*, which is localized in the chloroplast stroma, protects the plasma membrane remains unclear.

Some cold-induced soluble proteins are thought to exist on or near the plasma membrane under conditions associated with cold acclimation. For example, immunoelectron microscope analyses have suggested that WCOR410, which is the COR47 homologue in wheat, tends to localize near the plasma membrane during cold acclimation (Danyluk et al., 1998). COR47 belongs to a family of acidic SK-type dehydrins (Nylander et al., 2001), and an acidic dehydrin from maize (DHN1) can bind to phospholipid vesicles (Koag et al., 2003, 2009). ERD10 and ERD14 (Early Response to Dehydration), which are very similar to COR47, also accumulate on the plasma membrane (Kawamura and Uemura, 2003). Thus, some dehydrins may become more interactive with the plasma membrane under freezing conditions and contribute to the cryostability of the plasma membrane. Studies with overexpression mutants have shown that dehydrins enhanced freezing tolerance (Puukainen et al., 2004), apparently by preventing damage to the plasma membrane during freezing (Uemura et al., 2006).

### 5.5.3 Compatible solutes and freezing tolerance

Compatible solutes are thought to increase protein conformation stability and membrane integrity under conditions of low-temperature or extracellular freezing. In fact, the transgenic plants that can accumulate high concentrations of glycine betaine in chloroplasts or the cytoplasm show higher tolerance to drought, low-temperature, and freezing stresses than wild type plants (Sakamoto and Murata, 2011; Chen and Murata, 2011). Also, transgenic plants of *Arabidopsis* and petunia that accumulate high levels of raffinose tolerate drought stress better than wild type plants, and transgenic petunia plants have higher freezing tolerance (Taji et al., 2002; Pennycooke et al., 2003).

In contrast, Zuther et al. (2004) concluded that raffinose is not essential for freezing tolerance in *Arabidopsis*, because neither insertion mutants in which raffinose was completely absent nor overexpressing mutants that accumulated high levels of raffinose affected *Arabidopsis* freezing tolerance before or after cold acclimation. Thus, the roles of compatible solutes in freezing tolerance remain obscure, even today. However, in *in vitro* studies with liposomes, sugars including sucrose and raffinose protected liposome membranes from fusion during drying (Hincha et al., 2003; Cacela et al., 2006); therefore compatible solutes may mitigate the freeze-induced dehydration stress to the plasma membrane.

#### 5.5.4 Membrane cryodynamics and membrane resealing

So far, the cryostability of the plasma membrane has been mainly thought to be statically maintained by increases in hydrophilic substances and changes in membrane lipid composition. However, some reports have supported the hypothesis that plasma membrane dynamics during freezing confer cryostability. Yamazaki et al. (2008a) reported that plant freezing tolerance involved membrane resealing.

In animal cells, even after the plasma membrane is damaged, cells can rapidly reseal damaged sites, but the process is strictly dependent on extracellular calcium (Steinhardt et al., 1994). For membrane resealing, an exocytotic event or vesicle-vesicle fusion must occur as calcium flows from the extracellular space into the cytoplasm through the damaged site. Two membrane resealing models have been proposed (McNeil and Kirchhausen, 2005). One is the facilitated resealing model, in which the decrease in membrane tension caused by extracellular calcium-dependent exocytosis can facilitate self-resealing of the membrane at the disrupted site. Another is the patching model, which describes the resealing mechanism when cells experience much larger membrane disruptions. In this model, after the calcium influx triggers vesicle-vesicle fusion, large patch vesicles form and ultimately fuse to the plasma membrane in an exocytotic manner. Membrane resealing involves many kinds of proteins, including soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, synaptotagmin VII, annexin A1, dysferlin, and calpain (Steinhardt et al., 1994; Bi et al., 1995; Reddy et al., 2001; Bansal et al., 2003; Chakrabarti et al., 2003; Shen et al., 2005; McNeil et al., 2006; Mellgren et al., 2007).

During a freeze/thaw cycle, plant cells are believed to suffer mechanical stress induced by freeze-induced dehydration, thaw-induced rehydration, and ice crystal growth (Levitt, 1980), although whether the plasma membrane is mechanically punctured during freezing/thawing is unclear. In fact, physiological, immunochemical, and genetic studies using protoplasts and leaf sections with intact cell walls have illustrated that the tolerance of cells to mechanical stress associated with ice crystal growth, but not with freeze-induced dehydration or thaw-induced rehydration, depends considerably on the presence of extracellular calcium, which is related to membrane resealing. In addition, this mechanism involves the function of SYT1, which increases during cold acclimation (Yamazaki et al., 2008a).

#### 5.5.5 Other membrane cryodynamics

When mulberry cortical parenchyma cells acquire extreme freezing tolerance in winter and are subsequently frozen, for example at  $-5^{\circ}\text{C}$ , multiplex lamellae (MPL) form by the fusion of ER vesicles (Fujikawa and Takabe, 1996). MPL completely cover the area beneath the plasma membrane and are composed

of a parallel array of sheet-like ER cisternae. This cryodynamic process is completed within 10 min of freezing at  $-5^{\circ}\text{C}$  and is quickly reversed upon thawing. Because similar membrane dynamics of ER vesicles are caused by osmotic dehydration of the cortical tissues in winter, the ER cryodynamics may be due to freeze-induced dehydration. The freeze-induced formation of MPL has been hypothesized play a role in avoiding the close apposition of membranes, including the plasma membrane (Fujikawa and Takabe, 1996).

In protoplasts isolated from cold-acclimated *Arabidopsis* leaves, many vesicular structures appear in the cytoplasmic region near the plasma membrane just after extracellular freezing occurs (Yamazaki et al., 2008b). These structures, referred to as freeze-induced vesicular structures (FIVs), then develop horizontally near the plasma membrane as freezing continues. There is a strong correlation between increasing size of individual FIVs and decreased protoplast surface area during freezing. Occasionally, FIVs fuse with the plasma membrane, which may be necessary to relax the stress upon the plasma membrane during freezing. Vesicular structures resembling FIVs are also induced when protoplasts are mechanically pressed at room temperature. Because fewer FIVs form when protoplasts are treated with hyperosmotic solutions, FIV formation is associated with mechanical stress rather than dehydration stress. Even in epidermal cells with intact cell walls, the formation of ice crystals in the intercellular space leads to the formation of vesicle-like structures with similar properties to FIVs. To withstand the mechanical stress induced by extracellular freezing, cold-acclimated plant cells may mitigate tension in the plasma membrane by regulating its surface area.

In contrast, many filiform projections, referred to as exocytotic extrusions, develop on the surface areas of protoplasts isolated from cold-acclimated leaves of winter rye and *Arabidopsis* and treated with hyperosmotic solution (Dowgert and Steponkus, 1984; Steponkus et al., 1988; Yamazaki et al., 2008b). Exocytotic extrusions appeared even in protoplasts isolated from non-acclimated leaves of winter rye in which the proportion of di-unsaturated species of PC was artificially increased; this proportion increases naturally during cold acclimation in the plasma membranes spring oat, winter rye, and *Arabidopsis* (Steponkus et al., 1993; Uemura and Steponkus, 1994; Uemura et al., 1995). Exocytotic extrusions may be based on the physicochemical features of lipid composition in the plasma membrane (Steponkus and Lynch, 1989; Steponkus et al., 1993). Because fewer exocytotic extrusions develop when protoplasts are subjected to freezing than to hyperosmotic solution, the development of exocytotic extrusions may be caused by dehydration stress. However, whether and how the exocytotic extrusions observed in protoplasts are related to freezing tolerance in intact cell with cell walls remains unclear, although exocytotic extrusions are believed to enhance freezing tolerance of protoplasts isolated from cold-acclimated leaves as a way to avoid EIL (Dowgert and Steponkus, 1984).

### 5.6 Conclusion

In this chapter, we focused on plant low-temperature tolerances at the cellular level. However, even now, the detailed molecular mechanisms of low-temperature tolerances remain unresolved. This uncertainty is because few cell biological studies have examined living cells at low temperatures, including freezing temperatures. In addition, to better understand the low-temperature tolerance mechanisms of intact plants, we must consider not only the cellular level but also tissue and organ levels. For example, in freezing tolerance, some plants may regulate ice crystal formation within their bodies (Ishikawa et al., 1997; Ide et al., 1998; Pearce and Fuller, 2001), and consequently, the freeze-induced stresses should be different in each tissue and organ. In the future, both cellular and higher perspectives will be needed to elucidate plant low-temperature tolerances.

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## **6    Salinity tolerance**

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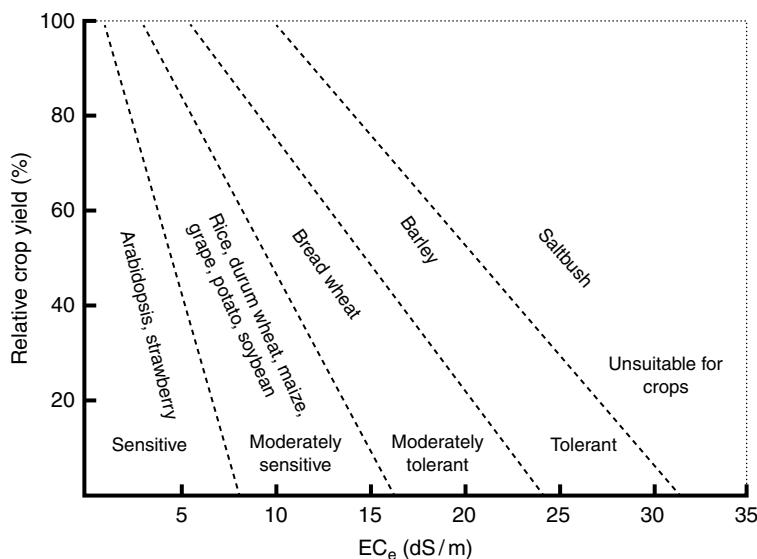
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Food and Wine, University of Adelaide, South Australia, Australia

### **6.1   Plant growth on saline soils**

Saline soils reduce plant growth and crop yields. It is estimated that 6–10%, or around 800 million ha, of the total land surface of the world is salt affected (Szabolcs, 1994; Eynard et al., 2005; Munns, 2010a). This area is predicted to increase as land continues to be cleared and irrigated with low-quality water to maintain global food supplies, accompanied by the detrimental and non-sustainable impacts of farming practices on soils.

Primary soil salinity is the result of natural processes and interactions between climate, weathering, geology, deposition, and redistribution of salts in groundwater systems (Jardine et al., 2011); for example, weathering of rocks releases sodium, potassium, magnesium and calcium chlorides, sulfates, carbonates and bicarbonates. Sodium chloride (NaCl) is the most soluble of these salts (Eynard et al., 2005; Szabolcs, 1994). Other sources of salinity include ocean salts carried inland by wind and rain that are deposited on soils and rainwater that contain between 6 and 50 mg/kg of sodium chloride (Munns and Tester, 2008; Munns, 2010b). These environmental effects lead to gradual increases in soil salinity over time.

Secondary soil salinity is caused by land clearing or irrigation. Clearing land of deep-rooted perennial native vegetation and replanting with shallow rooted crops and pastures results in rising water tables, bringing dissolved salts toward the soil surface. Groundwater often has high salt levels accumulated over a long period from primary processes, and this salinity impacts on plant growth when the water table is close to the soil surface (Rengasamy, 2002). About 2%, or 32 million of the 1,500 million ha farmed by dryland agriculture, is affected by secondary salinity (FAO, 2008). Of the 230 million ha of irrigated farmland, around 20% (45 million ha) is salt affected (FAO, 2008). The area of salt affected land is estimated to be increasing by 1–2% per year.



**Figure 6.1** Crop species can be described according to their sensitivity to salinity in the soil, ranging from sensitive to tolerant. As soil salinity increases, the yield of the crop decreases. For a salt sensitive species growing in soil with 4 dS/m (approximately 40mM NaCl), yield can be reduced by 50% where a salt tolerant species will have no yield penalty. Figure modified with permission of the American Society of Civil Engineers (ASCE) from Maas and Hoffman, 1977; Ayers and Westcott, 1985; and Munns and Tester, 2008.

(FAO, 2000). This is highly significant as yields of most irrigated crops can be as much as 4 times greater than those from dry land agriculture (FAO, 2008), and irrigated land provides approximately 40% of the world's food (FAO, 2000).

Soil salinity can be quantified by measuring the electrical conductivity ( $EC_e$ ) of a soil, which reflects the quantity of readily available ions in soil water. In general terms, saline soil is defined as having an  $EC_e$  of more than 4 dS/m, which is roughly equivalent to 40mM NaCl (Munns and Tester, 2008). Crop plants have varying sensitivities and yield reductions as a result of growing in saline conditions (Figure 6.1). Some crops are extremely sensitive to salt and struggle to generate a yield even in moderately saline environments, such as citrus (Maas and Hoffman, 1977; Storey and Walker, 1998) and rice (Maas and Hoffman, 1977; Aslam et al., 1993; Munns and Tester, 2008), while others are substantially more tolerant, like barley (Maas and Hoffman, 1977; Colmer et al., 2005; Munns and Tester, 2008; Figure 6.1 and Table 6.1). The sensitivity of a crop can also vary with variety and the developmental stage it has at exposure to salt stress (Eynard et al., 2005).

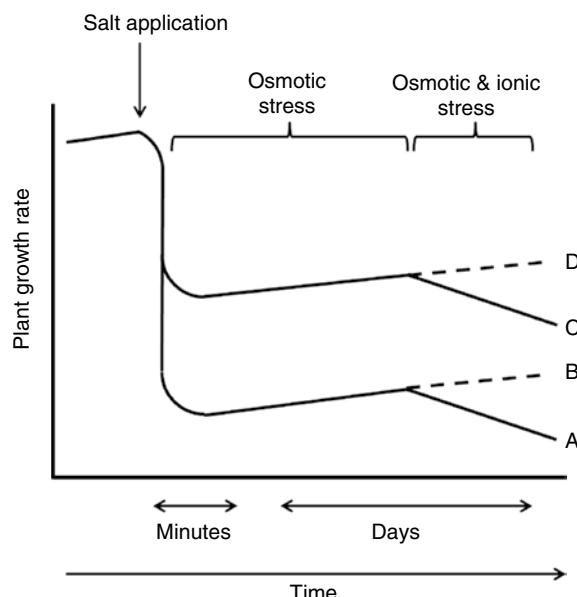
**Table 6.1** Yield potential.

Crop	Yield Potential		
	100% EC <sub>e</sub> (dS/m)	50% EC <sub>e</sub> (dS/m)	0% EC <sub>e</sub> (dS/m)
Barley ( <i>Hordeum vulgare</i> )	8.0	18	28
Wheat ( <i>Triticum aestivum</i> )	6.0	13	20
Wheat ( <i>Triticum turgidum sp durum</i> )	5.7	15	24
Soybean ( <i>Glycine max</i> )	5.0	7.5	10
Rice ( <i>Oryza sativa</i> )	3.0	7.2	7.6
Tomato ( <i>Lycopersicon esculentum</i> )	2.5	7.6	13
Alfalfa ( <i>Medicago sativa</i> )	2.0	8.8	16
Grapefruit ( <i>Citrus paradis</i> )	1.8	4.9	8.0
Orange ( <i>Citrus sinensis</i> )	1.7	4.8	8.0
Grape ( <i>Vitis sp.</i> )	1.5	6.7	12
Broccoli* ( <i>Brassica oleracea L. Italica</i> )	1.3	4.6	7.2

Note: In ideal conditions crop species have a yield potential of 100%. The impact of soil salinity on crop yields varies according to how sensitive the species or variety is to salt. Accordingly, there is a threshold value of soil salinity above which the yield of a crop reduces and a value where growth, flowering, and fruit or seed set is so low that no yield is expected (Grieve et al., 2007; Maas and Hoffman, 1977; Shannon and Grieve, 1998). Table adapted from Maas and Hoffman (1977), Ayers and Westcott (1985), and \*De Pascale et al. (2004). To convert from dS/m to mM NaCl, multiply by 10—note that this conversion is approximate and depends on a number of factors including soil type.

### 6.1.1 Effects of salt stress on plant growth

There are two groups of plants in terms of salinity tolerance; halophytes, which are plants native to areas with saline soils and glycophytes, that have a lower capacity to tolerate salinity. Halophytes can tolerate salt concentrations that kill 99% of other plants and many require high concentrations of salt (50–250 mM NaCl) to achieve optimal growth, concentrations that would kill most glycophytes (Flowers and Colmer, 2008). In glycophytic monocots, such as wheat, barley and rice, the major effects of salt stress are a reduction in number of tillers and total leaf area, which in turn reduces grain yield (Yeo et al., 1991; Colmer et al., 2005; Eynard et al., 2005; Munns and Tester, 2008). Glycophytic dicots such as soybean (Munns and Tester, 2008), cowpea (Lenis et al., 2011), and bean (Pitann et al., 2011) show reduced leaf area and number of branches per plant. Interestingly, root growth in cereals is less sensitive to salt exposure and recovers more quickly than shoot growth (Frensch and Hsiao, 1994; Munns, 2002). The plant response to salinity can be separated into two phases that are characterized as osmotic and ionic stress (Figure 6.2; Munns et al., 1995; Munns, 2002; Munns and Tester, 2008), and plants use different mechanisms to manage these stresses.



**Figure 6.2** Plant responses to salinity can be considered in two phases. The first phase is the immediate, osmotic response, which is mostly due to the more negative osmotic pressure of saline soil water. The second phase is ionic and slower, as it takes some time for salts to accumulate. It becomes evident once salts accumulate in the plant tissue to a level that generates toxic effects. Growth of plants is affected in both phases. Plants vary in their responses to salt and a plant can be categorized as having (A) osmotic and ionic sensitivity, (B) osmotic sensitivity and ionic tolerance, (C) osmotic tolerance and ionic sensitivity, and (D) osmotic and ionic tolerance. Figure modified from Munns and Tester, 2008.

### 6.1.2 Osmotic stress

When plants are exposed to salinity there is an immediate reduction in leaf expansion, shoot growth rates, and lateral bud development that is sustained for the whole growth period of the plant (Figure 6.2; Passioura and Munns, 2000; Fricke and Peters, 2002; Munns and Tester, 2008; Rahnama et al., 2011). This reduction in growth leads to delays in flowering time and reduced crop yield (Munns and Tester, 2008). In addition to these long-term effects, there is a transient loss of cell turgor that is caused by the sudden change in plant water relations. Dissolved salts in the soil water make the water potential more negative and therefore water uptake by the roots is more difficult (Munns, 2002; Fricke, 2004).

It is important to note, that the concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  that accumulate in the shoots over this period are not at levels that inhibit growth (Termaat and Munns, 1986; Yeo et al., 1991; Fricke, 2004; Hu et al., 2007; Munns and Tester, 2008), and the cellular and metabolic processes affected during the osmotic

stress period are similar to plant drought responses (Huang et al., 2012). The mechanisms behind this reduction in shoot growth are still unclear, although hormonal and non-hormonal root to shoot signaling pathways have been implicated (Davies et al., 2005; Miller et al., 2010; Huang et al., 2012; Wilkinson et al., 2012). This growth reduction does appear to be independent of water supply and nutrient availability (Munns et al., 2000a; Fricke and Peters, 2002).

In addition to reduced growth rates, osmotically stressed plants close stomata to manage water loss (Fricke, 2004; James et al., 2008). Stomatal closure restricts  $\text{CO}_2$  uptake, resulting in reduced carbon fixation and assimilation in leaf tissue. Carbohydrate production during photosynthesis is therefore reduced, which impacts on plant growth and crop yield. Some plants compensate for the reduction in  $\text{CO}_2$  by developing leaves that are smaller, thicker, and have more densely packed chloroplasts but this has a high energy cost to the plant (Munns and Tester, 2008; Lenis et al., 2011). Another consequence of stomatal closure is a reduction in latent heat loss as evapotranspiration of water from within the leaf and out through stomata is reduced (Nobel, 2009). Water is conserved but leaf temperature increases significantly and can be quantified using infra-red thermography and image analysis (Jones, 1999; Sirault et al., 2009). Closing of stomata also interrupts the soil-plant-atmosphere continuum, raising the water potential within the plant. Higher water potential means the plant has a lower capacity to take up water and nutrients from the soil (Nobel, 2009).

A consequence of reduced rates of photosynthesis is the build-up of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, which results from excess light energy from photosynthesis being transferred to oxygen acceptors other than water (Møller et al., 2007). Although ROS have a role in plant signaling and stress responses, including osmotic stress caused by salinity (Boursiac et al., 2008; Miller et al., 2010; Chen and Murata, 2011; Huang et al., 2012) high concentrations of ROS in cells results in lipid peroxidation, tissue damage and cell death (Wise and Naylor, 1987; Møller et al., 2007; Triantaphylidès et al., 2008).

### 6.1.3 Ionic stress

Ionic stress occurs once  $\text{Na}^+$  and  $\text{Cl}^-$  accumulate to toxic levels in tissues—this process takes time to occur (Figure 6.2). Most salt in the soil is excluded from the plant, but what salt is taken up by the roots is transported to the shoot in the transpiration stream (Munns and Passioura, 1984). As there are low levels of redistribution of salts via the phloem (Wolf et al., 1990; Tester and Davenport, 2003; Munns and Tester, 2008)  $\text{Na}^+$  and  $\text{Cl}^-$  can reach high concentrations in older leaf tissues, resulting in premature senescence (Munns and Tester, 2008; Munns, 2002). The majority of plants, including bread wheats, exclude about 98% of salt in the soil water (Munns, 2005). Those less efficient at excluding salts are rice, barley, and durum wheat, which still exclude at least 94% (Munns, 2005).

In most crop plants, including cereals,  $\text{Na}^+$  accumulates to toxic levels before  $\text{Cl}^-$  (Munns and Tester, 2008). Some crops such as soybean, citrus and grape vines, restrict  $\text{Na}^+$  more effectively within their roots and stems, and it is  $\text{Cl}^-$  that accumulates to levels that cause shoot damage (Flowers and Yeo, 1988; Storey and Walker, 1998; Teakle and Tyerman, 2010).

The degree of damage from ions, such as  $\text{Na}^+$  and  $\text{Cl}^-$ , depends on the rate of accumulation and how effective ion compartmentation is within cells (Tester and Davenport, 2003). There are adverse metabolic effects caused by high cytoplasmic concentrations of  $\text{Na}^+$  in plant cells, as  $\text{Na}^+$  competes with  $\text{K}^+$ , an essential element for many cellular functions. High levels of  $\text{Na}^+$  within the cell increase the  $\text{Na}^+:\text{K}^+$  ratio and reduce the availability of  $\text{K}^+$ , disrupting many enzymatic processes (Bhandal and Malik, 1988). Protein synthesis also requires  $\text{K}^+$  so that tRNA can bind to ribosomes (Wyn Jones et al., 1979), and when  $\text{K}^+$  concentrations are insufficient, protein synthesis is reduced (Blaha et al., 2000). In growing leaves, the cumulative salt effects are diluted as cells expand but once growth slows and ceases, toxic effects become evident (Munns, 1993; Munns, 2002).

## 6.2 Tolerance mechanisms

Plants have evolved a number of salinity tolerance strategies that allow them to survive and grow on saline soils. These multiple salt tolerance mechanisms can usually be categorized as “osmotic tolerance,” the ability to maintain growth under osmotic stress, and “ionic tolerance,” the ability to deal with the ionic component of salt stress.

### 6.2.1 Osmotic tolerance

Plants that are osmotically tolerant maintain shoot and root growth, and leaf stomatal conductance when exposed to soil salinity (Figure 6.2). This is useful where soil water is not a limiting factor for growth, but maintenance of growth and high stomatal conductance can be a problem in dryland agriculture when there is insufficient water. The mechanisms behind this tolerance are largely unknown but must be related to the ability to assimilate  $\text{CO}_2$ , to promote new growth, and maintain water uptake by the roots. Variation in osmotic tolerance within crop species is believed to exist but has been difficult to characterize based on growth as in the past it required destructive sampling (Yeo et al., 1991; Munns and James, 2003; Munns and Tester, 2008). New phenotyping methods, such as estimations of plant biomass from digital images (Rajendran et al., 2009) and/or the use of thermal imagery to determine leaf temperature (Sirault et al., 2009) as an indirect measurement of stomatal conductance, are now being used to determine the variation for osmotic tolerance in crop plants.

Many of the components of osmotic tolerance are envisioned to have similarities to strategies employed by plants during drought stress.

### 6.2.2 Ionic tolerance

How effectively a plant excludes salt from its tissues and how it tolerates accumulating salt are two elements of the slower, second phase of the salt-specific, ionic tolerance response to salinity (Figure 6.2). Those plants that primarily exclude salt from the leaf tissues, but not necessarily their roots, are described as using ion exclusion mechanisms. Plants that can accumulate high concentrations of salt in their aboveground biomass are frequently described as using ionic tissue tolerance mechanisms—even though most plants, including halophytes, exclude over 90% of the salt in the soil (Munns, 2005). As  $\text{Na}^+$  can accumulate to toxic levels in the shoot of most crop species before  $\text{Cl}^-$ , the majority of research has focused on  $\text{Na}^+$  exclusion and  $\text{Na}^+$  tissue tolerance.

### 6.2.3 Ion exclusion

Roots rarely accumulate high levels of  $\text{Na}^+$  and  $\text{Cl}^-$  (Munns, 2005), as the salts taken up are either exported back into the soil or transported to the shoot. In the shoots, evaporation of water across the leaf cuticle or through stomata results in net water movement from the root to the shoot—referred to as the *transpiration stream*. High transpiration rates result in greater concentrations of both  $\text{Na}^+$  and  $\text{Cl}^-$  in tissues (Hasegawa et al., 2000; Apse and Blumwald, 2007; Munns and Tester, 2008). Plants transpire approximately 50 times more water than is retained in the shoot (Munns et al., 2006). If the rate of water loss from leaves can be reduced, by closing stomatal pores for example, it will reduce transpiration and therefore reduce ion accumulation in the shoot. A relationship between salt exclusion and salinity tolerance has been established in rice (Zhu et al., 2001; Lee et al., 2003), *Lotus* (Teakle and Tyerman, 2010), *Medicago* (Sibole et al., 2003), durum wheat (Munns and James, 2003; Poustini and Siosemardeh, 2004), and barley (Forster, 2001; Wei et al., 2003; Garthwaite et al., 2005). For effective ion exclusion, Munns et al. (2006) suggest that salt tolerance depends on controlling salt transport at a number of key control points: (1) minimizing the uptake of ions from the soil into epidermal and cortical cells of roots and/or by maximizing the salt efflux back into the soil; (2) reducing the amount of  $\text{Na}^+$  and  $\text{Cl}^-$  being loaded into the root xylem, and therefore the transpiration stream; (3) maximizing salt retrieval from the xylem into tissues such as the roots, stem, or leaf sheath; and (4) increasing phloem loading of  $\text{Na}^+$  and  $\text{Cl}^-$  to transport it away from the leaves. If toxic levels of  $\text{Na}^+$  can be excluded or removed from the plant, premature senescence can be prevented and crop yields improved. As an example, a salt-tolerant durum wheat

genotype is better at excluding  $\text{Na}^+$  from the leaf blade by sequestering  $\text{Na}^+$  in the leaf sheath, than salt-sensitive durum varieties (Davenport et al., 2005).

#### 6.2.4 Ion tissue tolerance

Although most  $\text{Na}^+$  and  $\text{Cl}^-$  in the soil are excluded from the plant, salt still accumulates to toxic levels. For a plant to tolerate toxic levels of  $\text{Na}^+$  and  $\text{Cl}^-$  in tissue and maintain healthy growth, flowering and seed set, compartmentalisation of ions is required (Munns and Tester, 2008). At the cellular level it is important to maintain low levels of  $\text{Na}^+$  and  $\text{Cl}^-$  in the cytoplasm so metabolic processes are not affected. Additional cytoplasmic  $\text{K}^+$  concentrations relative to  $\text{Na}^+$  can alleviate the inhibition of metabolic processes resulting from high  $\text{Na}^+$  (Zhu, 2002), but no correlations between leaf  $\text{K}^+$  concentrations and salinity tolerance have been established (Munns and Tester, 2008). As minimal  $\text{Na}^+$  (Munns et al., 2006) and  $\text{Cl}^-$  (Teakle and Tyerman, 2010) are relocated from plant tissues via the phloem, one strategy that allows the accumulation of ions in tissues, but not in the cell's cytosol, is sequestration of the  $\text{Na}^+$  and  $\text{Cl}^-$  within the vacuole. Halophytes often accumulate high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in plant tissues (Flowers and Colmer, 2008; Amtmann and Beilby, 2010; Flowers et al., 2010a). Similar to salt-sensitive glycophytes, halophytes must minimize the build-up of  $\text{Na}^+$  and  $\text{Cl}^-$  in the cytosol of cells—the high concentrations of shoot  $\text{Na}^+$  and  $\text{Cl}^-$  in the leaves of halophytes are due to effective sequestration of the ions into the vacuoles (Flowers and Colmer, 2008; Flowers et al., 2010a). Some varieties of crops, such as several wheat species, show similar halophytic characteristics in that they more effectively accumulate ions in their vacuoles, thus have higher shoot  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations than salt-sensitive cultivars and exhibit good salt tolerance (Genc et al., 2007; Rajendran et al., 2009). However, cereals cannot accumulate the same high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  in their tissues as true halophytes (Colmer et al., 2006; Munns and Tester, 2008). Attempts have been made by breeding programs to introduce halophytic characteristics from wild, salt-tolerant species that are close relatives of crop plants, such as tall wheatgrass (*Thinopyrum*) and sea barleygrass (*Hordeum marinum*; Farooq et al., 1989; Flowers, 2004; Colmer et al., 2005; 2006); however, few salt-tolerant cereal varieties have been released by this approach (Colmer et al., 2006).

### 6.3 Identification of variation in salinity tolerance

#### 6.3.1 Variation in current crops

To improve crop salinity tolerance it is first necessary to identify variation in salt tolerance mechanisms within plants, noting those plants with increased

tolerance compared to current crop cultivars. For many crop species, large variation for salinity tolerance already exists, with some varieties better at maintaining yield under saline conditions. The selection and inclusion of these varieties in breeding programs will allow the generation of future salt-tolerant crop plants. Differences in yield between varieties is observed in many crops including bread wheat (Richards et al., 1987; Genc et al., 2007), durum wheat (Munns et al., 2000b; Munns and James, 2003; James et al., 2006), barley (Richards et al., 1987; Slavich et al., 1990), rice (Gregorio et al., 2002; Lee et al., 2003; Sahi et al., 2006), chickpea (Vadez et al., 2007; Flowers et al., 2010b), soybean (Yeo and Flowers, 1983; Lee et al., 2009), and citrus (Storey and Walker, 1998; Tozlu et al., 1999). For instance, the screening of 400 Iranian wheat varieties identified a number of accessions that were able to maintain high grain yields in salt stressed environments (Jafari-Shabestari et al., 1995). Similarly, a screen of 5,000 bread wheat accessions identified 29 that were able to grow and produce seed when exposed to 50% seawater (Kingsbury and Epstein, 1984).

The ability to maintain low shoot  $\text{Na}^+$  and high  $\text{K}^+$  concentrations is often been cited as an important salinity tolerance mechanism for wheat (Gorham et al., 1997; Colmer et al., 2006), particularly durum wheat (Dvorak et al., 1994; Husain et al., 2003; Munns and James, 2003). Variation is observed for  $\text{Na}^+$  exclusion in the leaves of durum wheat (Munns et al., 2000b; Husain et al., 2003; Munns and James, 2003) and bread wheat (Colmer et al., 2005; Munns et al., 2006; Bağci et al., 2007; Genc et al., 2007). In some cases this variation is relatively low with only a 1.5-fold difference in shoot  $\text{Na}^+$  accumulation across multiple cultivars (Shavrukov et al., 2009).  $\text{Na}^+$  exclusion is not the sole mechanisms for salinity tolerance in crops, however, and in some cases there is no clear link between  $\text{Na}^+$  exclusion from the shoot and crop salinity tolerance (Genc et al., 2007). In these instances, tolerance mechanisms other than  $\text{Na}^+$  exclusion must be responsible for the observed differences in salt tolerance. Variation for other salinity tolerance traits, such as the ability to germinate in saline environments (Ma et al., 2007), the degree of stomatal closure in response to osmotic stress (James et al., 2008), and improved grain yield under salt stress (Quarrie et al., 2005) is recorded.

### 6.3.2 Variation in near wild relatives

Unfortunately, while there is some variation for salt tolerance within our current crops, the narrowing of the genetic diversity within elite germplasm during plant breeding means there is reduced genetic variation within crops. It is estimated that of the total genetic variation observed in wild wheat and barley germplasm, only 15% and 40% has been captured in modern wheat and barley varieties, respectively (Langridge et al., 2006; Feuillet et al., 2008; Tester and Langridge, 2010). Centuries of traditional breeding for particular traits, such as

yield, have narrowed the total genetic diversity and as a result advantageous salt tolerance traits have been lost. Wide genetic diversity exists in plants that are either landraces or near wild relatives of modern day crops, as shown by studies comparing the diversity of molecular markers between cultivated crops and their relatives (Russell et al., 2004; Caldwell et al., 2006; Nevo and Chen, 2010). These, often untapped, resources of genetic variation are potential sources of novel salt stress mechanisms and/or genes, with their close genetic identity to current crops allowing candidate genes to be introduced into commercial lines by conventional breeding approaches (Hajjar and Hodgkin, 2007; Feuillet et al., 2008; Nevo and Chen, 2010; Tester and Langridge, 2010). The success of mining this genetic diversity is seen with the introduction of traits for improved disease and pest resistance into wheat from the wild wheat relatives *Aegilops speltoides*, *T. monococcum*, *T. uratu*, and *T. tauschii* (Hoisington et al., 1999; Hajjar and Hodgkin, 2007; Feuillet et al., 2008).

In the wild relatives of wheat, significant variation for shoot Na<sup>+</sup> accumulation is observed in *T. monococcum* (Shah et al., 1987; Gorham et al., 1991; Colmer et al., 2006; Rajendran et al., 2009; Shavrukov et al., 2009), *T. dicoccoides* (Nevo et al., 1992; Colmer et al., 2006; Shavrukov et al., 2010b), *A. tauschii* (Shah et al., 1987; Gorham, 1990; Schachtman et al., 1991; Colmer et al., 2006; Shavrukov et al., 2009), and *T. uratu* (Gorham et al., 1991; Colmer et al., 2006; Shavrukov et al., 2009). Wild relative accessions from dry and hot climates often exhibit significantly less Na<sup>+</sup> uptake than those from more rain fed environments (Nevo et al., 1992; Shavrukov et al., 2010b). Variation for other salt tolerance traits, including osmotic tolerance (Rajendran et al., 2009), yield and biomass production (Farooq et al., 1989; Schachtman et al., 1991; Nevo et al., 1993), Cl<sup>-</sup> accumulation (Datta et al., 1995), and Na<sup>+</sup> tissue tolerance (Rajendran et al., 2009) are observed in near wild relatives of wheat.

Similarly, the wild relatives of barley, such as *Hordeum spontaneum*, *H. bogdanii*, *H. marinum* and *H. intercedens* are found growing in a range of environments, such as deserts and mountainous regions, all with differences in water availability, temperature, and soil types (Nevo and Chen, 2010). Wild barley can be a source of novel genes and traits for improving the salinity tolerance of cultivated barley (Nevo et al., 1993; Garthwaite et al., 2005; Nevo and Chen, 2010). Traits for reduced shoot Na<sup>+</sup> (Pakniyat et al., 1997; Garthwaite et al., 2005; Islam et al., 2007; Malik et al., 2009), reduced shoot Cl<sup>-</sup> (Islam et al., 2007; Malik et al., 2009), improved leaf elongation rates (Cramer, 2003), and improved biomass under salt stress (Garthwaite et al., 2005; Yan et al., 2008) have already been identified in wild barley. Artificial amphiploids of wheat, produced by incorporating DNA from *H. marinum* have 39% and 36% of the shoot Na<sup>+</sup> and Cl<sup>-</sup> levels, respectively, when compared to bread wheat (Islam et al., 2007). Similar successes have also been observed by introducing salt tolerance traits from the halophytic tall wheatgrasses (as reviewed in Colmer et al., 2006).

Recently two genes encoding for the  $\text{Na}^+$  transporters *Nax1* (*TmHKT1;4*) and *Nax2* (*TmHKT1;5*) were introgressed from *T. monococcum* into the durum variety Tamaroi (Lindsay et al., 2004; James et al., 2006; Byrt et al., 2007). *Nax2* encodes a transporter responsible for retrieving  $\text{Na}^+$  from the xylem in the root, resulting in less  $\text{Na}^+$  translocated to the shoot (James et al., 2006). *Nax1* encodes a protein that removes  $\text{Na}^+$  from the transpiration stream in the shoot, resulting in increased partitioning of  $\text{Na}^+$  in the sheath away from the leaf blade (James et al., 2006). Recent field trials demonstrate the benefit of incorporating these genes into current wheat cultivars, with the durum cultivar Tamaroi exhibiting increased yield in saline soils when it contains the *Nax2* gene (James et al., 2012; Munns et al., 2012). Despite these promising results, a salt-tolerant wheat genotype with a gene derived from a wild relative is not yet available for commercial use by farmers.

### 6.3.3 Variation in model species

Model species, such as *Arabidopsis thaliana* and *Thellungiella salsuginea* (formerly *T. halophilla*), are widely used to gain understanding of important components of salinity tolerance and the genes behind them, due to their relatively simple genomes, ease of transformation and rapid lifecycles (Bressan et al., 2001; Amtmann et al., 2005). While *Arabidopsis* is a salt-sensitive glycophyte, and the extrapolation of results to cereals should be made with caution (as explained in Møller and Tester, [2007]), there is no denying its usefulness in research into salinity tolerance. A large number of *Arabidopsis* mapping populations are now available that are produced from crosses between ecotypes with large environmental and genetic distances (Lister and Dean, 1993; Alonso-Blanco et al., 1998; Loudet et al., 2002; Koornneef et al., 2004; Törjék et al., 2006; O'Neill et al., 2008; Kover et al., 2009). These populations are used to identify ecotypes with improved salinity tolerance and/or the identification of important traits and genes (Quesada et al., 2002; Rus et al., 2006; Buescher et al., 2010; Prinzenberg et al., 2010; Ren et al., 2010; Vallejo et al., 2010; Roy et al., 2012). In addition, because of *Arabidopsis*'s fast lifecycle and ease of genetic manipulation, artificially induced variation in salt tolerance, through the generation of mutant lines, has also lead to the discovery of important genes for salt tolerance, for example, the *SOS* genes (Wu et al., 1996; Liu et al., 2000). Due to these diverse sources of genetic variation, many of the important salt tolerance genes are either being discovered in *Arabidopsis* (e.g., the *SOS* genes [Wu et al., 1996]) or, if discovered in other species (for example *TaHKT2;1* (Schachtman and Schroeder, 1994)), the *Arabidopsis* homologues are more thoroughly characterized.

*T. salsuginea*, in contrast to its *Arabidopsis* relative, can tolerate extreme salinity, drought and cold, showing no symptoms of salt stress when grown at 300 mM NaCl (Oh et al., 2009) and able to maintain water relations at 600 mM

NaCl (the equivalent of seawater; Amtmann et al., 2005). Studies investigating the mechanisms involved in this plant's salt stress response may elucidate further important traits and genes for salinity tolerance.

#### *6.3.4 New phenomic approaches to identify variation in salinity tolerance*

To identify and further characterize diversity in salt tolerance mechanisms, such as osmotic and ionic tolerance, new rapid screening technologies to phenotype plants are required. Phenomics, the study of the growth, performance and composition of plants, has been described as a "high-throughput plant physiology" (Furbank and Tester, 2011). Non-destructive imaging and remote sensing technologies are being used in both controlled environments (Rajendran et al., 2009; Golzarian et al., 2011) and the field (Falkenberg et al., 2007; Qiu et al., 2009) to evaluate and phenotype crops for salinity tolerance traits that, until recently, have proven difficult to quantify. Parameters that can now be measured quickly and easily on a large number of plants include: maintenance of shoot growth immediately after salt stress, plant form and structure, leaf temperature (as an indirect measure of stomatal conductance), chlorophyll state, leaf water status and carbohydrate content (Rajendran et al., 2009; Sirault et al., 2009; Furbank and Tester, 2011). Use of these technologies will enable the identification of alternative sources variation in salinity tolerance in both model and crop species of plants.

### **6.4 Forward genetic approaches to identify salinity tolerant loci and candidate genes**

#### *6.4.1 QTL mapping*

To take advantage of natural variation in salinity tolerance between and within plant species, approaches are required that will allow the speedy identification of the genes behind important salt tolerance traits and then transfer these traits into current elite cultivars. Simply crossing a salt-tolerant accession with an elite high yielding cultivar, assessing the salinity tolerance of the offspring, obtaining viable seed and repeating the crossings with a parent to produce the next generation is labour intensive, expensive and slow. It also introduces undesirable traits that need to be removed over multiple generations.

One approach is to make use of molecular marker technologies that can speed up the production of cultivars by establishing a DNA marker that is closely linked to the desired salt tolerance phenotype. All varieties of crops and species have differences in their DNA—differences to which specific molecular markers can be designed. These differences can be extreme, such as deletions

or gene duplications or they can be subtle, a single change in a nucleotide of a gene. Molecular markers are designed to recognise these specific differences between the DNA of different organisms along their chromosomes, thereby allowing the production of a chromosome map establishing where these differences are. By phenotyping the offspring produced by crossing a salt-sensitive with a salt tolerance variety and then genotyping the offspring for molecular markers, it is possible to identify regions in the DNA that are linked to the salt tolerance trait. These regions are called *quantitative trait loci* (QTL). If a molecular marker is on a section of DNA that is close to the real gene that is responsible for the salt tolerance phenotype, breeders can screen the DNA of a plant to determine if it has the desired salt tolerance trait, rather than having to test it experimentally—a process that will speed up the selection process. Fine mapping of these QTL regions will also allow the identification of the specific gene responsible for encoding the salt tolerance trait, allowing a transgenic approach to be taken.

QTL for salinity tolerance are now identified in a large variety of crops (Bretó et al., 1994; Dubcovsky et al., 1996; Mano and Takeda, 1997; Tozlu et al., 1999; Koyama et al., 2001; Gregorio et al., 2002; Lee et al., 2004; Lindsay et al., 2004; Quarrie et al., 2005; Ren et al., 2005; Sahi et al., 2006; Ma et al., 2007; Villalta et al., 2008; Estañ et al., 2009; Genc et al., 2010; Shavrukov et al., 2010a; Ahmadi et al., 2011; Roy et al., 2011), and model species, such as *Arabidopsis* (Quesada et al., 2002; Buescher et al., 2010; Galpaz and Reymond, 2010; Ren et al., 2010; Vallejo et al., 2010; Table 6.2). While some of the QTL are over genes known to be involved in salinity tolerance (Ren et al., 2005; Rus et al., 2006; Genc et al., 2010; Shavrukov et al., 2010a), others have led to the discovery of novel genes (Ren et al., 2010). For some QTL the candidate genes have yet to be identified and/or confirmed (Quesada et al., 2002; Xue et al., 2009; Vallejo et al., 2010).

QTL for shoot Na<sup>+</sup> accumulation are frequently identified in mapping populations for a variety of plants (Koyama et al., 2001; Lin et al., 2004; Lindsay et al., 2004; Xue et al., 2009; Genc et al., 2010; Shavrukov et al., 2010a; Thomson et al., 2010; Ul Haq et al., 2010; Rivandi et al., 2011). These QTL can often be found over chromosomal regions containing the Na<sup>+</sup> transporter HKT, as observed in studies using *Arabidopsis* (Rus et al., 2006; Buescher et al., 2010; Prinzenberg et al., 2010; Roy et al., 2012), rice (Ren et al., 2005), and wheat (Byrt et al., 2007; Genc et al., 2010). Other QTL for salinity tolerance exist for germination rate (Mano and Takeda, 1997; Prasad et al., 2000; Ma et al., 2007), seedling vigour/survival (Zhang et al., 1995; Prasad et al., 2000; Lin et al., 2004; Lee et al., 2007), biomass production (Ellis et al., 1997; Mano and Takeda, 1997; Prasad et al., 2000; Koyama et al., 2001; Ellis et al., 2002; Lee et al., 2004; Ma et al., 2007), chlorophyll content (Ma et al., 2007; Genc et al., 2010; Thomson et al., 2010), and grain yield (Gong et al., 2001; Quarrie et al., 2005; Xue et al., 2009).

**Table 6.2** A selection of QTL identified from crops and the model plant species *Arabidopsis*, indicating the chromosomal position of valuable salt tolerance traits.

Process Involved	Trait Measured	Salt Tolerance Mechanism	Species	Chromosome	Reference
Germination	Germination rate		<i>Arabidopsis</i>	1, 4, 5	Vellejo et al. (2010)
			<i>Arabidopsis</i>	1, 2, 5	Galpaz and Reymond (2011)
			<i>Arabidopsis</i>	1, 2, 3, 4	Quesada et al. (2002)
			Rice	6, 7	Prasad et al. (2000)
			Barley	4H, 5H	Mano et al. (1997)
			Bread wheat	3A, 4D, 5A	Ma et al. (2007)
Shoot growth	Seedling vigour	Increased cell expansion; delayed senescence	<i>Arabidopsis</i>	1, 2, 3, 5	Galpaz and Reymond (2011)
			<i>Arabidopsis</i>	4, 5	Quesada et al. (2002)
			<i>Arabidopsis</i>	1, 3, 5	Ren et al. (2010)
			Rice	1, 3	Lee et al. (2007)
			Rice	7	Zhang et al. (1995)
			Rice	6	Prasad et al. (2000)
			Rice	1, 6, 7	Lin et al. (2004)
Shoot growth	Seedling survival	Increased cell expansion; delayed senescence			
Shoot growth	Tiller number	Increased cell expansion; delayed senescence	Rice	6	Gong et al. (2001)
			Barley	7H	Ellis et al. (2002)
			Barley	4H	Xue et al. (2009)
			Bread wheat	5A	Genc et al. (2010)
Shoot growth	Dry matter production	Increased cell expansion; delayed senescence	Rice	5, 6, 10	Prasad et al. (2000)
			Barley	6	Koyama et al. (2001)
			Barley	2H, 4H, 5H	Ellis et al. (2002)
			Barley	7H	Ellis et al. (1997)
			Barley	1H, 2H, 5H, 6H	Mano et al. (1997)
			Barley	2H	Xue et al. (2009)
			Bread wheat	1A, 3B	Ma et al. (2007)
Photosynthesis	Chlorophyll content	Avoidance/delay in ion toxicity in chloroplasts; decreased stomatal conductance	Rice	2, 3, 4	Thomson et al. (2010)
			Bread wheat	3D, 7A	Ma et al. (2007)
			Bread wheat	5B	Genc et al. (2010)

		Buescher et al. (2010)		
Shoot Na <sup>+</sup> accumulation	Increased osmotic adjustment; reduced Na <sup>+</sup> transport; reduced Na <sup>+</sup> exclusion	Arabidopsis Arabidopsis Tomato Citrus Rice Rice Rice Rice Barley Barley Durum wheat Bread wheat	1, 2, 4, 5 4 1, 3, 6, 7 1, 2, 3, 4, 5, 6, 9 4, 6 7 1 1, 4, 12 2H 7H 2A 2A, 2B, 7A	Rus et al. (2006) Villalba et al. (2008) Tozlu et al. (1999) Koyama et al. (2001) Lin et al. (2004) Thomson et al. (2010) Ul Haq et al. (2010) Xue et al. (2009) Shavrukov et al. (2010a) Lindsay et al. (2004) Genc et al. (2010)
Shoot K <sup>+</sup> concentration accumulation	Selective uptake of K <sup>+</sup> ; differential transport of K <sup>+</sup>	Rice Rice Tomato Bread wheat Bread wheat	1, 4 1 1, 5, 7 5A 5A, 5B, 5D	Koyama et al. (2001) Lin et al. (2004) Thomson et al. (2010) Villalba et al. (2008) Byrt et al. (2007) Genc et al. (2010)
Shoot Cl <sup>-</sup> accumulation	Increased osmotic adjustment; reduced Cl <sup>-</sup> transport; reduced Cl <sup>-</sup> exclusion	Citrus Unloading of Na <sup>+</sup> from xylem to reduce shoot Na <sup>+</sup> accumulation	1, 3, 4, 5, 6, 7, 8 Rice Rice Rice Barley Bread wheat Bread wheat	Tozlu et al. (1999) Koyama et al. (2001) Bonilla et al. (2002) Thomson et al. (2010) Ul Haq et al. (2010) Xue et al. (2009) Lindsay et al. (2004); Dubcovsky et al. (1996) Gong et al. (2001)
Ion transport	Na <sup>+</sup> :K <sup>+</sup> ratio	Rice Rice Rice Barley Bread wheat Bread wheat	1, 4 1 1, 9 6H 5A 4D	Xue et al. (2009) Quarrie et al. (2005) Estañ et al. (2009) Bretó et al. (1994)
Grain filling	Delayed senescence	Rice Barley Bread wheat	7 6H 1B, 2B, 3D, 4A, 4B	Gong et al. (2001)
Fruit production	Fruit yield	Delayed senescence Tomato Tomato	9, 11 1, 2, 3, 4, 10, 12	Xue et al. (2009) Quarrie et al. (2005) Estañ et al. (2009) Bretó et al. (1994)

Note: The trait measured and the likely mechanisms involved in tolerance are listed. Table modified from Roy et al. (2011).

With the identification of these QTL, it is imperative that tight molecular markers are generated for the breeders to use, and that the regions are fine mapped to the candidate gene responsible for the QTL.

QTL mapping, however, is only one approach to identify important salt tolerance genes through observation of plant responses to salinity stress. Other approaches such as transcriptomics, proteomics and metabolomics can be used to observe the adaptive response of a plant to salinity stress by measuring changes in the expression levels of genes, modifications to proteins and alterations in metabolites, respectively. By profiling alterations in the expression of thousands of genes, as well as protein and metabolite concentrations, key mechanisms and the genes behind the salt tolerance trait can be identified.

#### 6.4.2 Transcriptomics

In response to salt stress, plants will rely on multiple signaling pathways that result in the expression of genes and activation of proteins, which in turn will determine the plant's phenotype under salt stress. Data now exists on the gene expression profile of many plant species under salt stress. Careful analysis of these data will not only elucidate the function and regulation of the complex plant responses to salt stress, but will also allow the identification of genes of unknown function that may have important roles in salt tolerance. Microarrays are still the most common technique to profile the expression of thousands of genes within a plant (Jamil et al., 2011). However, other techniques such as real-time reverse transcription PCR and serial analysis of gene expression (SAGE and MPSS) can be used as well (Nakano et al., 2006; Vij and Tyagi, 2007; Guo et al., 2008; Jamil et al., 2011), with some, such as RNAseq (Filichkin et al., 2010; Haas and Zody, 2010; Severin et al., 2010; Zenoni et al., 2010) becoming more popular as sequencing technologies get cheaper.

Microarrays are used to study the global response of genes to salt stress in a variety of model and crop species including *Arabidopsis* (Kreps et al., 2002; Seki et al., 2002; Ouakfaoui and Miki, 2005; Kilian et al., 2007; Matsui et al., 2008; Popova et al., 2008; Yokotani et al., 2009), wheat (Mott and Wang, 2007), rice (Kawasaki et al., 2001; Walia et al., 2005; Ueda et al., 2006; Kumari et al., 2009; Senadheera et al., 2009; Cotsaftis et al., 2011), barley (Ozturk et al., 2002; Ueda et al., 2004; Ueda et al., 2006; Walia et al., 2006; Walia et al., 2007a; Walia et al., 2007b), poplar (Gu et al., 2004), and *Medicago truncatula* (Li et al., 2011a; Zahaf et al., 2012)—see Jamil et al. (2011) for a comprehensive list of microarray studies.

In rice, cDNA microarrays demonstrate that the salt-sensitive cultivar IR29 has a delay in timing in the response of key salinity-tolerant genes compared to the salt-tolerant Pokkali (Kawasaki et al., 2001). This faster response in Pokkali is linked to increased protein synthesis and stimulation of important signaling mechanisms (Kawasaki et al., 2001). Further studies show that compared to the

salt-tolerant rice cultivar FL478, which maintains a high K<sup>+</sup> to Na<sup>+</sup> ratio under salt stress, the salt-sensitive IR29 upregulates substantially more genes in the shoot under salt stress, particularly those involved in flavonoid synthesis (Walia et al., 2005). FL478 exhibits more changes in the levels of transcripts in the roots, including key genes encoding proteins involved in ion transport, such as the monovalent cation exchanger *OsCHX11*, the cyclic nucleotide gated channel *OsCNGC1*, the Ca<sup>2+</sup> channel *OsTPC1* and the Na<sup>+</sup> transporter *OsHKT1;5* (Senadheera et al., 2009; Cotsaftis et al., 2011). The differential expression of these transporters in the root is hypothesised to be partially responsible for reducing Na<sup>+</sup> influx into the root (Senadheera et al., 2009). Similar results, where there are substantial differences between the expression profile of shoot and root tissues between tolerant and salt-sensitive cultivars is seen in other species too, such as barley (Walia et al., 2007a).

Microarrays are also used to determine the effect of overexpressing or down-regulating specific genes in transgenic Arabidopsis and rice, and the effect this has on the expression profile of the plant under salt stress (Dai et al., 2007; Nakashima et al., 2007; Fang et al., 2008; Hu et al., 2008; Jung et al., 2008; Krishnaswamy et al., 2008; Matsui et al., 2008; Yokotani et al., 2009). These studies provide useful insights into plant metabolic processes and transcription factor responses, enabling the identification of key genes in plant signaling and survival under salt stress.

#### 6.4.3 Proteomics

Expression and regulation of salt tolerance genes during salinity stress is only part of the story. Expression profiling cannot determine the concentrations of the protein produced from the translation of the mRNA, or whether the protein undergoes post-translational modifications. They also do not give insight into important protein/protein interactions that ultimately affect the protein's function. To gain a complete picture of the mechanisms involved in the plant's response to salt it is necessary to examine the plant's proteome.

Mass spectrometry (MS) and two-dimensional gel electrophoresis (2DGE; Gottlieb et al., 2004) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF; Mann et al., 2001; Mann and Pandey, 2001) studies identify a number of salt stress-responsive proteins in a variety of tissues and plant species (Yan et al., 2005; Vij and Tyagi, 2007; Chang et al., 2012; Fatehi et al., 2012; Guo et al., 2012; Liu et al., 2012)—see Zhang et al. (2012) for a comprehensive list of proteomic studies. Often well-known stress-associated proteins are identified in these studies, particularly those involved with important salt-tolerant mechanisms such as altering plant cell wall properties, scavenging ROS, carbon fixation, and signal transduction (Yan et al., 2005; Witzel et al., 2009; Gao et al., 2011; Li et al., 2011b; Shi et al., 2011; Fatehi et al., 2012; Liu et al., 2012). A key step in the control of ROS production and damage is the

production of a range of enzymes to convert the ROS into less harmful forms. This includes the production of the proteins superoxide dismutase, peroxidases, thioredoxins, glutathionine and ascorbate peroxidases, which convert ROS into less damaging forms (reviewed in Møller et al. [2007]).

As of 2012, over 2,100 salt responsive proteins have been identified in the tissues of over 30 plant species (Zhang et al., 2011); the majority of these proteins (1500) are found to be induced by salt, the rest are reduced. The identities of these proteins are now captured by valuable online databases (Zhang et al., 2011). When examining the response of these proteins across different plant species some interesting trends can be observed. Photosynthetic proteins tend to increase in salt stressed glycophytes, such Arabidopsis, bread wheat and rice, while they decrease in more salt-tolerant species, for example *T. salsuginea* (Zhang et al., 2011). A similar pattern can be observed with proteins involved in carbohydrate and energy metabolism, suggesting that halophytes are more efficient with their photosynthesis and energy metabolism under salt stress than glycophytes (Zhang et al., 2011).

An exciting aspect of proteomics is the identification of novel proteins with as yet unknown functions, which when revealed are likely to suggest unique ways to improve plant salt tolerance.

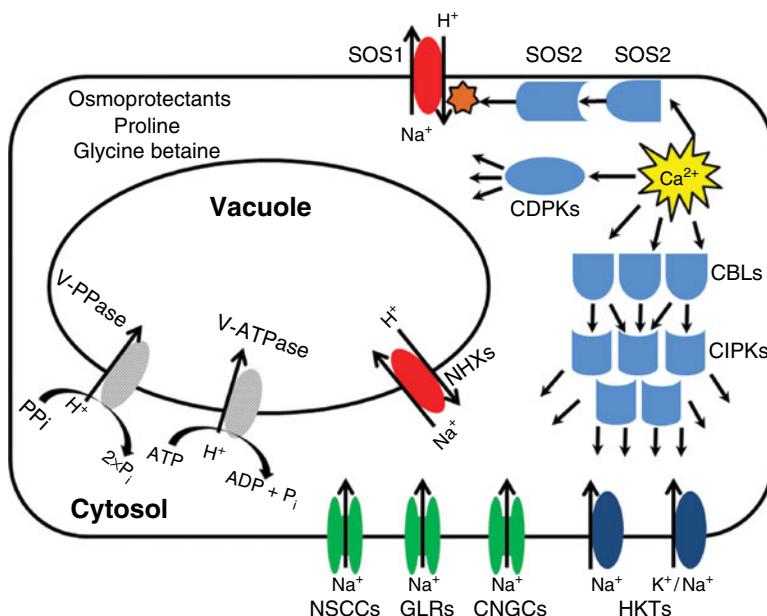
#### 6.4.4 Metabolomics

Metabolomic studies allow the profiling of a plant's metabolome before and after salinity stress, allowing the identification of key metabolites and processes important for the plant's response. During salt stress, the build-up of ions, either in the vacuole or in the apoplastic space between cells, requires the accumulation of solutes in the cytosol that have a role in both osmoprotection and osmotic adjustment (Hasegawa et al., 2000; Munns and Tester, 2008; Sanchez et al., 2008b; Widodo et al., 2009). These organic solutes, called *compatible solutes* due to their non-toxic effect on cellular processes, can be accumulated to high concentrations within the cytosol of a plant cell, helping to reduce water loss from the cytosol (Hasegawa et al., 2000; Munns and Tester, 2008). These solutes are hydrophilic and have a role in cellular protection as they can replace water on the surface of both proteins and membranes, so that their function is maintained (Rajendrakumar et al., 1997; Hasegawa et al., 2000; Takagi, 2008). Typically, compatible solutes are made up of sugars (e.g., fructose and sucrose), amino acids (e.g., proline), sugar alcohols (e.g., glycerol, methylated inositol), complex sugars (e.g., trehalose, raffinose, and fructans), and charged metabolites (e.g., glycine and betaine; Hasegawa et al., 2000; Chen and Murata, 2002; Munns and Tester, 2008; Sanchez et al., 2008b; Widodo et al., 2009; Chen and Murata, 2011). A disadvantage of compatible solutes is that they require a lot of energy to produce and maintain.

While changes in the levels of specific metabolites after salt application are well known, modern metabolomics can rapidly profile hundreds of small and large metabolites using a range of analytical technologies (such as gas chromatography coupled to mass spectrometry [GC-MS], liquid coupled to mass spectrometry [LC-MS] or nuclear magnetic resonance [NMR])—see Roessner and Bowne (2009) for a review. Increases in amino acids (e.g., proline), sugars (e.g., glucose, fructose and sucrose), and polyols (e.g., inositol) are commonly observed in metabolomic studies of *Arabidopsis* (Gong et al., 2005; Sanchez et al., 2008b), grape vine (Cramer et al., 2007), *Lotus japonicas* (Sanchez et al., 2008a), and barley (Widodo et al., 2009). Comparative metabolomic studies between salt-tolerant and salt-sensitive species (and varieties) has revealed that salt tolerant relatives of salt-sensitive plants have significantly higher concentrations of metabolites such as proline, sucrose and fructose, before salt stress, suggesting a constitutive adaptation in these plants (Gong et al., 2005; Sanchez et al., 2008b). The salt-tolerant *T. salsuginea* has significantly higher levels of proline, citrate, malate, sucrose, fructose and glucose compared to its salt-sensitive relative *Arabidopsis* when grown under control conditions (Gong et al., 2005). Despite having higher levels of key osmolytes, *T. salsuginea* shows a greater increase in other metabolites such as inositol and galactinol, after salt stress compared to *Arabidopsis* (Gong et al., 2005). Changes in the metabolites involved in photosynthesis and respiration are also observed—see the review of (Sanchez et al., 2008b).

## 6.5 Known candidate genes for salinity tolerance

One of the easiest tolerance mechanisms to study and control is the transport of  $\text{Na}^+$  and  $\text{Cl}^-$  through a plant. High concentrations of either  $\text{Na}^+$  and/or  $\text{Cl}^-$  in the shoot are frequently cited as being detrimental to the health of the plant, particularly for cereals (Flowers, 2004; Colmer et al., 2006; Munns et al., 2006; Gao et al., 2007; Munns and Tester, 2008; Amtmann and Beilby, 2010; Plett and Møller, 2010), and maintaining  $\text{K}^+$  homeostasis is important for plant survival (Maathuis and Amtmann, 1999; Kronzucker and Britto, 2011).  $\text{Na}^+$  influx into plant root cells is passive and is likely to occur through non-selective cation channels, cyclic nucleotide-gated channels and/or glutamate receptor-like channels (Demidchik and Maathuis, 2007; Plett and Møller, 2010). Consequently, the majority of research into improving plant salinity tolerance has focused on characterising genes encoding proteins involved in either reducing the amount of  $\text{Na}^+$  translocated from the root to the shoot, or on genes that encode proteins involved in sequestration of ions into vacuoles. A number of key genes are frequently identified as playing an important role in these processes; many are shown in Figure 6.3.



**Figure 6.3** A selection of well-characterized cellular processes involved in salt tolerance.  $\text{Na}^+$  can passively enter into plant cells through ion channels, such as non-selective cation channels (NSCCs), glutamate receptor-like channels (GLRs), cyclic-nucleotide gated channels (CNGCs), and the  $\text{Na}^+$  or  $\text{K}^+/\text{Na}^+$  HKT transporters.  $\text{Na}^+/\text{H}^+$  antiporters like SOS1 or NHXs transport  $\text{Na}^+$  out of the cell or into the vacuole and require the establishment of an  $\text{H}^+$  gradient by membrane bound PPases and ATPases. SOS2 and SOS3 regulate the activity of SOS1, ensuring the transporter is active during salt stress—SOS3 and SOS2 are members of the CBL/CIPK calcium signalling pathway. Both CDPKs and CBLs/CIPKs are involved in the  $\text{Ca}^{2+}$  dependent salt stress signalling pathways and regulate the cell response to salt stress by post-translationally modifying a variety of proteins, such as transporters. Finally, salt stressed cells accumulating high concentrations of  $\text{Na}^+$  produce proline and glycine betaine as osmoprotectants. For color details, please see color plate section.

#### 6.5.1 The high-affinity potassium transporter family

High-affinity potassium transporters (HKTs) have been demonstrated to mediate  $\text{Na}^+$  and/or  $\text{K}^+$  influx into plant cells (Schachtman and Schroeder, 1994; Uozumi et al., 2000; Su et al., 2003; Horie et al., 2009; Møller et al., 2009; Xue et al., 2011; Munns et al., 2012). The gene family can generally be divided into those encoding  $\text{Na}^+$ -specific transporter proteins (subfamily 1 genes) or  $\text{K}^+/\text{Na}^+$  transporter proteins (subfamily 2 genes) and this is often linked to whether there is a serine ( $\text{Na}^+$  selectivity) or a glycine ( $\text{K}^+$  and  $\text{Na}^+$  selectivity) at the filter position in pore loop A of the protein (Maser et al., 2002; Platten et al., 2006; Horie et al., 2009; Plett and Møller, 2010; Kronzucker and Britto, 2011). While members of subfamily 2 are suggested to be involved in  $\text{K}^+$  and  $\text{Na}^+$  nutrient acquisition from the soil, particularly when  $\text{K}^+$  is limiting (Horie et al., 2007;

Horie et al., 2009; Kronzucker and Britto, 2011), members of subfamily 1 encode proteins that facilitate the retrieval of  $\text{Na}^+$  from the xylem (Sunarpi et al., 2005; James et al., 2006; Davenport et al., 2007; Horie et al., 2009; Xue et al., 2011). Retrieval of  $\text{Na}^+$  from the xylem reduces the  $\text{Na}^+$  in the transpiration stream and reduces the accumulation of  $\text{Na}^+$  in the shoot (Ren et al., 2005; Horie et al., 2006; Byrt et al., 2007; Davenport et al., 2007; Horie et al., 2009). While this classification of *HKTs* is generally correct, it has recently become apparent that there are exceptions to the rule that members of subfamily 1 are solely  $\text{Na}^+$  transporters, with *TsHKT1;2* recently being discovered to have a higher affinity for  $\text{K}^+$  transport rather than  $\text{Na}^+$  in *T. salsuginea* (Ali et al., 2012). *HKTs* are identified in a number of plant species including wheat (Schachtman and Schroeder, 1994; Huang et al., 2006; Byrt et al., 2007), rice (Garcia de Blas et al., 2003; Ren et al., 2005; Jabnoune et al., 2009; Horie et al., 2011), *Arabidopsis* (Rus et al., 2001; Maser et al., 2002; Rus et al., 2004; Rus et al., 2006; Baxter et al., 2010), barley (Wang et al., 1998; Huang et al., 2008; Qiu et al., 2011), *Mesembryanthemum crystallinum* (Su et al., 2003), *Eucalyptus* (Fairbairn et al., 2000; Liu et al., 2001), *T. salsuginea* (Ali et al., 2012) *Puccinellia tenuiflora* (Ardie et al., 2010), and *Suaeda salsa* (Qun et al., 2008). Some species, such as *Arabidopsis*, have one *HKT* gene (*AtHKT1;1*, belonging to subfamily 1; Platten et al., 2006; Horie et al., 2009), while others have multiple genes that belong to both subfamilies, all with subtly different expression patterns and suspected function (Platten et al., 2006; Horie et al., 2009; Jabnoune et al., 2009; Cotsaftis et al., 2011; Horie et al., 2011).

### 6.5.2 The salt overly sensitive pathway

The salt overly sensitive (SOS) pathway is involved in mediating the response of plant cells to salt stress. Unlike the *HKT* genes, which only encode ion transporters, members of the *SOS* family are so named due to their effect on the salt sensitivity of *Arabidopsis* plants that have mutations in key genes involved in salinity tolerance (Wu et al., 1996; Liu and Zhu, 1998; Zhu et al., 1998; Shi et al., 2002b; Shi et al., 2003a). Different *SOS* genes encode for different classes of proteins. Initially, three proteins in the SOS pathway were identified: AtSOS1, a plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter (Wu et al., 1996; Shi et al., 2000; Shi et al., 2002a; Shi et al., 2003b); AtSOS2, a protein kinase that belongs to the Calcineurin B-like interacting protein kinase (CIPK) family (Zhu et al., 1998; Liu et al., 2000; Luan, 2009; Weinl and Kudla, 2009; Kudla et al., 2010); and AtSOS3 a  $\text{Ca}^{2+}$  binding protein belonging to the Calcineurin B-like proteins (CBL). These proteins have specific regions allowing them to interact with CIPKs, such as SOS2 (Zhu et al., 1998; Batistić and Kudla, 2004; Mahajan et al., 2008; Luan, 2009; Weinl and Kudla, 2009). During salt stress, vacuolar and apoplastic  $\text{Ca}^{2+}$  is released into a cell's cytosol where it binds to AtSOS3 (Halfter et al., 2000; Albrecht et al., 2001; Batistić and Kudla, 2004;

Mahajan et al., 2008; Luan, 2009; Weinl and Kudla, 2009). AtSOS3 then recruits AtSOS2 to the plasma membrane, which phosphorylates the  $\text{Na}^+/\text{H}^+$  antiporter AtSOS1 (Qiu et al., 2002; Mahajan et al., 2008; Luan, 2009; Weinl and Kudla, 2009; Das and Pandey, 2010), thereby activating the transporter and facilitating the movement of  $\text{Na}^+$  out of the cell. This activation of AtSOS1 is likely to involve another calcium binding protein, SCaBP8, in addition to the phosphorylation of AtSOS1 by the AtSOS2/3 complex (Lin et al., 2009). A further two members of the salt overly sensitive family are also identified: AtSOS4, a pyridoxal kinase important for root hair development (Shi et al., 2002b; Shi and Zhu, 2002); and AtSOS5, a putative cell surface adhesion protein that is required for cell expansion (Shi et al., 2003a).

Members of the SOS family are now identified in a number of different species including Arabidopsis (Wu et al., 1996; Halfter et al., 2000; Liu et al., 2000; Shi et al., 2000; Qiu et al., 2002), rice (Kolukisaoglu et al., 2004; Martinez-Atienza et al., 2007; Mullan et al., 2007), wheat (Mullan et al., 2007; Cuin et al., 2011), poplar (Wu et al., 2007; Tang et al., 2010), barley (Rivandi et al., 2011), tomato (Villalta et al., 2008; Olias et al., 2009; Sun et al., 2010), *Cymodocea nodosa* (Blanca et al., 2007), *M. crystallinum* (Cosentino et al., 2010), *Brassica* (Wang et al., 2004; Kushwaha et al., 2011; Chakraborty et al., 2012), and *T. salsuginea* (Vera-Estrella et al., 2005; Oh et al., 2009). They are shown to be significantly upregulated under salt stress (Liu et al., 2000; Shi et al., 2000; Shi et al., 2002a; Martinez-Atienza et al., 2007; Wu et al., 2007; Oh et al., 2009; Cosentino et al., 2010; Jha et al., 2010; Kushwaha et al., 2011; Chakraborty et al., 2012).

### 6.5.3 Vacuolar $\text{Na}^+/\text{H}^+$ antiporters and vacuolar pyrophosphatases

While HKT and SOS1 are important in controlling the movement of  $\text{Na}^+$  into and out of the cell, members of the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter (*NHX*) family are important in the compartmentation of  $\text{Na}^+$  into the cell's vacuole (Gaxiola et al., 1999; Aharon et al., 2003; Bassil et al., 2011b; Fukuda et al., 2011). The proteins have been shown to transport  $\text{Na}^+$  and/or  $\text{K}^+$  across membranes in exchange for a proton ( $\text{H}^+$ ; Gaxiola et al., 1999; Bassil et al., 2011a; Bassil et al., 2011b). The *NHX* genes are therefore important in tissue tolerance mechanisms as they remove toxic ions like  $\text{Na}^+$  from the cytosol and compartmentalize them in the vacuole before it has detrimental effects on metabolism. The expression of *NHX* genes increases under salt stress (Fukuda et al., 2004b; Fukuda et al., 2011; Guan et al., 2011; Jha et al., 2011) and the gene is identified in a variety of plant species including Arabidopsis (Apse et al., 1999; Apse et al., 2003; Bassil et al., 2011a; Bassil et al., 2011b), tomato (Wilson and Shannon, 1995), barley (Garbarino and DuPont, 1989; Fukuda et al., 2004a), wheat (Mullan et al., 2007; Yu et al., 2007; Liu et al., 2010; Zhang et al., 2012), rice (Fukuda et al., 2004b; Mullan et al., 2007; Fukuda et al., 2011), maize (Zörb et al., 2005), cotton (Wu et al., 2004; Liu et al., 2010), grape vine

(Ismail et al., 2012), poplar (Silva et al., 2010), *Medicago* (Zahran et al., 2007), *Salicornia brachiate* (Jha et al., 2011), *Halostachys caspica* (Guan et al., 2011), and sunflower (Ballesteros et al., 1997).

A second gene family, important in the sequestration of  $\text{Na}^+$  into the vacuole, but does not encode for proteins directly responsible for physical transport of  $\text{Na}^+$  across the membrane, are the vacuolar  $\text{H}^+$  pyrophosphatase genes. These genes encode for proteins that use the energy released from the breakdown of inorganic pyrophosphatase (PPi) to pump proteins into the vacuole (Apse et al., 1999; Gaxiola et al., 1999; Gaxiola et al., 2001). During salt stress proton pumping into the vacuole establishes an electro-chemical gradient that can then be used by  $\text{Na}^+/\text{H}^+$  antiporters, such as the NHXs to move  $\text{Na}^+$  into the vacuole (Gaxiola et al., 2001). As PPi is produced as a by-product from a number of metabolic pathways its use as an energy donor to pump  $\text{H}^+$  into vacuoles allows other high energy containing compounds, such as ATP, to be used for other metabolic processes—this is particularly important when plants are growing under adverse environmental conditions such as salinity. A number of vacuolar  $\text{H}^+$  pyrophosphatase genes are identified, the first being *Arabidopsis AVP1* (Apse et al., 1999; Gaxiola et al., 1999; Gaxiola et al., 2001), and they are also identified in barley (Shavrukov et al., 2010a), wheat (Mullan et al., 2007), rice (Mullan et al., 2007), and others.

#### 6.5.4 Osmoprotectants

Control over the transport of ions is only one out of numerous mechanisms for salinity tolerance, and candidate genes are identified for other processes that are important in maintaining growth under saline conditions. As noted earlier, the accumulation of ions, either in the vacuole or in the apoplastic space between cells, requires the accumulation of solutes in the cytosol that have a role in both osmoprotection and osmotic adjustment (Hasegawa et al., 2000; Flowers and Colmer, 2008; Munns and Tester, 2008). Proline and glycine betaine (GB) are often seen to accumulate in plant tissues in response to salt application (Hasegawa et al., 2000; Ashraf and Foolad, 2007; Flowers and Colmer, 2008; Munns and Tester, 2008; Sanchez et al., 2008b; Widodo et al., 2009), and the mechanisms for proline and GB synthesis are now well known (Hare et al., 1999; Ashraf and Foolad, 2007; Székely et al., 2008). Transcriptomic analysis indicates a positive relationship between the expression of genes involved in proline synthesis, such as  $\Delta^1$ -pyrroline-5-carboxylate reductase (P5CR) and  $\Delta^1$ -pyrroline-5-carboxylate synthetase, and increasing salt stress (Yoshiba et al., 1995; Knight et al., 1997; Strizhov et al., 1997; Hare et al., 1999; Székely et al., 2008).

#### 6.5.5 Calcium signaling pathways

Many aspects of plant growth and development are mediated by the  $\text{Ca}^{2+}$  ion. Environmental cues are perceived by receptors on the plasma membrane, which

activates a  $\text{Ca}^{2+}$  signaling cascade, resulting in the regulation of gene expression and protein activities (Luan, 2009; Weinl and Kudla, 2009; Das and Pandey, 2010; Kudla et al., 2010). It is hypothesised that there are different  $\text{Ca}^{2+}$  “signatures” for each stress that define the plant’s cell-specific response to that stress (Webb et al., 1996; Kiegle et al., 2000; Tracy et al., 2008; Kudla et al., 2010; Batistić and Kudla, 2012). Salt application to the roots of *Arabidopsis* results in a two-phase calcium signature in the cells of the root—a transient spike in  $\text{Ca}^{2+}$  concentration in the cytosol, followed by an oscillation in  $\text{Ca}^{2+}$  concentrations (Kiegle et al., 2000). The  $\text{Ca}^{2+}$  signal is then relayed within minutes from the root to other tissues that have yet to be exposed to the salt (Tracy et al., 2008). This  $\text{Ca}^{2+}$ -specific salt response is subtly different to the alteration in cytoplasmic concentrations of  $\text{Ca}^{2+}$  observed when the root is exposed to other stresses, such as cold or osmotic stress (Kiegle et al., 2000). Once a  $\text{Ca}^{2+}$  signature has been initiated, calcium binding proteins, such as CDPKs (Harper et al., 2004; Batistić and Kudla, 2012), CBLs (Albrecht et al., 2003; Cheong et al., 2003; Luan, 2009; Weinl and Kudla, 2009), Calmodulins (CaM; Kim et al., 2009; Kudla et al., 2010; Reddy et al., 2011; Batistić and Kudla, 2012), and Calmodulin-like proteins (CMLs; Kim et al., 2009; Reddy et al., 2011; Batistić and Kudla, 2012), are then critical in regulating the response of downstream processes, such as the activation of protein kinases and the control of transcriptional factors (Finkler et al., 2007; Kim et al., 2009; Reddy et al., 2011; Batistić and Kudla, 2012). Some of these proteins, such as the CDPKs combine two functions into one protein. They bind  $\text{Ca}^{2+}$ , have a response activity (e.g., kinase activity), and are called *sensor responders*, while others like the CaMs and CMLs interact with specific targets to perform the desired response and are *sensor relays* (Sanders et al., 2002; Kudla et al., 2010; Batistić and Kudla, 2012). A large number of these calcium binding proteins are shown to be important in the control of a plant’s response to salt. Numerous members of the CDPK, CBL and CIPK family are identified in several plant species as being important control proteins during salt stress, such as OsCPK7 (Saijo et al., 1997), AtCBL1 (Cheong et al., 2003), AtCBL10 (Kim et al., 2007), OsCIPK31 (Piao et al., 2010), HvCBL4 (Rivandi et al., 2011), CaCIPK6 (Tripathi et al., 2009), and AtCIPK16 (Roy et al., 2012). The most famous of these are SOS3 (AtCBL4) and SOS2 (AtCIPK24) in *Arabidopsis* (Halfter et al., 2000; Liu et al., 2000; Qiu et al., 2002; Mahajan et al., 2008; Luan, 2009; Weinl and Kudla, 2009). Refer to Kudla et al. (2010) and Batistić and Kudla (2012) for a comprehensive review on  $\text{Ca}^{2+}$  signaling in plants.

## 6.6 Prospects for generating transgenic crops

A transgenic approach for increasing the salinity tolerance of crops is an attractive alternative to selective breeding, particularly when multiple and often undesirable genes are introduced through traditional breeding practices. In the

course of selection for an adventitious trait like salinity tolerance, an elite high yielding commercial cultivar is bred with another cultivar (or wild accession) that has the desired salt tolerance trait. The salt-tolerant cultivar may not have been subject to the same selection pressures for other important crop traits such as grain yield, grain quality, flowering time, plant shape etc. as the elite cultivar. Breeding the two together results in progeny with mixed DNA, but introduces a potential problem where non-beneficial, commercially undesirable traits are introduced into the elite cultivar along with desirable salt tolerance traits. This phenomenon is called *linkage drag* and often limits the use of certain cultivars and wild relatives in breeding programs (Feuillet et al., 2008). While it is possible to remove the disadvantageous traits by backcrossing to the elite parent line, this remains a long and time-consuming process that can take many years/decades. The problem is exacerbated when crosses are performed between elite cultivars and near wild relatives—it can be extremely difficult to get recombination events between the elite cultivar DNA and the wild relative DNA, and large sections of undesirable DNA containing genes for undesirable traits remain in the elite cultivar.

Marker assisted selection increases the speed of selective breeding, however, it is still necessary to grow the progeny of these crosses through several generations to achieve recombination, leaving the genes responsible for the desirable trait within the genome and eliminating the genes responsible for undesirable yield penalty traits. With many genes having now been identified as being important for salinity tolerance, novel genes can be directly introduced into the target crop, without the introduction of genes detrimental to yield phenotypes. Alternatively the expression of native genes within the crop can be altered. In theory, directly transforming a gene of choice into elite cultivars could significantly increase the speed at which these crops become available to farmers, although this still has to be shown in practice. Candidate genes for transformation into crops to improve salinity tolerance include those involved with the transport of ions, transcription factors, production of compatible solutes and protectors of metabolism, such as enzymes involved in detoxifying reactive oxygen species.

A key feature of this process is not only the selection of important candidate genes for transformation but also selecting the correct promoter that will regulate both temporal and/or cell-type-specific expression of the gene. Many genes can be expressed constitutively using promoters from important cellular housekeeping genes such as ubiquitin that are expressed in every cell in the plant (Christensen et al., 1992). Promoters, such as the 35S promoter from the cauliflower mosaic virus (Odell et al., 1985), can also be used to drive high expression of genes in all cell types. Constitutive expression, however, is not often desired, as the expression of the gene may only be wanted in specific cell types and/or only active during salt stress. For these genes, stress inducible and cell-type-specific promoters are required.

### 6.6.1 Overexpression of genes involved with the transport of ions

To date, perhaps the greatest success is the generation of transgenic plants with altered  $\text{Na}^+$  transport properties, particularly with genes that encode proteins facilitating better compartmentation of  $\text{Na}^+$  into the vacuole. Expression of genes belonging to the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter and vacuolar  $\text{H}^+$  pyrophosphatase families result in significant improvements in crop salinity tolerance, measured under both greenhouse and field conditions. Constitutive overexpression of the *Arabidopsis AtNHX1* gene in *Arabidopsis* (Apse et al., 1999; Apse et al., 2003; Liu et al., 2010), tomato (Zhang and Blumwald, 2001; Leidi et al., 2010), wheat (Xue et al., 2004), cotton (He et al., 2005), poplar (Qiao et al., 2011), kiwifruit (Tian et al., 2011), *Brassica napus* (Zhang et al., 2001), and fescue (Zhao et al., 2007) results in plants with increased salinity tolerance through the ability to alter either the accumulation of  $\text{Na}^+$  or  $\text{K}^+$  in the vacuoles of shoots and/or roots. Importantly from a consumer point of view,  $\text{Na}^+$  accumulation only occurs in green tissue and not in fruit or seed, at least in the case for tomato (Zhang and Blumwald, 2001). Homologues of *AtNHX1* are found in a range of crop plants and the constitutive overexpressing of these genes in model plant species, such as *Arabidopsis* (Liu et al., 2010; Guan et al., 2011), tobacco (Jha et al., 2011), alfalfa (Zhang et al., 2012), and rice (Fukuda et al., 2004b) also increases salinity tolerance. What has significant implications for the generation of crops is that increasing *AtNHX1* expression in *Arabidopsis* results in significant alterations in the expression of other salinity tolerance genes (Sottosanto et al., 2004; Sottosanto et al., 2007). Therefore it may not be necessary to transform a plant with multiple genes involved in salinity tolerance, as the expression of one key gene that regulates others may be enough.

Constitutive expression of vacuolar  $\text{H}^+$  pyrophosphatases, such as *AVP1*, improves salinity tolerance of plants. Expression of *AVP1* in alfalfa results in plants that maintain greater shoot biomass than wild type plants when grown under high saline conditions (Bao et al., 2009). Similar results are observed when *AVP1* is expressed in bentgrass (*Agrostis stolonifera* L.), where the transgenic plants are able to survive up to 300 mM NaCl, levels of salinity that significantly reduce the growth of non-transformed bentgrass (Li et al., 2010). Enhanced results are observed when *AVP1*, in conjunction with an *NHX* gene, are co-transformed into *Arabidopsis*, rice, and tobacco (Zhao et al., 2006; Brini et al., 2007; Duan et al., 2007).

Increases in salinity tolerance are also seen in plants that overexpress components of the SOS pathway. Transgenic *Arabidopsis* exhibit greater biomass, reduced concentration of  $\text{Na}^+$  in the shoot, and reduced senescence than wild type plants when grown in high saline conditions (Shi et al., 2003b; Yang et al., 2009). The increase in salt tolerance is attributed to increasing the efflux of  $\text{Na}^+$  from cells. It is not always necessary, however, to generate a salt-tolerant plant by manipulating the expression of a protein that is directly involved in the

transport of ions. The salinity tolerance of crops could be potentially increased by altering the expression of genes involved in stress signaling, the production of compatible solutes, or the activation of key genes in tolerance pathways.

#### 6.6.2 Manipulation of genes involved in signaling pathways

Enhanced expression of genes involved in signaling pathways, such as the CBL/CIPK pathway is shown to increase the salt tolerance of *Arabidopsis* (Cheong et al., 2003; Zhao et al., 2009; Cheong et al., 2010; Wang et al., 2012), rice (Xiang et al., 2007), tomato (Wang et al., 2012), and tobacco (Tripathi et al., 2009), presumably by enhancing and regulating the signaling and tolerance mechanisms within individual cells. Overexpression of transcriptions factors, such as *alfin* in alfalfa, *DREB1A*, *TaMYB2A*, *MbDREB1*, *OrbHLH2* and *OsDREB1A* in *Arabidopsis*, *OsDREB1A* and *TREF1* in rice, and *CgDREBa* in chrysanthemum, results in plants with increased root and/or shoot biomass under salt stressed conditions (Kasuga et al., 1999; Winicov, 2000; Dubouzet et al., 2003; Gao et al., 2008; Zhou et al., 2009; Chen et al., 2011; Mao et al., 2011; Yang et al., 2011). Care must be taken, however, as alterations in transcription factor expression, particularly constitutive overexpression, can lead to undesirable phenotypes (Liu et al., 1998; Kasuga et al., 1999; Morran et al., 2011) and often best results are observed when transcription factors are under control of a stress inducible promoter (Kasuga et al., 1999; Mallikarjuna et al., 2011).

#### 6.6.3 Altering the expression of genes involved in compatible solute synthesis

Similarly, the overexpression of genes involved in the synthesis of compatible solutes, such as *P5CR* and *P5CS*, which are involved in proline production, and betaine aldehyde dehydrogenase, which is involved in GB production, result in plants with improved performance under salt stress (Kishor et al., 1995; Hong et al., 2000; Yang et al., 2008; Fan et al., 2012). However, as with transcription factors, stress inducible expression of these genes is often desirable to avoid reductions in growth under control conditions (Sheveleva et al., 1997; Vendruscolo et al., 2007).

#### 6.6.4 The need for cell-type- and temporal-specific expression

In some cases constitutive expression of genes known to be important for salinity tolerance can be detrimental rather than beneficial and only by carefully controlling the gene's expression can beneficial effects be observed. Manipulation of *HKT* expression frequently results in alterations in a plant shoot Na<sup>+</sup> concentration. In *Arabidopsis*, both naturally occurring ecotypes and mutant lines with reduced *AtHKT1;1* expression exhibit increased shoot Na<sup>+</sup> accumulation (Rus et al., 2006; Baxter et al., 2010). Unfortunately, from a genetic

engineering approach, simply overexpressing *AtHKT1;1* does not result in reduced shoot Na<sup>+</sup> accumulation. Instead, as AtHKT1;1 moves Na<sup>+</sup> into cells, constitutive overexpression throughout the whole plant results in increased shoot Na<sup>+</sup> concentrations and increased salt sensitivity (Rus et al., 2004; Møller et al., 2009), potentially due to an increase in the influx of Na<sup>+</sup> from the soil to the root.

As each tissue and cell in a plant is adapted for a specific purpose, the expression levels of genes will be different from tissue to tissue and cell to cell. Genes that are involved in the uptake of nutrients from soils will not necessarily be expressed in leaf or floral tissues, while genes involved in photosynthesis processes, such as those that encode for Rubisco, will not be expressed in tissues that are not exposed to light, for example, roots. While constitutive expression appears to work well for transporters involved in the sequestration of Na<sup>+</sup> and Cl<sup>-</sup> into vacuoles it is important to manipulate processes that occur in specific tissues or cell types by altering the expression of the genes involved only in that cell type.

Retrieval of Na<sup>+</sup> from the stele by HKTs is a process that occurs in a specific cell type; therefore *HKT* genes should only be expressed in those cells. Transgenic Arabidopsis and rice plants have been developed that expressed *AtHKT1;1* in a specific cell type in the root. Unlike plants that have constitutive expression of *AtHKT1;1*, the cell-type-specific expression of this gene results in significant reductions in shoot Na<sup>+</sup> accumulation and increased salt tolerance, due to reduced root to shoot Na<sup>+</sup> transport (Møller et al., 2009; Plett et al., 2010).

In addition to location, not all genes are expressed at the same time—those involved with abiotic stress tolerance are not expressed when the plant is growing in non-stressed conditions. Therefore it is important to consider using stress-inducible promoters in addition to spatial promoters to control the activity of transgenes when developing a transgenic crop plant. This way the plant does not waste critical energy reserves to generate and maintain proteins it only requires when stressed. Constitutive overexpression of genes can also lead to severe growth abnormalities and stunting (Romero et al., 1997; Morran et al., 2011). Transgenic rice overexpressing the compatible solute trehalose have increased growth, with reduced shoot Na<sup>+</sup> concentrations, but only when the transgene is under the control of a stress inducible promoter (Romero et al., 1997). Constitutive expression of trehalose leads to severe stunting.

It is now imperative that more promoters of genes are identified that will allow cell-type and/or stress inducible expression in crops to occur. It is also important that the specific DNA sequences, which allow the desired pattern of expression, are identified to facilitate the construction of designer promoters that can control the inserted transgene in the manner desired.

## 6.7 Conclusion

Crop plants show substantial yield reductions when grown on saline soils due to the interaction of multiple stresses. To improve yield production on marginal saline land, improvements in the salt tolerance mechanisms of crops are required. To do this, approaches are needed that allow the fast transfer of important traits for salinity tolerance into present crop cultivars. This can be achieved through a selective breeding approach, making use of natural variation in crops and their near wild relatives, or through the generation of transgenic plants transformed with key genes in salinity tolerance pathways. While progress has been made in recent years to identify these traits and genes, what is needed is the transfer of the research outcomes into crops that farmers can grow profitably in the field. Numerous candidate genes are now known to be important for salinity tolerance, but few of these genes have been transferred into crop plants and tested in field conditions. The genes must now be introduced into crops, using either traditional breeding approaches or genetic engineering, and field evaluation carried out. This will not only determine if a salt-tolerant plant has been generated that can grow and maintain yield in saline field conditions, but importantly the plant will exhibit no yield penalty when soil salinity is low or absent. It is also becoming apparent that plants use multiple salt tolerance mechanisms. Therefore, both breeding and genetic engineering approaches must ensure that multiple tolerance mechanisms are introduced into elite breeding lines, allowing plants to respond to different salt stresses. For genetic engineering, while many candidate genes are known, a research priority is the identification of promoter elements that will allow the expression of these genes to be regulated so they are only expressed in specific cell types and under specific conditions. While we know a lot about what happens to the aboveground plant tissues, further research is also required to phenotype roots under salinity stress in their natural soil conditions. Recent advances in infrared imaging and magnetic resonance imaging (Furbank and Tester, 2011) are providing opportunities to gain insight into this dark world. In addition, more research is needed to help elucidate the genes behind salinity tolerance traits that have not yet been extensively studied.

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## 7 Molecular and physiological mechanisms of plant tolerance to toxic metals

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### 7.1 Introduction

Heavy metals are defined as metals with a density greater than 5 g/cm<sup>3</sup> (Holleman and Wiberg, 1985). This definition encompasses 53 of the 118 elements on the periodic table. Of these 53 elements, some are essential to plants as micronutrients, including iron (Fe), copper (Cu), zinc (Zn), and manganese (Mn). Because many of these essential metals are also highly reactive, they can cause toxicity to plants if accumulated in the plant to levels above what is required for essentiality. Additionally, other heavy metals are similar to essential micronutrient metals with regards to their ionic properties, and they can enter plants via transporters that normally function in micronutrient transport. Thus heavy metals such as cadmium (Cd), lead (Pb), and mercury (Hg) will cause toxicity symptoms in plants when found in elevated levels in the soil primarily due to anthropogenic activities. Finally, aluminum (Al) is quite toxic to plant roots in highly acidic soils ( $\text{pH} < 5$ ) where it is solubilized from aluminosilicate clays into the soil solution as the rhizotoxic ion Al<sup>3+</sup>. Because acid soils are quite prevalent worldwide, where it is estimated that up to 50% of the world's potentially arable lands are highly acidic, Al toxicity is a major limitation to agriculture worldwide (Uexküll and Mutert, 1995). Thus, crop mechanisms that underlie Al tolerance are probably the best characterized plant metal tolerance mechanisms, both at the molecular and physiological levels. Therefore, although Al is a light metal, not a heavy metal, we also will review the current understanding of crop Al tolerance genes and mechanisms.

There are two general strategies plants employ to deal with toxic metals in the soil: exclusion of the metal from the root system or the entire plant, which is an avoidance strategy, or true tolerance of the metal after it has been accumulated in the plant. Plant metal-exclusion strategies can involve several different mechanisms, including (1) the transport of the metal back out of the root after

it has entered the root symplasm, with the metal efflux involving transport of metal either in its ionic form or as a metal-ligand complex with the metal chelated to an organic compound; (2) exclusion of the metal from the shoot via sequestration in the root; or (3) the secretion of a chelating organic compound from the root into the rhizosphere, where it can chelate the metal and prevent its entry into the root. Thus the exclusion strategy is aimed at either limiting the levels of the toxic metal entering the plant and/or sequestering the metal before it is transported to the aerial tissues, primarily to protect photosynthetic processes, which are quite vulnerable to disruption by toxic heavy metals (Küpper et al., 2007). The main compounds secreted by plants to deal with heavy metal or Al toxicity are usually in the form of the organic acids citrate and malate but may also be non-protein peptides such as phytosiderophores that primarily function in Fe uptake in the grasses (Ma et al., 2001; Dubbin and Louise Ander, 2003) or the sulfur-rich peptide phytochelatin (Cobbett and Goldsbrough, 2002). Mechanisms associated with strategies based on the tolerance of the toxic metal in the plant usually involve metal sequestration in plant organelles, usually the vacuole and/or the cell wall. A general strategy used by non-accumulating plant species, which includes most crop species, involves the sequestration of the metal in root-cell vacuoles to prevent the metal from reaching the sensitive photosynthetic machinery in the leaf (Audet and Charest, 2008). However, in highly specialized heavy metal hyperaccumulating species, a significant fraction of the toxic metal is actually transported to the shoot via the xylem and stored in leaf epidermal cell vacuoles, with greater accumulation in epidermal cells that are generally not photosynthetic. The most well studied of these hyperaccumulators are *Noccaea caerulescens* (formally known as *Thlaspi caerulescens*) and *Arabidopsis halleri*. These plant species are able to tolerate up to 30,000 ppm Zn and 10,000 ppm Cd DW in shoots before metal toxicity symptoms are observed. These levels are 100 times greater than what is accumulated in your typical non-accumulating plant species, which starts to exhibit toxicity symptoms when leaf Zn concentrations exceed approximately 300 ppm DW in the aerial tissues (Marschner, 1995). However, there are also plant species that hyperaccumulate other toxic metals, such as the fern *Pteris vittata* with its ability to hyperaccumulate arsenic (Ma et al., 2001) and a number of Ni hyperaccumulating plant species that have evolved on serpentine soils, with *Alyssum lesbiacum* as one of the most widely studied Ni hyperaccumulators (Kramer et al., 1996).

Recent progress has been made in the understanding of the molecular basis for the heavy metal tolerance in plants. It appears that a common feature is an alteration in the expression of genes involved in the homeostasis and transport of the metal in question. Metal hyperaccumulation often is associated with increased expression of genes encoding existing transporters of that metal. This can be through the increased expression of the gene(s) in a new tissue or at different developmental stages to cope with the heavy metal stress. Other times there is copy number variation for metal transporter genes such that

multiple copies of the gene result in increased abundance of the specific metal transporter protein. An example of this is the triplication of the *HMA4* gene in the Zn/Cd hyperaccumulator *Arabidopsis halleri* (Hanikenne et al., 2008). *AhHMA4* is a heavy metal ATPase involved in the loading of Zn and Cd into the xylem for transport to the aerial portions of the plant. Three copies of the *AhHMA4* gene exist in the *A. halleri* genome, leading to increased expression of *AhHMA4* and elevated Zn and Cd transport to the aerial tissues, which is an important component of Zn/Cd hyperaccumulation in the shoot. As discussed later in this chapter, there are also a number of other metal transporter gene families involved in the movement of the different toxic metals throughout the plant. Some of the most well-studied transporters involved in metal tolerance include the Al activated malate transporters (ALMTs), multidrug and toxic compound extrusion transporters (MATEs), zinc iron permeases (ZIPs), heavy metal associated transporters (HMAs), metal tolerance protein transporters (MTPs), natural resistance to macrophage protein transporters (Nramps), and the yellow stripe-like family of transporters (YSLs). In addition to metal transporter genes, other genes encoding enzymes involved in the production of organic compounds that can chelate and detoxify or be involved in cell-to-cell movement of the metal in the plant might also play a role in metal tolerance, and two such enzymes are phytochelatin synthase and nicotianamine synthase (Cobbet and Goldsbrough, 2002; Mari et al., 2006). Hence the list of genes potentially involved in metal tolerance in plants is quite extensive.

It also has been found that different plant species have evolved different mechanisms for tolerance to the same metal. For example, Al tolerance can involve exclusion of the rhizotoxic Al<sup>3+</sup> from the growing root tip, a strategy used by a number of monocot species including wheat, sorghum, and maize (see, e.g., Kochian et al., 2004), or it can involve true tolerance of accumulated Al in the root or shoot, such as Al hyperaccumulation to more than 1% shoot dry weight, by species such as tea (*Camellia sinensis*; Matsumoto et al., 1976), buckwheat (*Fagopyrum esculentum*; Ma et al., 1997a), and hydrangea (*Hydrangea macrophylla*; Ma et al., 1997b), or tolerance to Al accumulated in the root in rice (Xia et al., 2010).

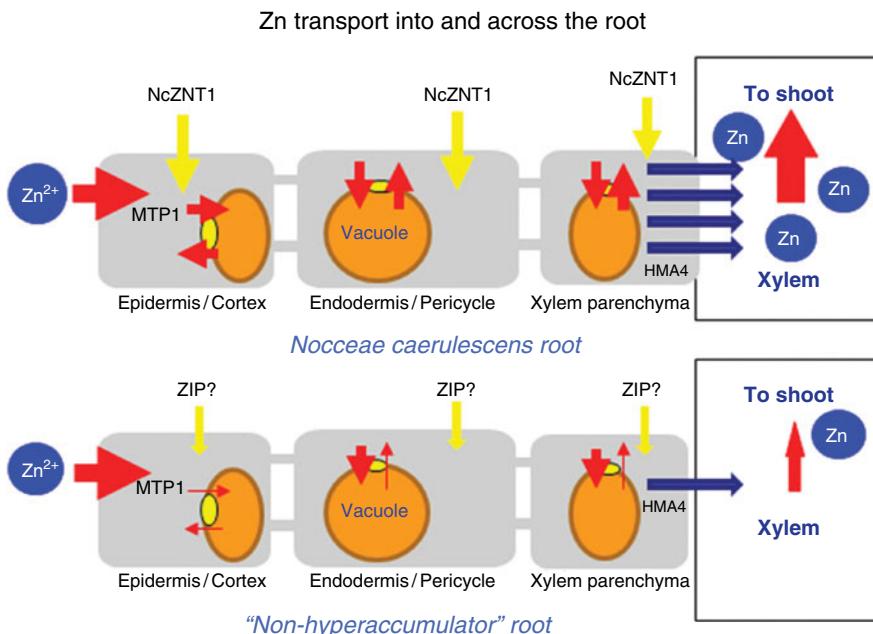
In this chapter, we will focus on plant tolerance to Zn and, to a lesser extent, Cd, with regards to heavy metal tolerance, and we also will review our understanding of crop tolerance to Al toxicity on acid soils. These will be used as examples of the different strategies plants employ to tolerate a range of toxic metals in the soil.

## 7.2 Plant Zn tolerance

### 7.2.1 Physiology of Zn tolerance

Zn tolerance in plants is fairly well understood due to research on two Zn/Cd hyperaccumulating plant species, *Noccaea caerulescens* (formally known as *Thlaspi caerulescens*) and *Arabidopsis halleri*. Both appear to employ similar

mechanisms to hyperaccumulate and tolerate high concentrations of Zn and Cd in the shoot. The metal transport component of hyperaccumulation appears to involve at least four different Zn transport strategies. The first is a large influx across the root cell plasma membrane, the second is reduced metal sequestration in the root vacuole, the third increased loading into the xylem for transport to the shoots, and finally, the fourth involves increased metal influx across the leaf cell plasma membrane and sequestration in the leaf vacuole (Lasat et al., 1998). The three transport steps that appear to be enhanced or altered in the root of Zn hyperaccumulators are summarized in the model depicted in Figure 7.1.



**Figure 7.1** Model of Zn transport into and across the root of the Zn/Cd hyperaccumulator, *Noccea caerulescens*. This is a speculative model for Zn<sup>2+</sup> transport from the soil into the root, radial Zn transport to the center of the root, and Zn loading into the xylem. The model depicts differences between the Zn hyperaccumulator, *Noccea caerulescens*, compared with a “typical” non-accumulating plant species. Elevated root Zn<sup>2+</sup> influx into the root from the soil is depicted for *N. caerulescens* (larger green arrows compared with root Zn<sup>2+</sup> influx in the non-hyperaccumulator). It has been suggested that NcZNT1 may be the transporter facilitating this uptake, but recent localization of the Arabidopsis homolog of the *TcZNT1* gene suggests it may also be involved in metal loading into the stele. Hence we also show NcZNT1 facilitating Zn influx from the apoplast into cells of the pericycle and other cells within the stele. The model also indicates there is less vacuolar sequestration of Zn in roots of *N. caerulescens*. Thus in the hyperaccumulator, there would be a larger pool of mobile Zn in the root that can more readily move through the endodermis and pericycle to the xylem parenchyma. We also show the elevated Zn loading into the xylem in *N. caerulescens*, via the Zn/Cd ATPase, HMA4, for subsequent transport to the shoots. For color details, please see color plate section.

Zn uptake kinetic analysis for *N. caerulescens* compared with a closely related non-accumulator species, *N. arvense*, found that the concentration-dependent affinity of Zn transport into the root was similar between the two species ( $K_m$  values of 6  $\mu\text{M}$  for *N. caerulescens* and 8  $\mu\text{M}$  for *N. arvense*); however, the maximal Zn uptake ( $V_{max}$ ) in *N. caerulescens* was approximately 6 times higher (Lasat et al., 1996). This suggests that the Zn transporters in roots of *N. caerulescens* are not more efficient at transporting Zn than in non-accumulators; instead, it appears that the density of Zn transporters in the root-cell plasma membrane is much higher in *N. caerulescens* versus *N. arvense*. In *Noccaea caerulescens*, the regulation of root Zn uptake by plant Zn status appears to be altered compared to what occurs in *N. arvense*. In *N. arvense*, like other non-accumulators such as the model plant species, *Arabidopsis thaliana*, root Zn uptake increases as the plant transitions from Zn sufficiency to deficiency. However, *N. caerulescens* maintains its elevated root Zn influx under both Zn deficiency and sufficiency. Only when *N. caerulescens* is grown on very high Zn concentrations (nutrient solutions with 50–1000  $\mu\text{M}$  Zn) does the  $V_{max}$  for Zn uptake decrease, but is still maintained at Zn influx values higher than what is seen in non-accumulators (Talukdar and Aarts, 2008). This Zn influx into the root has been suggested to be mediated by the high-affinity Zn transporter NcZNT1, which is highly specific for Zn transport (Pence et al., 2000). *NcZNT1* is one of the most highly expressed genes in the roots of *Noccaea caerulescens* (Pence et al., 2000). The expression of *NcZNT1* in the root mirrors the response of root Zn influx to changing plant Zn status, in that *NcZNT1* expression is very high in roots of Zn deficient and Zn sufficient plants, but its expression is reduced when the plants are grown on very high levels of Zn. These findings, along with reports of elevated expression of several other micronutrient transporters in *N. caerulescens* (Papoyan et al., 2004; Plaza et al., 2007; Talukdar and Aarts, 2008; Ó Lochlainn et al., 2011), have led us to speculate that the regulation of expression of micronutrient/heavy metal transporters by plant metal status is altered in metal hyperaccumulators, resulting in higher expression of several different transporters involved in the Zn/Cd transport pathway from the soil to the shoot.

Once Zn enters the root, it can either be stored in the root vacuole or transported radially through the root to be loaded into the xylem for transport to the shoots (see Figure 7.1). Root Zn compartmentation via efflux analysis was studied in both *Noccaea caerulescens* and *N. arvense* (Lasat et al., 1998). It was found that *N. arvense* stored approximately 2.5 times more Zn in the root vacuole compared to *N. caerulescens* and this vacuolar Zn was released from the vacuole 2 times more slowly. Longer-term studies showed that as much as 6 times more Zn was sequestered in the root-cell vacuoles of *N. arvense* compared to *N. caerulescens* over a 46-hour time period (Lasat et al., 1998). These findings indicate that along with much higher rates of Zn entry into the root, the hyperaccumulator species also maintains the root Zn in a more mobile pool that can be more readily moved to the xylem.

The next step regarding the movement of Zn from the soil to the shoot is the loading of Zn from xylem parenchyma into xylem vessels for translocation to the shoot (Figure 7.1). Xylem sap from *N. caerulescens* was found to contain approximately a five-fold higher Zn concentration than xylem sap from *N. arvense*, for plants grown on the same level of Zn. This is similar to the six-fold greater root Zn influx reported in *N. caerulescens* compared to *N. arvense* (Lasat et al., 1996). The dramatically greater metal loading into the xylem is a hallmark of metal hyperaccumulators and is believed to be mediated via the activity of the P-type ATPase, *HMA4*, which will be discussed in greater detail in the next section on the molecular biology of Zn accumulation and tolerance.

In the shoot, the storage of Zn to very high levels appears to require some coordination between different cell types. The highest concentrations of Zn and Cd are found in leaf epidermal cells, with Zn concentrations 4 times higher than what is measured in leaf mesophyll cells (Küpper et al., 1999). This preferred storage in leaf epidermal cells may be associated with the avoidance of high levels of heavy metals in photosynthetically active cells, as the photosynthetic apparatus is quite sensitive to heavy metals (Küpper et al., 2007). Epidermal cells (except for guard cells) lack chloroplasts, which makes them a preferential site for Zn and Cd accumulation. Although these metals are accumulated to much higher levels in the epidermis, a significant fraction of the total leaf metal accumulation still occurs in the mesophyll, as a larger fraction of the leaf biomass is associated with the mesophyll. It has been calculated that 65–70% of the total Zn in the leaves is in the mesophyll (Ma et al., 2005).

It appears that the high level of tolerance to Zn and Cd is due to a plant-wide cellular tolerance to these metals and not just the cells and tissues where the metal is preferentially stored. Investigation of Zn/Cd tolerance in suspension cells cultures of *N. caerulescens* compared with suspensions cell cultures derived from *Arabidopsis thaliana* showed that at the cellular level, *N. caerulescens* suspension cells were indeed more Zn and Cd tolerant than *Arabidopsis* suspension cells (Klein et al., 2008). However, it was interesting that the *N. caerulescens* cell lines accumulated less Zn and Cd than the *Arabidopsis* cell lines, most likely due to a greater metal efflux. This finding led the authors to hypothesize that the *N. caerulescens* suspension cells represent cells of the Zn/Cd transport pathway between the root epidermis and leaf, which appear to function to keep the Zn/Cd in a mobile pool that is readily translocated to the shoot.

Another interesting aspect of the relationship between plant metal status and plant heavy metal tolerance and accumulation comes from the discovery that when *N. caerulescens* was grown on very high Zn levels, the plants were more Cd tolerant and accumulated more Cd. The converse was also true, in that high Cd-grown plants also accumulated more Zn than low Cd-grown plants (Papoyan et al., 2007). This stimulated shoot metal accumulation was associated with

enhanced root metal influx, and xylem transport of these metals from the root to shoot. The authors speculated that as xylem loading is a key step in the hyperaccumulation process, the enhanced xylem loading triggered by exposure to high heavy metal levels for extended periods may translate into improved heavy metal tolerance, as the metals are more efficiently translocated to the shoots where highly effective metal tolerance mechanisms operate.

To better dissect and further understand the role that the roots and shoots both play in the Zn/Cd hyperaccumulation phenotype, a recent study using reciprocal grafting between *N. caerulescens* and a closely related non accumulator, *T. perfoliatum*, was performed to study the role of roots and shoots on the ability of *N. caerulescens* seedlings to hyperaccumulate Zn (de Guimarães et al., 2009). In this study it was shown that when *T. perfoliatum* root stock was grafted to a shoot scion of *N. caerulescens*, the grafted shoot no longer hyperaccumulated Zn, but rather accumulated Zn to levels seen in the non-accumulating *T. perfoliatum* shoots. However, when the reciprocal graft was made, with *T. perfoliatum* shoots on *N. caerulescens* roots, the *T. perfoliatum* shoots hyperaccumulated Zn when the grafted seedlings were grown on lower Zn levels. This ability to accumulate large levels of Zn in the shoots was reduced at higher Zn levels, as Zn toxicity started to occur in the aerial tissues, indicating that other tolerance mechanisms must be operating in the shoots of *N. caerulescens* compared with *T. perfoliatum*.

### 7.2.2 Molecular biology of Zn tolerance

Researchers have begun to understand some aspects of the transporters that play a role in Zn/Cd transport at the four suggested “altered” sites of metal transport associated with metal hyperaccumulation. Recent knowledge on how plants tolerate high levels of heavy metals in the soil suggests that two components of increased tolerance to Zn and Cd in hyperaccumulator plants involve enhanced gene expression. The first is that increased transporter expression is often associated with increased gene copy number; this seems to be the case in several different plant species and was first proposed for the Zn and Cd hyperaccumulating species, *Arabidopsis halleri*, by Talke et al. (2006). The other is more speculative, but circumstantial evidence suggests there are *cis*-acting DNA sequences involved in the higher expression of a number of metal related genes in hyperaccumulators. Such a genomic region was suggested to exist in the recent work from Milner et al. (2012) in *N. caerulescens*; however, the exact sequence causing this elevated expression remains to be determined.

The gene thought to be involved in the first step of hyperaccumulation, increased root Zn/Cd influx, is believed to be *NcZNT1*, which encodes a high-affinity Zn uptake transporter that is highly expressed in the roots of *Noccea caerulescens*. *NcZNT1* has been suggested to be a major player in the uptake of Zn from the soil to the root, with strong expression in the epidermis and cortex

of the root (Milner et al., 2012). Because of the strong NcZNT1 expression in the root stele, it also has been suggested by Milner et al. (2012) that this transporter may be involved in Zn translocation to the shoot. NcZNT1 was previously suggested to also be involved in root low-affinity Cd uptake (Pence et al., 2000). However, the more recent study of Milner et al. (2012) showed that the low-affinity Cd influx only occurred in the absence of Zn. Thus under normal soil solution conditions where free Zn<sup>2+</sup> ions are going to be present, it is likely that NcZNT1 mediates root Zn and not Cd uptake. NcZNT1 may be involved in the first and third transport steps of hyperaccumulation depicted in Figure 7.1. That is, NcZNT1 appears to be involved in the increased uptake of Zn into the root from the soil, and also in the reabsorption of Zn from the xylem in the shoot. The closest ortholog of NcZNT1 in *Arabidopsis thaliana* is AtZIP4. With regards to the elevated expression of NcZNT1 in roots and shoots, it was found that when expression of an NcZNT1 promoter-reporter and a AtZIP4 promoter-reporter were both stably transformed in *Arabidopsis thaliana*, expression of the NcZNT1 promoter-reporter was considerably higher than expression driven by the AtZIP4 promoter (Milner and Kochian, unpublished results). This result suggests that something specific in the promoter sequence of NcZNT1 leads to higher expression of this gene not only in *N. caerulescens* but also in the non-hyperaccumulator, *Arabidopsis thaliana*. The promoter sequence for this enhancer region believed to be involved in gene “hyperexpression” has been mapped to less than 100 bp; however, the exact sequence leading to the increased expression has not yet been identified.

Another metal transporter that has received significant attention for its role in metal hyperaccumulation in *N. caerulescens* and *A. halleri* is HMA4, which was first identified in *N. caerulescens* via a yeast complementation screening from a cDNA library comprised of Noccaea cDNAs. Two separate groups identified NcHMA4 based on its ability to increase Cd tolerance when expressed in yeast (Bernard et al., 2004; Papoyan and Kochian, 2004). NcHMA4 is a member of the P-type ATPase superfamily, and more specifically, the P<sub>IB</sub> subfamily of ATPases that transport heavy metals. NcHMA4 was found to be expressed primarily in roots and to a lesser extent in shoots and its expression is induced by both Zn deficiency and high Zn treatments, as well as in response to high Cd (Papoyan and Kochian, 2004). HMA4 has now been studied in several different plant species and is now known to be a plasma membrane transporter involved in the transport of divalent cations, mainly Zn and Cd, into the xylem, which is a key transport process for movement of metals to the shoot (Hussain et al., 2004; Papoyan and Kochian, 2004; Verret et al., 2004; Sinclair et al., 2007). When AtHMA4 and its close homolog AtHMA2 were knocked out in *Arabidopsis thaliana* plants, this led to poor growth on normal levels of Zn, with the plants being unable to effectively transport Zn to the aerial portions of the plant (Verret et al., 2004). Support for HMA4’s role in Zn and Cd hyperaccumulation comes from a QTL mapping study in a population generated from a cross between

*A. halleri* and *A. lyrata* (a related non-accumulator), which identified a major Cd tolerance QTL co-located with *AhHMA4* (Courbot et al., 2007). Recent studies in the two different metal hyperaccumulators, first *A. halleri* by Hanikenne et al. (2008) and more recently different ecotypes of *Noccaea caerulescens*, demonstrated a strong correlation between the level of accumulation of Zn and Cd in the shoot and the number of copies of *HMA4* in the genome (Craciun et al., 2012). In both *A. halleri* and *N. caerulescens*, multiple *HMA4* repeats were found in tandem, leading to increased *HMA4* expression and Zn and Cd accumulation (Hanikenne et al., 2008; Ó Lochlainn et al., 2011; Craciun et al., 2012). *HMA4* is believed to be the main player in the third step in Zn and Cd hyperaccumulation, which is the increased loading into the xylem for transport to the shoots.

A transporter that appears to play a significant role in the final step of Zn hyperaccumulation, loading of Zn into leaf cells, is MTP1, or Metal Tolerance Protein 1. MTP1 has been best characterized in *Arabidopsis thaliana* and is involved in the transport of Zn into the vacuole in both roots and leaves (Kobae et al., 2004; Debrosses-Fonrouge et al., 2005). In *A. thaliana*, AtMTP1 is a member of the Cation Diffusion Facilitator or CDF family, with 8 CDFs found in the *A. thaliana* genome. CDF transporters are involved in the transport of divalent cations out of the cytosol, by either transporting the metals into the vacuole or out of the cell. MTP proteins that mediate Zn transport have also been in some cases been given other names including ZAT (Zinc transporter of *A. thaliana*), ZTP (Zn Tolerance Protein) and CDF (Cation Diffusion Facilitator), depending on the lab that identified the transporter in a specific plant species.

In *A. thaliana*, loss of *AtMTP1* expression leads to increased Zn sensitivity when plants are grown on high levels of Zn (Kawachi et al., 2009). Functional analysis of Zn transport mediated by AtMTP1 has shown that Zn transport by AtMTP1 into the vacuole is pH dependent and most likely AtMTP1 is an antiporter transporting H<sup>+</sup> into the cytosol and Zn<sup>2+</sup> or other divalent cations into the vacuole (Kawachi et al., 2008). It should be noted that not all members of the CDF family in higher plants are tonoplast localized, with *Noccaea goesingense* *MTP1* being plasma membrane localized and involved in the transport of Ni ions out of the cell, which is associated with enhanced Ni tolerance (Kim et al., 2004). Another important finding regarding AtMTP1 is that a histidine (HIS)-rich region, once believed to be involved in Zn binding, appears to instead play a role in the regulation of Zn transport (Kawachi et al., 2008). When half of the HIS-rich cytosolic loop of MTP1 located between transmembrane domains 4 and 5 was deleted, increases in Zn transport by as much as ten-fold were measured when expressed in yeast. However, if more of the AtMTP1 cytosolic loop between transmembrane domains 4 and 5 was removed, then Zn transport function was lost (Kawachi et al., 2008). The authors speculated that the loss of a portion of the HIS region led to increased Zn transport

because this resulted in the Zn ions being bound to the protein for shorter periods of time, allowing them to more quickly move through the transporter “pore” region.

In the Zn/Cd hyperaccumulator' *A. halleri*, there are multiple copies of *AhMTP1* and this is associated with the increased tolerance and Zn accumulation seen in this plant species (Shahzad et al., 2010). The authors also found differences in the expression of each paralog of *MTP1* in *A. halleri*, which suggests other regulatory factors are also controlling the elevated expression seen in the hyperaccumulator species.

Another transporter that appears to be involved in Zn and Cd accumulation in the shoot is *HMA3*. *HMA3*, like *HMA2* and *HMA4*, transports divalent cations. However, *HMA3* is a tonoplast transporter while *HMA2* and *HMA4* are plasma membrane localized and, as described above, are involved in metal loading from xylem parenchyma into the xylem in the root. In *Arabidopsis thaliana*, *AtHMA3* mediates the transport of Zn and Cd (and also Pb), leading to increased Zn and Cd accumulation and tolerance in plants where *AtHMA3* has been over-expressed (Gravot et al., 2004; Morel et al., 2009). However, not all ecotypes of *A. thaliana* contain a functional *HMA3* gene, with Col-0 being one of the ecotypes lacking a functional *HMA3*. It was found that *A. thaliana* populations with a functional *HMA3* gene have a greater tolerance to Cd than those lacking *HMA3* (Morel et al., 2009).

In *Noccaea caerulescens*, there are two significant differences in *NcHMA3* in reference to *AtHMA3*. First, *NcHMA3* only transports Cd, and not Zn or Pb. The other major difference that like *NcHMA4*, different ecotypes of *Noccaea caerulescens* contain different numbers of copies of *NcHMA3*, with those populations with greater genome copy numbers exhibiting increased Cd hyper-accumulation (Ueno et al., 2011). In the Ganges ecotype of *N. caerulescens*, there were found to be five genomic copies of *NcHMA3*, whereas in the Prayon ecotype, *HMA3* is a single copy gene. This copy number difference results in a three-fold increase in *NcHMA3* expression in the shoots and a five-fold increase in root *NcHMA3* expression in Gange *versus* Prayon. *NcHMA3* is still expressed to significantly higher levels in Prayon than in the related non-accumulator, *A. thaliana*, with basal expression in the Prayon ecotype harboring 1 copy of *NcHMA3* still 10 times higher than *AtHMA3* expression in *Arabidopsis thaliana* (Milner and Kochian, unpublished data).

### 7.2.3 Role of metal-binding ligands in Zn tolerance

Physiological and biochemical studies have attempted to identify elevated levels of metal binding ligands that would be expected to be associated with the significantly increased shoot Zn and Cd levels in hyperaccumulators, and also the lower but still elevated levels of Zn/Cd found in roots and shoots of non-accumulators grown on toxic Zn/Cd levels. Many of these studies have focused

on organic acids, amino acids, and phytochelatins as candidate metal-binding ligands. It is interesting to note that several studies have not found a correlation between increased Zn and Cd accumulation in *N. caerulescens* and elevated levels of shoot phytochelatins or organic acids (Ebbs et al., 2002; Hernández-Allica et al., 2006). However, several other molecular studies have provided some circumstantial evidence that other organic compounds may play a role as metal-binding ligands. Using a yeast complementation approach, it was found that several different *N. caerulescens* metallothionein genes conferred significant increases in yeast Cd tolerance (Papoyan and Kochian, 2004). Metallothioneins are cysteine rich, low molecular weight, metal binding proteins that can form mercaptide bonds with various metals and have been implicated in metal homeostasis, primarily in mammals (Cobbett and Goldsbrough, 2002). Metallothioneins have been found in a wide range of organisms crossing a number of kingdoms, with all of the plant MT's falling in one of two main subclasses. For these two main groups, classification is based on where the cysteine residues thought to be involved in the binding of the various metal ions are located (Cobbett and Goldsbrough, 2002). However, the exact role that MTs play in plant metal tolerance and metal homeostasis remains to be determined.

Several studies have focused attention on the role of the non-protein amino acid, nicotianamine, in metal tolerance of *Arabidopsis* and *N. caerulescens*. Nicotianamine (NA) has been shown to be a chelator of several micronutrient metals and has been suggested to be involved in the movement of micronutrients and heavy metals throughout the plant (Stephan and Scholz, 1993). In ecotypes of *N. caerulescens* that also hyperaccumulate Ni, Mari et al. (2006) found via a yeast complementation assay for Ni tolerance that the gene encoding nicotianamine synthase, *NcNAS1*, conferred high levels of Ni tolerance when expressed in yeast. *NcNAS1* was found to be expressed only in the shoots and induced in as little as 6 hours after treatment of Ni. However, after this same 6-hour exposure to Ni, high levels of nicotianamine were found in the roots and NA-Ni complexes were also found in the xylem sap. This led the authors to speculate that in response to Ni, NA is translocated to the roots where it chelates the absorbed Ni and facilitates its transport to the shoot. Further evidence for a role for *NcNAS1* in Ni tolerance came from studies where *NcNAS1* was over expressed in transgenic *A. thaliana* plants (Pianelli et al., 2005). This resulted in significant increases in both plant Ni tolerance and Ni accumulation in the shoot. These findings suggest that a number of transporters need to be involved in the movement of both free NA and the NA-metal complexes in the plant. One possible family of transporters for this role is the Yellow Stripe Like family, where the first member of this family was identified as the putative Fe-phytosiderophore uptake transporter in maize roots, while other members have been hypothesized to be involved in transport of NA-metal complexes (Curie et al., 2001).

### 7.3 Plant Cd tolerance

Cd accumulation in *N. caerulescens* and *A. halleri* may utilize the same mechanisms that these plants use for Zn accumulation. The one component of Zn/Cd hyperaccumulation where this might not be the case is root Cd influx. To date no Cd-specific transporters have been identified, and it is generally believed Cd is transported into and within the plant via transporters functioning to transport the essential micronutrients. Because of the ionic similarities between Zn<sup>2+</sup> and Cd<sup>2+</sup>, it has long been assumed that Cd is absorbed by plant roots via Zn and Fe transporters. In a number of plant species, the high-affinity Fe uptake transporter IRT1 can transport physiologically relevant levels of Cd into the root from complex nutrient solutions that mimic soil solution (Korshunova et al., 1999; Cohen et al., 2004; Plaza et al., 2007). However, as detailed above, it appears that contrary to an earlier result from our lab indicating the Zn uptake transporter, NcZNT1, might also mediate root Cd influx (Pence et al., 2000), findings from more detailed recent research indicates that although NcZNT1 can mediate yeast Cd influx from a simple salt (CaCl<sub>2</sub>) solution, when low micromolar levels of Zn are included in the uptake solution, this Cd influx disappears. These results suggest that NcZNT1 primarily functions in Zn uptake and is at best a low-affinity Cd transporter. This finding suggests that possibly Fe transporters may be the major route for Cd entry into the plant. Recent research on the Nramp family of transporters has shown some members of this that were believed to be Fe transporters can also transport physiologically relevant levels of Cd and may be involved in many aspects of Cd translocation through the plant. It is also possible that Mn transporting Nramps also can transport Cd. The recent publication by Cailliatte et al. (2010) showed that AtNramp1 is a high-affinity Mn transporter with a broad substrate range and can mediate Cd transport. Other plant Nramps, namely Nramp3 and Nramp4, can also transport Cd but are localized to the vacuole and are involved in the remobilization of heavy metals from the vacuole back into the cytosol (Lanquar et al., 2005).

### 7.4 Plant aluminum tolerance

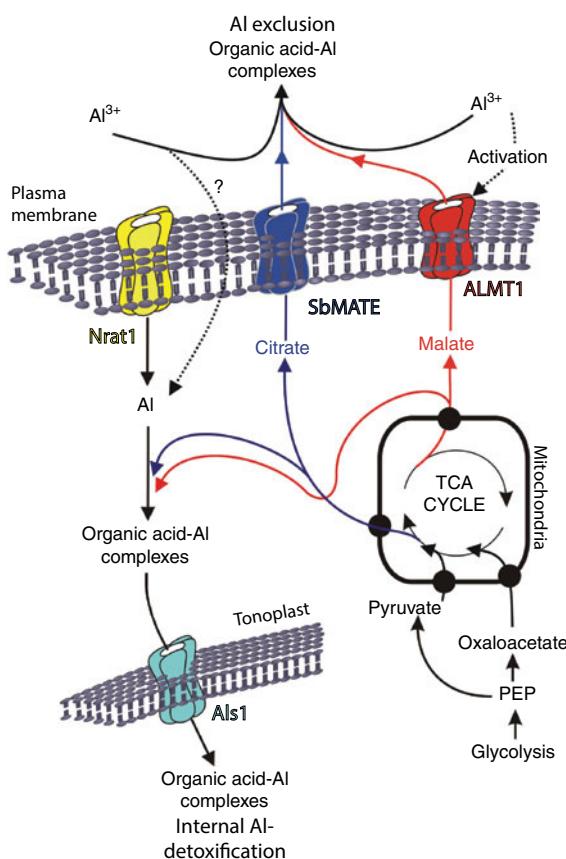
#### 7.4.1 Physiology of Al tolerance

Aluminum toxicity is the major constraint to crop production on highly acid soils (soil pH<5), which are estimated to comprise over 50% of the world's arable land (Uexküll and Mutert, 1995). On these acid soils, Al is solubilized from aluminosilicate clays into the soil solution as the rhizotoxic Al<sup>3+</sup> ion, causing a rapid inhibition of root growth that leads to a reduced and stunted root system, limiting the ability of a plant to acquire both water and nutrients. There

is considerable evidence in the literature that many plant species employ an Al tolerance mechanism based on the exclusion of toxic Al<sup>3+</sup> ions from the growing root tip. This mechanism is based on the chelation of Al<sup>3+</sup> by organic acid anions effluxed from cells of the root apex (the site of Al toxicity), with the plant employing specialized, plasma membrane-localized transporters (Ma et al., 2001; Kochian et al., 2004; Delhaize et al., 2007). This Al exclusion mechanism has been shown to operate in a number of cereal plant species, including barley, wheat, maize, and sorghum, as well as dicot crop species such as soybean, snapbean, and rice bean, and also the model plant species, *Arabidopsis thaliana* (Miyakawa et al., 1991; Delhaize et al. 1993b; Pellet et al., 1995; Yang et al., 2000; Hoekenga et al., 2006; Furukawa et al., 2007; Magalhaes et al., 2007; Yang et al., 2011).

Because Al is a very reactive metal and can interact with many possible toxicity sites in the root tip cell wall and symplasm, potential mechanisms of Al toxicity are numerous and include inhibition of cell elongation and cell division via Al interaction with cell wall proteins and cell wall carbohydrates, Al interactions with the root cell plasma membrane, cytoplasmic interactions including the cytoskeleton, Ca signaling and G proteins, and Al interactions with nucleic acids (see, e.g., Kochian, 1995). Al-induced inhibition of root growth is rapid, and can be detected within minutes of Al exposure, indicating that Al inhibition of cell elongation and not cell division is the primary initial Al toxicity symptom. Also, it has been shown that Al must interact with the root tip to elicit inhibition of root growth (Ryan et al., 1993), which makes it clear that Al tolerance mechanisms must reside in the root tip. The general features of the physiological basis for an Al tolerance mechanism based on Al exclusion from the root tip have been well described in the literature and are summarized in Figure 7.2; they include: (1) Al induces or activates root exudation of Al chelating organic acids. The primary organic acids released are malate (wheat), citrate (maize, sorghum, soybean, ricebean, snapbean), and oxalate (buckwheat). Also, in a few species including *Arabidopsis* and some very Al-tolerant wheat varieties, both malate and citrate are excreted from the root. (2) The Al-activated organic acid release is localized to the root tip. This minimizes unnecessary exudation of citrate or malate, which are valuable C resources for the plant. Thus this process is highly regulated so that it only occurs within the tissues damaged by Al (the root tip) and only in the presence of the toxic metal Al. (3) It appears that the primary process underlying this tolerance mechanism is Al activation of organic acid transport and not Al effects on organic acid synthesis (see, e.g., Delhaize et al., 1993a, 1993b).

A second mechanism of Al tolerance involves processes that detoxify and inactivate Al absorbed by the plant. Some of the evidence for these true Al tolerance mechanisms comes from work on two Al accumulating species, *Hydrangea* and buckwheat. *Hydrangea* is an ornamental plant whose floral



**Figure 7.2** Physiological mechanisms of aluminum (Al) tolerance. The model depicts the well-characterized root tip Al exclusion and less well-studied internal Al detoxification mechanisms of Al tolerance. The Al exclusion mechanism involves the transport of organic acids (OA) across the root-cell plasma membrane into the rhizosphere via an Al-gated anion channel for malate (ALMT1) or an Al-activated citrate efflux transporter (MATE). Activation of the ALMT channels appears to be due to direct activation of the transport protein by Al. Al activation of the MATE may be more indirect and could involve Al interacting with a second membrane-bound receptor protein that associates with the MATE protein, or by Al entering the cytosol and triggering MATE activation. It is known that Al also triggers changes in expression of genes involved in Al tolerance. The internal Al detoxification involves the entry of Al via an Al transporter that is known to be OsNrat1 in the rice root but has not been identified in Al accumulators such as buckwheat and *Hydrangea*, where the Al is sequestered in the leaf vacuole. For these shoot Al accumulators, the tolerance mechanism involves chelation of cytoplasmic Al by organic acids with the subsequent sequestration into the vacuole. Here we also suggest that in the rice root, possibly Al transported into the root cell by OsNrat1 might be transported into the vacuole by OsALS1 mediated by the transport of Al complexed with organic acids. For color details, please see color plate section.

sepals change from red to blue when the soil is acidified. This change in sepal color is the result of sepal Al accumulation where the Al complexes with delphinidin-3-glucoside and 3-caffeylquinic acid, forming a blue complex. *Hydrangea* is an Al accumulator plant and leaf Al concentrations can exceed

3000 ppm Al (Ma et al., 1997b). While Al is complexed with the above-mentioned phenolics in the sepals, in the leaves the accumulated Al exists primarily as a 1:1 Al-citrate complex, based on  $^{27}\text{Al}$ -NMR spectroscopy. In a pH 7 cytosol, the Al-citrate complex is quite stable and should not be phytotoxic. The same researchers also showed that a component of Al tolerance in another Al accumulator plant, buckwheat, involves Al-activated oxalate exudation from the root apex (Zheng et al., 1998). Buckwheat also accumulates Al in leaves to as high as 15,000 ppm when grown on acid soils (Ma and Hiradate, 2000), and much of the accumulated Al is complexed with oxalate in a 1:3 Al:oxalate complex (Ma et al., 1998). In another study, protoplasts and vacuoles were isolated from buckwheat leaves that had accumulated Al; it was found that most of the Al in the leaf protoplast exists as a 1:3 Al:oxalate complex, which was sequestered in the vacuole (Shen et al., 2002). Therefore, as depicted in the model in Figure 7.2, this mechanism appears to involve Al chelation in the cytosol and subsequent transport of the Al-organic acid complex into the vacuole.

Rice is the most Al-tolerant cereal and its high Al tolerance is not correlated with Al exclusion from the root tip or Al-activated release of Al chelating organic acids (Famoso et al., 2010). It is clear that unlike other cereals that have been extensively studied for Al tolerance (barely, wheat, sorghum, maize, rye), rice employs true Al tolerance mechanisms and not Al exclusion mediated by root organic acid exudation. Furthermore, the genetics of rice Al tolerance is far more complex than the genetics of Al tolerance in the other cereals. A single gene can explain much of the variation in wheat, barley and sorghum Al tolerance mapping populations (Garvin and Carver, 2003). In rice, Al tolerance is a complex quantitative trait that can involve a number of different loci. For example, Famoso et al. (2011) conducted a genome-wide association study of rice Al tolerance and identified more than 40 different regions in the rice genome associated with Al tolerance.

Research by Ma and coworkers have identified a number of different rice Al tolerance genes using forward genetics approaches based on map-based cloning of Al sensitive rice mutants. One of these tolerance genes is *OsNrat1*, which encodes a unique Nramp metal transporter that specifically mediates Al uptake across the plasma membrane of cells of the rice root tip (Xia et al., 2010). This transporter appears to work in conjunction with a second Al transporter, *OsALS1*, which mediates transport of the absorbed Al from the cytosol to the vacuole, where it is stored and detoxified (Huang et al., 2011). This is a novel Al tolerance mechanism, and appears to involve protection of the root cell wall from Al. Because rhizotoxic  $\text{Al}^{3+}$  is a trivalent cation, the numerous negative sites in the root cell wall bind  $\text{Al}^{3+}$  with high affinity. Hence, the majority of the root-associated Al resides in the cell wall. It appears that *OsNrat1* and *OsALS1* function together to remove some of this Al from the cell wall and store it in the root cell vacuole.

#### 7.4.2 Molecular biology of Al tolerance

A number of Al tolerance genes have been identified and a surprising number of these genes encode different types of ion transporters. The first Al tolerance gene cloned was *TaALMT1*. ALMT stands for Al-activated Malate Transporter; it was cloned in wheat (Sasaki et al., 2004). *TaALMT1* is the first identified member of the ALMT family cloned, underlies the Al tolerance locus,  $Alt_{BH}$ , and is an Al activated plasma membrane anion channel that facilitates the efflux of malate from the root tip, which underlies the root tip Al exclusion tolerance mechanism in wheat (Sasaki et al., 2004; Yamaguchi et al., 2005). The ALMT family is a small group of transporters unique to plants that all have five to seven transmembrane domains, with the transmembrane domains located within the N terminal end of the protein followed by a large soluble C terminal region (Delhaize et al., 2007). While the ALMT family is named for its founding member's role in Al tolerance, it has since become clear that not all members of the family play a role in Al tolerance, with other family members being involved in anion homeostasis, osmotic adjustment and guard cell function (Sasaki et al., 2004; Kovermann et al., 2007; Piñeros et al., 2008a; Gruber et al., 2010; Meyer et al., 2010, 2011). Electrophysiological analysis of *TaALMT1* expressed in *Xenopus* oocytes showed that the transport protein contains both the malate-permeable pore and the Al-activation domain, and the transporter can also mediate the transport of inorganic mineral anions including  $\text{NO}_3^-$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$  (Piñeros et al., 2008b).

The second family of Al tolerance proteins to be identified is the large and diverse Multidrug and Toxic Compound Extrusion or MATE family of transporters. This family of transporters mediates the transport of a wide range of different organic solutes from the cytoplasm either out of the cell or into the cell vacuole. The first member of the MATE family found to be involved in Al tolerance was identified via mapped based cloning of the major sorghum Al tolerance locus,  $Alt_{SB}$  (Magalhaes et al., 2004). The gene underlying the  $Alt_{SB}$  locus was named SbMATE and encodes the sorghum root tip plasma membrane citrate efflux transporter (Magalhaes et al., 2007). At about the same time that SbMATE was identified, Furukawa et al. (2007) found that HvMATE was a similar MATE protein that underlies barley root citrate exudation responsible for barley Al tolerance. Since these discoveries, other MATE proteins from Arabidopsis, wheat, maize, ricebean, and rice have been identified that play a role in Al tolerance in these species by also functioning as root citrate efflux transporters (Liu et al., 2009; Ryan et al., 2009; Maron et al., 2010; Yang et al., 2011; Yokosho et al., 2011).

As mentioned above, the superior Al tolerance in rice is a more genetically complex trait than Al tolerance in the other cereals, and the majority of the Al tolerance in rice is not based on root organic acid efflux. Ma's group has identified several different rice Al tolerance genes based on the cloning of Al sensitive

mutations in rice. The first of these rice genes was *OsSTAR1*, which encodes a protein with a nucleotide-binding domain for an ABC transporter, which interacts with a second rice protein encoded by *OsSTAR2*. Together, these two proteins form an ABC transporter complex (Huang et al., 2009). It appears that OSSTAR1/STAR2 mediate the transport of UDP-glucose into endomembrane vesicles, which fuse with the plasma membrane, releasing the UDP-glucose into the apoplast where it can modify cell wall composition. It has been suggested that this transporter functions to modify the carbohydrate composition of the cell wall such that it alters the wall binding of Al.

Another rice Al tolerance gene identified via map-based cloning of an Al sensitive mutation was *ART1*, which encodes a Zn-finger transcription factor that Ma's group found regulates the Al-induced expression of a suite of other candidate rice Al tolerance genes (Yamaji et al., 2009). When *ART1* expression was knocked down, rice Al tolerance was significantly diminished. *ART1* was found to be constitutively expressed in the root tip and its expression is not influenced by Al exposure. Comparative microarray analysis of rice root gene expression between wild type and *ART1* knockouts identified 31 genes whose Al-induced expression appears to be regulated by *ART1*. This list of genes includes *STAR1* and *STAR2*. Some of the other *ART1* regulated genes are homologues of other cereal Al tolerance genes. This list of genes is an excellent resource that Ma's group is using to identify other novel rice Al tolerance genes and mechanisms. One of the genes regulated by *ART1* is *Nrat1*, which we discussed above as a rice Al tolerance gene that is involved in a novel Al tolerance mechanism involving removal of toxic Al from the cell wall by transporting it into the root cytoplasm, where the vacuolar transporter, ALS1 then mediates its sequestration in the vacuole (Xia et al., 2010; Huang et al., 2011).

As Ma's group was not able to locate any of these rice genes to previously identified Al tolerance QTL, it was not clear if these genes explain the variation of rice Al tolerance that can be used to facilitate breeding for improved rice Al tolerance. The role of these genes in rice Al tolerance diversity subsequently came from a genome-wide association study (GWAS) of rice Al tolerance (Famoso et al., 2011). This involved the phenotyping of a panel of 383 diverse domesticated rice lines for Al tolerance. The panel was also genotyped with approximately 37,000 SNPs and GWAS was conducted on rice Al tolerance. Forty eight regions of the rice genome were found to be associated with rice Al tolerance and many of these associations were subpopulation specific. Three of the Al tolerance regions corresponded to the locations of *ART1*, *STAR2* and *Nrat1*, strongly suggesting that these tolerance genes play a role in genetic variation in rice Al tolerance. Haplotype analysis for *Nrat1* identified an Al sensitive haplotype that explained 40% of the variation in Al tolerance in the *aus* subpopulation. Detailed sequencing of *Nrat1* alleles identified three non-synonymous mutations within the *Nrat1* coding region that were predictive of Al sensitivity. These finding suggest that causal polymorphisms in the *Nrat1* coding region

may result in differences in Nrat1 Al transport properties, such that the Al sensitive Nrat1 transporter is less effective at transporting Al.

### 7.5 Conclusion

Plants have evolved a myriad of adaptive mechanisms based on a number of genes to deal with the different toxic metals they encounter in soils worldwide. These genes encode a range of different metal and organic compound transporters and enzyme pathways for the synthesis of metal detoxifying ligands. Overall, our understanding of this field indicates that plants have evolved at least two main strategies to deal with toxic metal stress: (1) exclusion of the metal from the plant or from the shoot; and (2) tolerance mechanisms that allow the plant to accumulate or even in some cases hyperaccumulate the metal in the plant. Within these two general metal tolerance strategies, there are a number of variants and subtle adaptations that different plant species have made to adapt to different environmental niches. In this chapter, we have provided an overview of these strategies by focusing first on plant tolerance to an essential micronutrient that is also a heavy metal, Zn. We also focused on a toxic metal, Al, which is very abundant in the soil and only becomes a problem to plants when the soil pH drops to pH 5 or below. It is interesting to note that both Zn and Al tolerance appears to depend on the integrated function of metal transporters and the synthesis and transport of metal detoxifying organic ligands. There is still much to be discovered about these tolerance mechanisms, particularly with regard to how these processes are regulated and coordinated. Significant progress already has been made that should set the stage for further discoveries, and more importantly, the translation of the knowledge gained from this basic research into the generation of crops that can provide increased yields on metal-intoxicated soils while minimizing metal entry into the food chain. Also, these discoveries may allow for the generation of high biomass plants designed to remediate heavy metal contaminated processes via green technologies such as phytoremediation.

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## **8 Epigenetic regulation of abiotic stress responses in plants**

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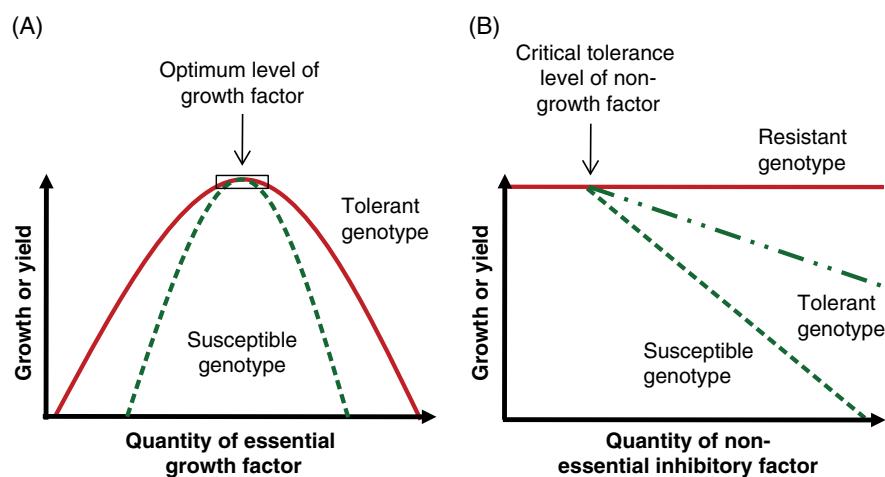
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### **8.1 Introduction**

Environmental factors such as water, temperature, light, and nutrients are essential for plant growth and development. Environments that supply optimal levels of these factors are best suited for plant productivity. However, most agricultural lands are deficient or toxic in one or more of these essential growth factors. The stresses caused by deficiency or toxicity of these growth factors are called *abiotic stresses*. These include water-deficit (drought)/waterlogging stress, temperature extremes, nutrient deficiency/toxicity stresses, and low/high light stresses. Some non-essential inhibitory environmental factors, such as excess salts in the soil solution (salinity), heavy metals, UV radiation, or air pollution, also cause stress to plants when their levels exceed certain thresholds. Growth and development responses of plants depend on the nature of stress (Figure 8.1). Plants have evolved several adaptive mechanisms to survive and reproduce under these stress conditions. The mechanisms for tolerance to these two kinds of abiotic stresses differ at large but have some common networks. These mechanisms require precise reprogramming of gene expression patterns. The molecular genetic and physiological bases of abiotic stress responses of plants have been reviewed extensively (Zhu, 2002; Chinnusamy et al., 2004, 2010; Cramer et al., 2011; Qin et al., 2011; Sunkar et al., 2012) and summarized in the previous chapters of this book. During the past decade, significant progress has been made in unraveling epigenetic mechanisms involved in gene regulation. An understanding of the mechanisms of establishment of epigenetic codes and their relationship with gene repression under abiotic



**Figure 8.1** Growth response of plants to environmental stresses. (A) Response to essential growth factors. The decline in growth and yield of tolerant genotypes is less as compared with susceptible genotypes as the quantity of essential growth factors decreases or increases from the optimum levels. (B) Response to non-essential inhibitory factors. No decline in growth and yield of resistant genotypes. The decline in growth and yield of tolerant genotypes is less as compared with susceptible genotypes as the quantity of growth inhibitory factors increases above the critical minimum levels.

stresses is important for developing stress-tolerant crop plants. This chapter focuses on epigenetic mechanisms involved in abiotic stress responses of plants.

## 8.2 Epigenetic controls of gene expression

Epigenetics is defined as the study of DNA sequence-independent changes in gene function that are mitotically and/or meiotically heritable (Wu and Morris, 2001). Often the definition of epigenetics includes chromosomal marks that are transient, as in the case of DNA repair or cell-cycle phases, and stable marks that are maintained across multiple cell generations (Bird, 2007). Genome architecture and gene expression depends upon the sequence of genome and its organization into varying degrees of packaging. The DNA is packaged into an organized structure called *chromatin*. Nucleosome, the basic unit of chromatin, consists of about 200 bp DNA, an octamer histone-core complex (H2A, H2B, H3, and H4), and the linker histone H1. The N-terminal regions (20–35 residues) of core histones undergo various post-translational modifications such as acetylation, methylation, phosphorylation, sumoylation, ubiquitination, etc. The cytosine residue of DNA also undergoes methylation and demethylation. Histone variants, histone post-translational modifications and DNA methylation determine nucleosome arrangement and compactness of chromatin. For instance, tightly packed heterochromatin region is less transcribed or not transcribed, while less packed euchromatin is amenable for RNA polymerase II mediated gene transcription. The combination of histone modifications,

histone variants, nucleosome repositioning and DNA methylation constitute the epigenetic code. The epigenetic code in turn determines the chromatin structure and gene expression (Chinnusamy and Zhu, 2009a,b; Law and Jacobsen, 2010; Meyer, 2011; Zhang and Zhu, 2011; Pecinka and Mittelsten Scheid, 2012).

### 8.2.1 Establishment of histone code

Post-translational modifications of N-terminal regions of histones lead to changes in chromatin structure and affinity for chromatin-associated proteins, and thereby regulate gene expression. For detailed mechanisms of establishment of histone codes and erasure of histone modifications, the readers may refer to specialized reviews (Zhang, 2003; Peterson and Laniel, 2004; Couture and Trievle, 2006; Houben et al., 2007; Garcia-Dominguez and Reyes, 2009; Deal and Henikoff, 2011; Messner and Hottiger, 2011). The enzymes involved in establishment and erasure of various histone post-translational modifications and their relationship with gene expression are summarized in Table 8.1.

### 8.2.2 DNA cytosine methylation

DNA methylation is the major heritable epigenetic modification. The cytosine residue of DNA is methylated at the 5' position. Although 5-methylcytosine in the CG sequence context is predominant in animals, cytosine methylation occurs in all sequence contexts in plants, that is, CG, CHG, and CHH (H=A, T, or C; Feng et al., 2010). Epigenome sequencing revealed that CG, CHG and CHH methylation accounts for 24, 6.7 and 1.7%, respectively in *Arabidopsis* (Cokus et al., 2008). DNA cytosine methyltransferases catalyze cytosine methylation by transfer of an activated methyl group from S-adenosyl methionine (SAM) to the 5' position of the cytosine. DNA methyltransferases are classified into three families in *Arabidopsis*, namely the DNA methyltransferase (MET) family (homolog of vertebrate DNMT1), chromomethylase (CMTs, plant specific) family, and DOMAINS REARRANGED METHYLASE (DRM; the canonical methyltransferase motifs are organized in a novel order) family (homolog of vertebrate DNMT3). In *Arabidopsis*, *de novo* methylation is mainly established by DRM1 and DRM2 (Cao and Jacobsen, 2002a; Cao et al., 2003). However, MET1 and CMT1 also catalyze establishment of *de novo* cytosine methylation (Cao and Jacobsen, 2002b; Aufsatz et al., 2004). The symmetric CG and CHG methylation are maintained by MET1 (Cokus et al., 2008; Lister et al., 2008) and CMT3, respectively, while the asymmetric CHH methylation is maintained by the CMT3 (Bartee et al., 2001; Cao et al., 2003), DRM1, and DRM2 (Cao et al., 2003; Lister et al., 2008).

#### *RNA-directed DNA methylation*

Small RNAs, mainly small interfering RNAs (siRNAs) and in some cases microRNAs (miRNAs), direct sequence specific *de novo* methylation of DNA.

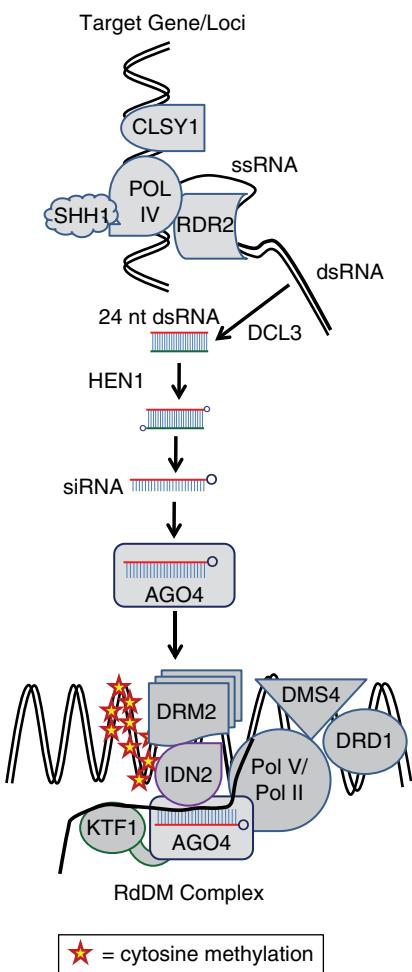
**Table 8.1** Histone modifications and gene expression

Histone Modification	Common N-tail Residues Modified	Establishment	Erasure	Gene Expression
Acetylation	H2A (K 4, 5, 7)	Histone acetyltransferases (HATs) transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine	Histone deacetylases (HDACs)	Acetylation leads to transcriptional activation
	H2B (K 5, 11, 12, 15, 16, 20)			
	H3 (K 4, 9, 14, 18, 23, 27)			
	H4 (K 5, 8, 12, 16)			
Methylation	H3 (K4, 9, 27, 36) H4K20 H3 R3	Histone lysine methyltransferases (HKMTases) and protein arginine methyltransferase (PRMT) transfer methyl group from S-adenosyl-L-methionine to ε-amino group of lysine and guanidino group of arginine, respectively	Lysine-specific demethylase1 (LSD1)/ KDM1) and Jumonji C (JmjC) domain-containing proteins	H3K4 trimethylation and H3K36 dimethylation activate gene expression. H3K9 and H3K27 dimethylation repress gene expression. H4R3 asymmetric dimethylation represses gene expression
Ubiquitination	H2A H2B (monoubiquitination)	E3 ubiquitin ligases such as BRE1 (Brefeldin A-sensitivity protein 1) homologs (Histone Monoubiquitination1 and HUB2), and E2 ubiquitin conjugases enzymes such as RAD6 homologs (Ubiquitin Carrier Protein1, UBC2, and UBC3) ubiquitinate H2	Ubiquitin-specific thiol proteases, Ubatain-like deubiquitinase (OTLD1)	Activation of transcription, often associated with H3K4me3 (Krichevsky et al., 2011)
SUMOylation	H2A.Z H2B H4	Small ubiquitin-like modifier (SUMO) E3-ligases	SUMO-specific proteases	Transcriptional repression, often associated with HDACs
Phosphorylation	Hydroxyl groups of serine and threonine in H2A, H2B, H3, and H4	Aurora family kinases	Phosphatase 1 (PP1) family enzymes	Activation of transcription
Biotinylation	H1, H2A, H2B, H3, and H4	Biotinidases (histone biotinyl transferases) transfer biotin to ε-amino group of lysine	Biotinidase	Transcriptional repression. Mainly studied in animals
ADP-Ribosylation	H1, H2A, H2B, H3, and H4	Guanidino group arginine and carboxyl group glutamic acid are ribosylated by mono- or poly ADP-ribosyl transferases (ARTs)	ADP ribosyl hydrolases (ARHs) and PAR glycohydrolases (PARGs)	Activation of transcription in animals

This pathway is called the *RNA-directed DNA methylation (RdDM) pathway*. It was discovered initially in viroid-infected tobacco plants (Wassenegger et al., 1994). Transcriptional gene silencing was found to be caused by heritable promoter methylation in tobacco (Park et al., 1996). The promoter methylation was caused by small RNAs of 23 nt derived from double-stranded RNA (dsRNA; Mette et al., 2000). Later studies identified the cellular machinery involved in biogenesis of small RNAs and RdDM. The plant-specific DNA-dependent RNA polymerase IV (Pol IV/Pol IVa/Nuclear RNA Polymerase D, NRPD) transcribes inverted repeats, pseudogenes, promoters and heterochromatic loci to generate aberrant transcripts in plants. *Arabidopsis* and maize mutants of Pol IV are defective in siRNA accumulation and DNA methylation (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005; Erhard et al., 2009; He et al., 2009a) suggesting that the Pol IV transcripts are necessary for siRNA accumulation. The RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) converts Pol IV transcripts into dsRNAs (Xie et al., 2004; Alleman et al., 2006). RDR2 physically interacts with Pol IV, and this interaction is necessary for RDR2 activity. This suggests a coupling of Pol IV transcription with RDR2 dependent dsRNA production at target loci (Haag et al., 2012). A homeodomain protein, SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) is also present in Pol IV complex and is required for *de novo* and maintenance DNA methylation (Law et al., 2011).

The Dicer-like family of ribonucleases III catalyzes the cleavage of dsRNAs to produce ~21–24 nt small RNA duplex (Chan et al., 2004; Xie et al., 2004), which is then methylated at 2'-OH group of the three-terminal nucleotide by a methyltransferase, HUA ENHANCER-1 (HEN1) in the nucleus (Xie et al., 2004). This duplex is unpaired and siRNAs are loaded onto Argonaute (AGO4/6; Zilberman et al., 2003; Baulcombe, 2004; Henderson and Jacobsen, 2007; Zheng et al., 2007). MicroRNA::miRNA target mRNA hybrid derived siRNAs and miRNAs can also mediate RdDM in plants (Chellappan et al., 2010; Khraiwesh et al., 2010; Wu et al., 2010).

The core complex of RdDM consists of AGO4/6, DNA-dependent RNA polymerase V (Pol V), DRM2, and several other proteins (Figure 8.2). Transcription factors such as RNA-DIRECTED DNA METHYLATION 4 (RDM4)/DEFECTIVE IN MERISTEM SILENCING 4 (DMS4) may help recruit Pol V (He et al., 2009b; Kanno et al., 2010). The nascent non-coding transcripts produced by Pol V recruit siRNA-bound AGO4 through Watson-Crick base pairing (Wierzbicki et al., 2008). Comparative analysis of genomic sites of Pol V occupancy, siRNA deep sequencing and methylcytosine mapping of wild type (WT) and mutants of *Pol IV*, *Pol V*, or *Pol IV/V* double mutants revealed that Pol IV and Pol V are required for guiding CHH methylation (Wierzbicki et al., 2012; Zhang and Zhu, 2012). RNA binding proteins, namely the KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1)/SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L; He et al., 2009c) and



**Figure 8.2** RNA-directed DNA methylation in plants. The SNF2 chromatin remodeling protein CLASSY 1 (CLSY1) facilitates chromatin decondensation and access of RNA Pol IV to the target loci. Pol IV transcripts are converted into dsRNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). These dsRNAs serve as substrate for DICER-LIKE 3 (DCL3) catalyzed production of 24-nt small RNA duplex. HUA ENHANCER-1 (HEN1), an RNA methyltransferase, catalyses the methylation of the 2'-OH group of three-terminal nucleotides in the small-RNA duplex. SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) is required for Pol IV dependent siRNA production. The 24nt siRNAs are loaded onto Argonaute 4 (AGO4) protein. DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1, chromatin-remodelling factor) and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) facilitate Pol V/II access to the target loci. Transcription factors such as RNA-DIRECTED DNA METHYLATION4 (RDM4)/DMS4 help recruit RNA polymerase V. Pol V transcribes target loci to produce ssRNAs. These transcripts serve as scaffolding for recruitment of complementary siRNA bound AGO4. The KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1)/SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L) binds to nascent scaffold transcript RNA and recruits AGO4-bound siRNAs to the RdDM effector complex. INVOLVED IN DE NOVO 2 (IDN2)/RNA-dependent DNA methylation 12 (RDM12) binds to the hybrid siRNA-nascent scaffold transcripts and aids recruitment or retention of *de novo* DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). DRM2 catalyzes cytosine methylation in DNA sequences complementary to siRNAs. For color details, please see color plate section.

INVOLVED IN DE NOVO 2 (IDN2)/RNA-dependent DNA methylation 12 (RDM12), facilitate the assembly of RdDM complex at target loci (Ausin et al., 2009; Zheng et al., 2009). This complex then catalyzes *de novo* methylation. The RDM1, a single-stranded methyl-DNA binding protein is associated with RNA polymerase II (Pol II), Pol V, AGO4, and DRM2, and thus appears to be a critical component of the RdDM effector complex (Gao et al., 2010). The chromatin remodeling proteins, namely DDM1 (DECREASED DNA METHYLATION1; Jeddeloh et al., 1999) and DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1; Wierzbicki et al., 2008), facilitate Pol V transcription and RdDM in heterochromatin, transposon, and probably other target loci (Figure 8.2).

Dynamic regulation of DNA methylation in response to developmental and environmental cues, and removal of non-target methylation require an active DNA demethylation machinery. 5-methylcytosine DNA glycosylases, namely Repressor of Silencing 1 (ROS1), DEMETER (DME), DML2, and DML3, catalyze DNA demethylation by a base-excision-repair mechanism in *Arabidopsis* (Choi et al., 2002; Gong et al., 2002; Zhu, 2009). The 5-methylcytosine removal by ROS1 leads to a single-nucleotide gap flanked by 3'- and 5'-phosphate termini in the DNA backbone. The DNA phosphatase ZDP removes the blocking 3' phosphate and thus allows subsequent repair by DNA polymerization and ligation steps (Martínez-Macías et al., 2012). ROS1 is involved in demethylation in most tissues, while DME catalyzes demethylation primarily in the central cell of the female gametophyte and developing seeds (Choi et al., 2002; Gong et al., 2002; Ortega-Galisteo et al., 2008). In *Arabidopsis*, ROS3, an RNA-recognition motif (RRM)-containing protein, appears to bind to small-activating-RNAs (saRNAs) and direct ROS1 for sequence-specific demethylation (Zheng et al., 2008; Zhu, 2009).

DNA methylation in repetitive regions and transposons leads to silencing. Promoter methylation results in transcriptional gene silencing (TGS), while CG methylation in the gene body regions may prevent cryptic transcription, recombination, or transposon insertion within the genes in plants (Zilberman et al., 2007; Zhu, 2008). DNA methylation is involved in transposon silencing (Bucher et al., 2012; Ibarra et al., 2012; Lisch, 2012), nucleolar dominance (Tucker et al., 2010), paramutation (Hollick, 2012), imprinting (Bauer and Fischer, 2011), germline reprogramming (Creasey and Martienssen, 2010), polyploidy (Ng et al., 2012), development (Feng et al., 2010; Furner and Matzke, 2011; Mayfield et al., 2011; Zhang and Zhu, 2011), and stress responses (Chinnusamy et al., 2008, 2009a, 2009b; Gutzat and Scheid, 2012).

#### *Histone modification-mediated DNA methylation*

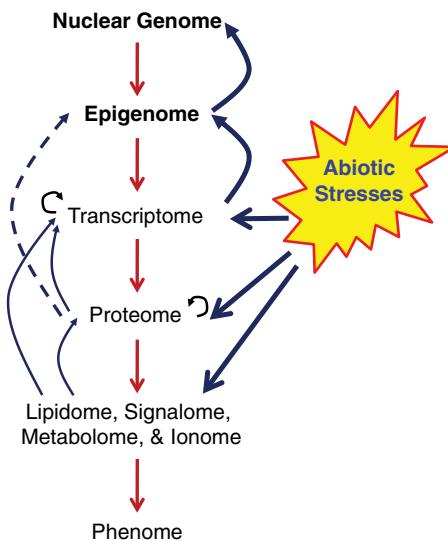
Histone modifications and DNA methylation lead to specific epigenetic codes that regulate gene expression. Often a specific histone modification can lead to a change in DNA methylation and DNA methylation also leads to establishment

of a specific histone modification pattern. Thus, histone code and DNA methylation can regulate each other. Histone modifying enzymes with or without methylated DNA binding domain mediate reestablishment of methylation or demethylation. The DNA 5-methylcytosine binding SUVAR (SUVH4/KRYPTONITE [KYP], SUVH5, SUVH6) family proteins with the SRA (SET and RING Associated) domain bind to hemimethylated DNA and catalyze H3H9 dimethylation (H3K9me2). The DNA methyltransferase CMT3 binds to methylated H3 (Lindroth et al., 2004) and catalyzes CHG DNA methylation. The KYP/SUVH4 and SUVH9 facilitate maintenance of non-CG methylation through DRM2 (Jackson et al., 2002; Malagnac et al., 2002), while VARIANT IN METHYLATION (VIM)/ORTHRUS family of SRA-RING domain proteins help maintain CG methylation. The Jumonji C (JmjC) domain containing JM14, a histone lysine demethylase, catalyzes demethylation of H3K4me3 and facilitates CHH methylation by the RdDM pathway in *Arabidopsis* (Searle et al., 2010). Morpheus' molecule (MOM1), a CHD3 chromatin remodeling ATPase, is required for TGS but is not required to maintain DNA methylation. MOM1 recognizes methylated DNA and induces repressive histone modifications at RdDM target loci (Numa et al., 2010; Wierzbicki, 2010).

DNA demethylation at specific loci requires permissive histone marks such as H3K18 and H3K23 acetylation. IDM1 (Increased DNA Methylation 1), a histone acetyltransferase with an MBD domain, a PHD finger domain, and an N-acetyltransferase domain, binds to methylated DNA at chromatin sites lacking histone H3K4 di- or trimethylation and catalyzes acetylation of H3. This leads to a permissible chromatin structure for binding of 5-methylcytosine DNA glycosylases and DNA demethylation (Qian et al., 2012). Conversely, deacetylation of H3K9 and H3K14 by the histone deacetylase 6 (HDA6) is required for maintenance of RdDM-mediated CHG methylation of promoters and rRNA silencing in nucleolar dominance (Earley et al., 2010) and regulates heterochromatin silencing possibly by recruiting MET1 (To et al., 2011b). The RdDM pathway is required for maintaining ROS1 demethylase expression, and thus has also an antisilencing function in *Arabidopsis* (Li et al., 2012).

### 8.3 Epigenetic regulation of abiotic stress responses

Abiotic stresses lead to profound expression changes of genes coding for various signaling proteins, effectors involved in stress acclimation and epigenetic modifications. The transcriptome and proteome changes induced by abiotic stresses can lead to transposon activation and genome instability. Stress-induced transposons may activate gene expression or serve as a source for siRNA generation and repress gene expression. Thus, transcriptome and proteome changes lead to changes in epigenetic marks at different loci and establishment



**Figure 8.3** Abiotic stress-induced epigenome and its expression under stress. Abiotic stresses induce changes in membrane lipid, protein, metabolite, and so forth that lead to altered activity of proteins. The altered protein activity changes transcriptome and transposon expression. This in turn results in altered expression levels of genes involved in epigenetic modification. The balance between activities of proteins involved in establishment and erasure of epigenetic code in a target loci on chromatin determines establishment of a specific epigenetic state. The epigenome, thus established, facilitates/represses stress responsive gene expression and stress tolerance.

of stress epigenome (Figure 8.3). Developmental and environmental cues induced changes in epigenetic marks give rise to several epigenomes from the single genome. Change in the epigenetic codes lead to reprogramming of gene expression. The following sections briefly summarize the abiotic stress-induced changes in histone modification, histone variants and DNA methylation patterns and their significance in acclimation responses and stress tolerance of plants.

### 8.3.1 Stress regulation of genes for histone modification and RdDM

Abiotic stresses regulate the expression of several genes involved in the establishment and erasure of epigenetic marks. Abiotic stresses such as salt, cold and drought stresses upregulated *OsAGO2* expression in rice (Kapoor et al., 2008). In rice, cold and salt stresses significantly upregulated the expression of *OsCMT2* in seedlings. In contrast, *OsCMT3* was significantly downregulated in rice seedlings under salt and dehydration stresses (Sharma et al., 2009). Heavy metal stress significantly downregulated DNA methyltransferases (*MET1-2* and *CMT3-2*) and SWI/SNF chromatin remodelers (*DDM1a* and *DDM1b*) but upregulated DNA methyltransferases (*CMT3-1*, *DRM2-1*, and *DRM2-2*) and DNA glycosylase (*DME*) in rice. These expression patterns were consistent

with active demethylation of CHG sites under heavy metal stress and maintenance of demethylated state of CHG in their progenies (Ou et al., 2012). In soybean, salt, cold and dehydration stresses altered the expression of *DCL2a*, *DCL2b*, *DCL3a*, *DRM1* and *MET1* genes (Curtin et al., 2012). These results show that abiotic stresses induce changes in expression of genes coding for enzymes and other proteins as well as non-coding RNAs involved in histone modification, chromatin remodeling, DNA methylation and DNA demethylation. Microarray analysis of cold stress treated *Arabidopsis* seedlings showed upregulation of *NRPD1*, *HAC5* (histone acetyltransferase 5, a GCN5-related N-acetyltransferase family member), and histone deacetylases (Lee et al., 2005). In the moss *Physcomitrella patens*, abscisic acid (ABA)-induced accumulation of miR1026 can induce hypermethylation of the *PpbHLH* gene at CHG sites, probably through RdDM (Khraiwesh et al., 2010). These results suggest that stress-induced changes in genes coding for histone variants, and genes involved in histone modifications, RdDM, and DNA demethylation lead to changes in epigenetic marks. Stress-induced epigenome then affects the expression pattern of genes involved in stress tolerance (Figure 8.3).

Mutants of genes involved in establishment and erasure of epigenetic marks show impaired stress responses. This further supports the role of stress-induced changes in epigenome in stress tolerance. Some of the examples of mutations or genetic alteration in genes coding for proteins involved in histone modifications, chromatin remodeling and DNA methylation and their association with abiotic stress tolerance are listed in Table 8.2.

### 8.3.2 Gene regulation mediated by stress-induced histone modifications

Studies conducted during the past decade have demonstrated the important role of histone modification in regulation of abiotic stress responsive gene expression. In the previous section, the stress responses of some of the mutants impaired in histone modification were summarized (Table 8.2). Genome-wide analysis of histone methylation by using chromatin immunoprecipitation and deep sequencing (ChIP-Seq) revealed that about 90% of annotated *Arabidopsis* genes carry H3K4me marks, and these are dynamically regulated in several stress responsive genes under drought stress in *Arabidopsis* (van Dijk et al., 2010). Stress-induced changes in histone modifications are covered in recent reviews (Kim et al., 2010; Luo et al., 2012a). Hence, here we discuss some examples of the histone modification that is critical for stress response of plants.

#### *ABA signaling*

In *Arabidopsis*, abiotic stress signaling is mediated by the plant stress hormone ABA-dependent pathway and ABA-independent pathways. Histone modifications play important role in ABA-regulated expression of genes during

**Table 8.2** Abiotic stress response of mutants/transgenics of genes involved in establishment and erasure of epigenetic marks.

Gene	Function	Remarks
<b>Histone modification</b>		
<i>SDG102 (SET DOMAIN GROUP 102)</i>	Histone methyltransferase with a SET domain	Maize RNAi lines showed hypersensitivity to UV-B stress (Casati et al., 2008)
<i>SDG8 (SET DOMAIN GROUP 8)</i>	Histone methyltransferase with a SET domain	<i>Arabidopsis SDG8</i> regulates a subset of genes in jasmonic acid (JA) and/or ethylene signaling involved in biotic stress response. <i>sdg8-1</i> mutants showed impairment in H3L36 methylation and reduced resistance to necrotrophic fungal pathogens (Berr et al., 2010)
<i>AtHDA6</i> (Histone Deacetylase 6)	Histone deacetylation	<i>AtHDA6</i> is upregulated by cold stress. <i>hda6</i> ( <i>axe1-2</i> ) mutants of <i>Arabidopsis</i> are impaired in acquired freezing tolerance (To et al., 2011a)
<i>HD2C</i> (Histone Deacetylase 2C)	Histone deacetylation	<i>axe1-5</i> mutant and <i>HDA6</i> RNAi lines showed hypersensitivity to ABA and salt stress (Chen et al., 2010) Interacts with <i>HDA6</i> ; <i>hd2c</i> mutants showed enhanced expression of PP2CA genes <i>ABI1</i> and <i>ABI2</i> , the negative regulators of ABA signaling, and were hypersensitive to ABA and NaCl during germination (Luo et al., 2012b)
<b>Chromatin remodeling</b>		
<i>PICKLE (PKL)</i>	Putative chromatin modifier	<i>Arabidopsis pk1</i> mutants are ABA hypersensitive and showed enhanced levels of ABA-induced expression of <i>ABI3</i> and <i>ABI5</i> genes during germination (Perruc et al., 2007)
<i>CHR12</i> (SNF2/Brahma-type chromatin-remodeling protein)	Chromatin remodeling	<i>Arabidopsis</i> plants overexpressing <i>CHR12</i> showed enhanced growth arrest, while knock-out line exhibited less growth inhibition under stress as compared with WT plants (Mlynarova et al., 2007)
<i>SWI3B (SWITCH SUBUNIT 3)</i>	<i>Arabidopsis</i> homolog of the yeast <i>SWI3</i> subunit of SWI/SNF chromatin-remodeling complex	<i>SWI3B</i> interact with HAB1 PP2C; <i>swi3b</i> mutant of <i>Arabidopsis</i> showed a reduced sensitivity to ABA during germination and reduced expression of ABA and abiotic stress-responsive genes <i>RAB18</i> and <i>RD29B</i> (Saez et al., 2008)
<i>CHC101</i> (an SWIB domain-containing protein)	Chromatin remodeling	Maize RNAi lines showed hypersensitivity to UV-B stress (Casati et al., 2008)

(Continued)

Table 8.2 (Cont'd).

Gene	Function	Remarks
<i>MSII (MULTICOPY SUPPRESSOR OF IRA1)</i>	A subunit of Polycomb protein complex 2, and chromatin assembly factor 1	A subset of ABA-dependent genes involved in drought and salt stress responses are upregulated in MSII co-suppression lines of <i>Arabidopsis</i> . These plants accumulated more proline and were more tolerant to drought stress (Alexandre et al., 2009)
<i>MBD101</i> (methyl-CpG binding protein)	Chromatin modification	Maize RNAi lines showed hypersensitivity to UV-B stress (Casati et al., 2008)
<i>ARP6</i>	A subunit of the SWR1 chromatin remodeling complex	ARP6 mediates the replacement of H2A with H2AZ histone. <i>Arabidopsis arp6-10</i> mutant showed early flowering and enhanced <i>HSP70</i> expression (Kumar and Wigge, 2010)
<i>DDM1 (DECREASED DNA METHYLATION 1)</i>	SWI2/SNF2 family of adenosine triphosphate-dependent chromatin remodeling factor	<i>Arabidopsis ddm1</i> mutants were hypersensitive to methyl methane sulfonate (MMS) and NaCl stresses (Yao et al., 2012)
<b>DNA methylation</b>		
<i>MET1</i> (DNA methyltransferase)	DNA cytosine methylation (CG and CHG)	<i>Arabidopsis met1</i> mutant is hypersensitive to salt stress (Baek et al., 2011)
<i>DCL</i> (Dicer-like) 2, 3, and 4	RNase III involved in biogenesis siRNAs, which are required for RdDM	<i>dcl2</i> and <i>dcl3</i> mutants of <i>Arabidopsis</i> are impaired in transgenerational stress memory and stress tolerance (Boyko et al., 2010)

development and stress responses (Chinnusamy et al., 2008). The ABA-insensitive (ABI) protein phosphatase 2Cs (ABI1 and ABI2) negatively regulate stress responsive gene expression and stomatal closure by inhibiting the activity of SnRK2 protein phosphatases (Klingler et al., 2010). Loss of function of *PP2Cs* results in constitutive activation of ABA signaling and enhanced tolerance to drought (Rubio et al., 2009). Hence, proper regulation of expression levels of PP2Cs is important for stress responses of plants. ABA and salt stress enhanced histone modifications associated with activation of transcription (H3K9 and H3K14 acetylation and H3K4 trimethylation), and decreased the repressive H3K9 dimethylation in *ABI1* and *ABI2* genes in *Arabidopsis*. The *hda6* mutants and *HDA6*-RNAi lines exhibited reduced expression of these genes under salt stress and ABA, and exhibited hypersensitivity to ABA and salt stress (Chen et al., 2010). Mutations in the *Arabidopsis HDA6 (RPD3, REDUCED POTASSIUM DEFICIENCY 3-type HDAC)* resulted in reduced tolerance to multiple abiotic stresses and impaired ABA signaling (Table 8.2). Another histone deacetylase, HDA19, interacts with AtSin3 (a homolog of human global corepressor of transcription). The AtSin3 interacts with AtERF7 (ETHYLIC LINE RESPONSE FACTOR 7). These interactions enhance the transcriptional repression activity of AtERF7. Silencing of *AtERF7* and *AtSin3* expression resulted in ABA hypersensitivity during germination, while overexpression of *AtERF7* reduced ABA sensitivity in stomatal closure, and enhanced drought susceptibility (Song et al., 2005). Further, *hda19-1* mutant of *Arabidopsis* exhibited reduced expression of ABA-responsive genes (*ABI1*, *ABI2*, *KAT1*, *KAT2*, and *RD29B*) and hypersensitivity to ABA and salt stress (Chen and Wu, 2010).

The type 2C protein phosphatase HYPERSENSITIVE TO ABA1 (HAB1), a negative regulator of ABA signaling, interacts with SWI3B, an *Arabidopsis* homolog of the yeast SWI3 subunit of SWI/SNF chromatin-remodeling complexes. Chromatin immunoprecipitation (ChIP) showed that HAB1 is removed from the vicinity of *RD29B* and *RAB18* promoters by ABA treatment. Further, *swi3b* mutant of *Arabidopsis* showed a reduced sensitivity to ABA-mediated inhibition of germination and reduced expression of the ABA and abiotic stress-responsive genes such as *RAB18* and *RD29B* (Saez et al., 2008). These results clearly show that HDA-mediated alterations in histone modification are critical for ABA signaling and stress tolerance.

#### *CBF pathway of cold acclimation*

The C-Repeat Binding Factor (CBF)/Dehydration Responsive Element Binding Factor 1 (DREB1) pathway regulates genes important for cold acclimation in diverse plant species (Chinnusamy et al., 2010). This pathway regulates effector genes involved in stress protection such as LEA proteins, osmoprotectant accumulation, ROS detoxification, and growth regulation. Chromatin modification appears to be important for regulation of cold responsive genes by the

CBF/DREB1 pathway. GCN5 (general control non-derepressible) is the catalytic subunit of the Spt-Ada-Gcn5 acetyltransferase (SAGA) involved in histone acetylation. This histone acetyltransferase interacts with transcriptional adaptor (ADA), and regulates H2B and H3 acetylation. *Arabidopsis* ADA2B interacts with GCN5 and CBF1/DREB1B *in vitro*, suggesting the role of ADA/SAGA-like complexes in facilitating the access of CBF1 to its target *COR* genes promoters, and thus in enhancing transcription. *Arabidopsis ada2* mutants and *gcn5* mutants showed delayed and reduced expression of some *COR* genes. The higher levels of basal freezing tolerance of *ada2b-1* mutant suggest that ADA2B is important in repressing basal freezing tolerance pathway genes during normal growth (Vlachonasios et al., 2003).

Cold stress decreases H3K27me3 in the promoters of CBF pathway target cold responsive (*COR*) genes such as the *COR15A* (Cold-regulated 15A) and *GOLS* (Galactinol Synthase) genes. The *COR15A* encodes a plastid-targeted polypeptide with cryoprotective properties (Artus et al., 1996; Nakayama et al., 2007). Constitutive overexpression of *COR15A* enhanced freezing tolerance of *Arabidopsis* (Artus et al., 1996). *GOLS* catalyzes the first step in biosynthesis of Raffinose family oligosaccharides (RFO), which are important for abiotic stress tolerance (Taji et al., 2002). The decrease in H3K27me3 in the promoters is associated with the induction *COR15A* and *GOLS3* genes under cold stress. Although transcription of these genes was returned to the basal level within a day when the plants were returned to normal temperature but H3K27me3 levels did not increase (Kwon et al., 2009). Drought stress also induces the *AtGOLS2* gene by enhancing H3K9 acetylation and H3K4me3. Upon recovery from drought stress, H3K9ac pattern was reestablished quickly, while H3K4me3 pattern was not reinstated to the original levels (Kim et al., 2012). These results suggest the possibility of the histone hypomethylation status serving as a short-term stress memory.

#### *DREB2A pathway*

Drought and salinity stress upregulate the expression of Dehydration Responsive Element Binding Factor (DREB2A) transcription factor gene in *Arabidopsis*. Further, these stresses activate and stabilize DREB2A protein. Transgenic *Arabidopsis* plants overexpressing a constitutively active form of DREB2A (DREB2A-CA) protein resulted in the expression of several stress responsive genes and enhanced drought and heat stress tolerance (Sakuma et al., 2006). DREB2A transcription pathway is important for regulating several drought, salt and heat stress responsive genes (Qin et al., 2011). Salinity stress triggered rapid and transient upregulation of histone H3 Ser-10 phosphorylation (H3S10P), which was associated with salt stress-induced upregulation of the *DREB2A* gene in *Arabidopsis* T87 cells (Sokol et al., 2007). In addition, salt stress enhanced H3K4 trimethylation and H3 acetylation, but decreased H3K9

dimethylation in *DREB2A* chromatin. These histone modifications resulted in transcriptional activation of *DREB2A* under salt stress. The *hda6* mutant of *Arabidopsis* impaired in these histone modifications showed a very low stress-induced expression of *DREB2A* and exhibited reduced salt tolerance (Chen et al., 2010).

#### *FLC* flowering pathway

Flowering under appropriate environmental conditions is important for the successful reproduction of plants. Hence, plants have evolved elaborate photoperiodic and vernalization pathways to regulate flowering time (Dennis and Peacock, 2007; Yaish et al., 2011). The floral integrator FLOWERING LOCUS C (FLC) encodes a MADS-box transcription factor. FLC represses the initiation of flowering by repressing *FLOWERING LOCUST (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* genes that promote flowering. A connection between cold stress-induced gene expression and flowering time was revealed by a mutant screen aimed at identification of regulators of *COR* genes in *Arabidopsis*. One of the mutant thus identified, *altered cold-responsive gene expression1 (acg1)*, is a null allele of the *FVE* gene. *FVE* encodes a component of a histone deacetylase (HDAC) complex involved in transcriptional repression. Under cold stress, ACG1 negatively regulates the CBF/DREB pathway and *FLC* expression (Kim et al., 2004). ChIP analysis revealed that *FVE* directly binds to *FLC* and *COR15A* chromatin (Jeon and Kim, 2011). In contrast to *FVE*, HDA6 negatively regulate *FLC* expression as evident from significant upregulation of *FLC* in *hda6 (axe1-5)* mutant and HDA6 RNAi lines (Wu et al., 2008). *HDA6* is upregulated by cold stress in *Arabidopsis* (To et al., 2011a), which in turn may lead to repression of *FLC* expression.

The SWR1 ATP-dependent chromatin remodeling complex deposits H2A.Z (a histone H2A variant) in the nucleosomes flanking the transcription start site of actively transcribed genes. Reduction in H2A.Z occupancy at the *FLC* chromatin reduced the expression of *FLC* and caused early flowering (Deal et al., 2007). The H2A.Z perceives ambient temperature changes, and H2A.Z nucleosome occupancy decreases with increasing temperature. Thus, high temperature leads to early flowering in *Arabidopsis* (Kumar and Wigge, 2010).

In addition to temperature stresses, salinity stress also alters *FLC* expression. The floral initiator SHK1 KINASE BINDING PROTEIN1 (SKB1) associates with chromatin and increases the histone H4 Arg3 symmetric dimethylation (H4R3sme2) levels. SKB1 mediated H4R3sme2 resulted in suppression of expression of *FLC* and stress-responsive genes such as *RD29A*, *RD29B*, *HAB1*, *MEK1* (MAP Kinase Kinase 1), and *MEKK3* (MAP Kinase Kinase Kinase 3). The *skb1* mutant showed hypersensitivity to ABA and salt stresses, suggesting that SKB1 is a negative regulator of ABA and salt stress signaling. Salt stress

reduces H4R3sme2, and thus releases SKB1 from *FLC* chromatin. This leads to enhanced expression of *FLC* and delayed flowering under salt stress (Zhang et al., 2011).

### 8.3.3 Gene regulation mediated by stress-induced changes in DNA methylation

Abiotic stresses may also regulate stress responsive gene expression through induction of hypo and hyper-methylation of DNA (Chinnusamy and Zhu, 2009a). DNA methylation-sensitive amplified polymorphisms (MSAPs) analysis showed drought stress-induced DNA methylation changes accounted for 12.1% of the total site-specific methylation differences in the rice genome. Drought-induced DNA methylation showed a significant level of developmental and tissue specificity and association with stress tolerance (Wang et al., 2011). MSAPs analysis showed significant alteration in DNA methylation patterns in rice under cold (Pan et al., 2011), salt (Bilichak et al., 2012; Karan et al., 2012), N deficiency (Kou et al., 2011), and heavy metal (Ou et al., 2012) stresses. Similar examples are available in several other plants. Here, we focus on stress-induced changes in DNA methylation and its association with expression of genes critical for stress responses.

The *ABSCISIC ACID STRESS RIPENING1* (*ASR1*) encodes a plant-specific Zn-dependent DNA binding protein. ABA, salt and water-deficit stresses upregulate the expression of *ASR1*. It was originally cloned from tomato and latter found that several monocot and dicot plants encode homologs of *ASR1* (Iusem et al., 1993, Cakir et al., 2003). Transgenic tobacco and *Arabidopsis* plants overexpressing tomato *ASR1* (Kalifa et al., 2004) and the lilly ortholog *LLA23* (Yang et al., 2005), respectively, exhibited enhanced salinity and drought stress tolerance. Overexpression of *ZmASR1* improved the kernel yield of transgenic maize by 7 and 17% under well-watered and water-limited conditions, respectively, in the field (Virlouvet et al., 2011). Bisulphite sequencing of *ASR1* in tomato leaf revealed significant levels of all the three types of DNA methylation in tomato leaves under well-watered conditions. The CHH methylation was preferentially in the intron. Drought stress induced demethylation of CHH sites with concomitant decrease in the repressive H3K27me3 enhanced the expression of *ASR1* (González et al., 2011). In moss *Physcomitrella patens*, ABA induced the accumulation of miR1026, hypermethylation of the *PpbHLH* (miR1026 target gene) gene at CG sites and thus decreased *PpbHLH* expression (Khraiwesh et al., 2010). These results suggest that ABA regulates DNA methylation, which in turn regulates stress-responsive gene expression.

The salt overly sensitive (SOS) pathway regulates Na<sup>+</sup> exclusion from the cytosol and is critical for salt tolerance of plants. The SOS3 calcium sensor protein activates SOS2 Ser/Thr protein kinase, which then phosphorylates SOS1

$\text{Na}^+/\text{H}^+$  antiporter that excludes  $\text{Na}^+$  from the cytosol (Chinnusamy et al., 2005). The high-affinity  $\text{K}^+$  transporter (HKT1) also mediates  $\text{Na}^+$  influx under salinity stress (Rus et al., 2001). A suppressor screening for *sos3* mutation led to the isolation of *hkt1-1* mutant. The promoter of *HKT1* has a putative siRNA target region, which is heavily methylated at CHG and CHH sites. The *rdr2* and *met1* mutants impaired in DNA methylation showed an enhanced expression of *AtHKT1*. The *met1* mutant also showed hypersensitivity to salt stress (Baek et al., 2011).

The stomatal density in the leaf controls the rate of transpiration and thus, stress-induced reduction in stomatal density is an important tolerance mechanism to avoid cellular dehydration. *Arabidopsis Ler* plants grown in low relative humidity showed hypermethylation and repression of two genes in the stomatal development pathway, *SPEECHLESS* and *FAMA*. This resulted in reduced stomatal density and thus better adaptability to atmospheric water deficit stress caused by low RH. The DNA methylation in these genes appears to be established by DRM2 through RdDM pathway and maintained by MET1 under low RH (Tricker et al., 2012).

#### 8.3.4 Stress-induced transposon regulation

Transposable elements contribute to a substantial proportion of plant genomes (14% in *Arabidopsis* and rice; 60% in maize). Epigenetic mechanisms maintain transposons in a silent state (Lisch, 2012). Abiotic stresses activate transposons. Cold stress activated *Ac/Ds* transposon in maize (Steward et al., 2000), *Tam3* in *Antirrhinum majus* (Hashida et al., 2006), and mPing in rice (Naito et al., 2009). Plant hormones ethylene, methyl jasmonate, salicylic acid, and 2, 4-dichlorophenoxyacetic acid and salt stress induced *TLC1*, a member of the long terminal repeat (LTR) retrotransposon family in *Lycopersicon chilense* (Tapia et al., 2005). Interestingly, the expression of *Arabidopsis AtCopeg1* (*Copia*-like retrotransposons) was induced by cytokinin, but was repressed by ABA (Duan et al., 2008). Heat stress activated a copia-type retrotransposon *ONSEN* (Japanese “hot spring”) in *Arabidopsis*. Further, *ONSEN* insertions conferred heat responsiveness to nearby genes. siRNA biogenesis mutants showed high levels of *ONSEN* activation under heat stress, and a high frequency of new *ONSEN* insertions was found in the progeny of stressed plants deficient in siRNAs, but not in WT plants. These results suggest that stress-induced transposons are reset to their basal levels during recovery and the RdDM pathway plays a crucial role in the resetting (Ito et al., 2011; Matsunaga et al., 2012). Transposon activation affects genome stability and gene expression. Transposon insertion in gene coding region leads to gene inactivation, while it can give promoter activity that leads to sense- or antisense-strand transcription depending upon the site of insertion, which in turn can activate or silence gene expression.

#### 8.4 Transgenerational inheritance and adaptive value of epigenetic modifications

Environment-induced epigenetic changes can be mitotically inherited, as evident from transmission of vernalization induced epigenetic memory at the *FLC* locus (Sheldon et al., 2008). However, these epigenetic modifications are reset during meiosis. Recent studies showed that abiotic stresses such as UV-B, UV-C, extreme temperatures, oxidative stress, salt stress, and osmotic stress induced increase in homologous recombinations (SHR) in the *Arabidopsis* plants (Molinier et al., 2006; Pecinka et al., 2009; Boyko et al., 2010). However, only low and apparent stochastic increases in SHR were inherited in the progeny of stress treated plants (Pecinka et al., 2009). The *dcl2* and *dcl3* mutants were impaired in stress-induced HRF and DNA methylation, suggesting that stress-induced changes in DNA methylation and its transgenerational inheritance depend on RdDM (Boyko et al., 2010). High salt stress, nutrient deficiency stress, and the plant hormones salicylic acid and jasmonic acid induced changes in DNA methylation (hypo as well as hyper) in dandelions (*Taraxicum officinale*). The majority (>70%) of the stress-induced methylation patterns were inherited in apomictic seed-derived progenies of the stressed plants (Verhoeven et al., 2010). Besides DNA methylation changes, stress-induced histone acetylation also may be transgenerationally inherited. In *Arabidopsis*, UV-B, heat and freezing stresses released a transcriptionally silenced GUS transgene through histone H3 acetylation, and this effect was inherited as a dominant trait through two successive generations (Lang-Mladek et al., 2010).

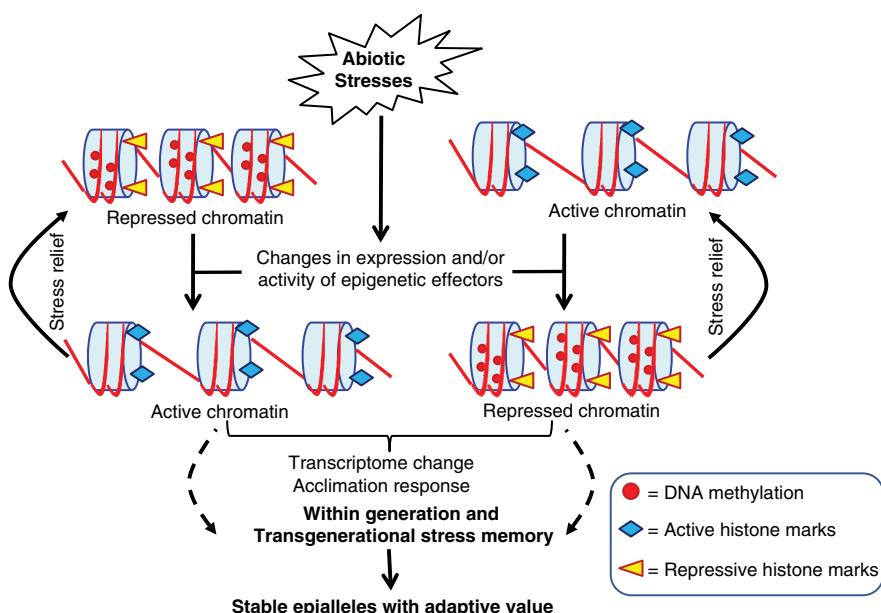
The adaptive value of stress-induced epialleles was examined in the progenies of stress treated plants. Exposure of *Arabidopsis* plants to NaCl stress resulted in change in DNA methylation at several loci, and these patterns were inherited to the progenies. Hypermethylation, enrichment of H3K9me2 and depletion of H3K9ac histones correlated with repression of gene expression in the progeny of salt-stressed plants (Bilichak et al., 2012). Progeny of salt stressed *Arabidopsis* plants displayed hypermethylation in transposons and genes involved in stress responses. These progenies also exhibited a higher tolerance to salt stress (Boyko et al., 2010). Under salt (NaCl) stress, chlorine ions induce genotoxic stress. This leads to transcriptional activation of *AtRad51* and downregulation of *AtKu70*, and thus higher somatic recombination rate. This trait was inherited to the progenies. These progenies were more tolerant to salt and genotoxic stresses (Boyko et al., 2011).

Imposition of N deficiency to rice plants in a hydroponic culture changed DNA methylation in leaves, and at least 50% of these methylation sites were inherited to the progenies. The S2 progenies that inherited modified methylation patterns also exhibited enhanced tolerance to N deficiency-stress (Kou et al., 2011). MSAP analysis of heavy metal induced DNA methylation pattern in rice revealed that heavy metal stress induced demethylation in some CHG

loci, and this demethylated state is inherited via both maternal and paternal germline to the progenies of stressed plants. These progenies also showed enhanced tolerance to the respective heavy metal stress (Ou et al., 2012).

### 8.5 Conclusion

From the above discussion, it can be concluded that environmental stress-induced epigenetic changes are important for reprogramming the transcriptome for stress responses. Abiotic stresses and secondary stress signals such as ABA, jasmonic acid, salicylic acid, and reactive oxygen species modulate the expression and/or activity epigenetic effectors (histone variants, histone modifiers, chromatin remodelers, RdDM components, DNA methyltransferase, and DNA demethylases). Thus, stress induces activation of repressed chromatin at some loci, while it inactivates active chromatin in some other loci. These epigenetic modifications lead to transcriptome changes and stress responses. Most of these epigenetic modifications are reset once the plants return to non-stress conditions. Some of the epigenetic modifications may be inherited mitotically and even meiotically to provide within generation and transgenerational stress memory and adaptive advantages to the progenies (Figure 8.4).



**Figure 8.4** Epigenetic regulation of stress tolerance. Abiotic stresses alter epigenetic state, which determines stress responsive gene expression. Transient chromatin modifications mediate acclimation response. Heritable epigenetic modifications provide within generation and transgenerational stress memory. For color details, please see color plate section.

Stress-induced epigenetic changes appear to play important roles in stress tolerance and development under stress. Stress memory may help the plant to adapt to stress in the ensuing season. Reduction in growth and development is one of the important strategies of plants to survive and reproduce under abiotic stress conditions. Depending upon the stress-induced epiallele, the growth and development of progenies of stressed plants might be restrained even under non-stress conditions. However, even if the crop is not exposed to stress in the ensuing season, it may yield less due to the stress memory. Often in the developing world, farmers use seeds harvested during the previous year to raise their new crop. Therefore, transgenerational stress memory might be advantageous or disadvantageous depending upon the environment.

Most of our understanding of stress-induced epigenetic modifications, inheritance, and their adaptive values has been generated from laboratory studies with model plants. The rate of stress development and the time available for acclimation of plants differ significantly between lab and field conditions. Under lab conditions, plants are often exposed to sudden and acute levels of a single stress, while in field conditions, combinations of abiotic and biotic stresses occur simultaneously. Abiotic stresses often change the levels of plant hormones such as ABA, ethylene, salicylic acid, and JA that act antagonistically or synergistically under different stresses. Hence, emphasis needs to be given to unravel the epigenetic mechanisms involved in stress combinations that occur in field conditions in crop plants. Abiotic stress-induced histone and DNA modifications have been observed in several cases, but their role in regulation of genes critical for stress tolerance, inheritance, and transgenerational adaptive value needs further study in crop plants. This will help lay the foundation for rational strategies for genetic improvement of economically important plants by establishing or erasing epialleles.

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## 9 Genomics of plant abiotic stress tolerance

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### 9.1 Genomics in plant research—an introduction

As much as biology has benefited from new instrumentation during the last 100 years, the current and ongoing revolution in biological thought is unprecedented. Progress has been brought about by a package of platforms known as next-generation sequencing (NGS) tools applied to genomes and transcriptomes (Egan et al., 2012; Niedringhaus et al., 2011). The advantages of NGS are often referred to as a revolution; in fact this revolution has been a long time coming. Beginning in the late 1970s there were several distinct breakthroughs in DNA sequencing. This was followed by methods to precisely amplify and manipulate desired gene or transcript fragments. The 1990s saw intense activities in tool development and the sequencing of ever more complex genomes. By the year 2000, plant scientists could begin to analyze the genome of *Arabidopsis thaliana*, which became the quintessential plant model species (*Arabidopsis* Genome Initiative, 2000). This significant advance proved to be a launching pad aimed at the cataloging, mutating, and characterizing of genes present in this genome, an effort that, in the end, took much longer than the 10 years originally (and optimistically) allocated to it. In fact, characterizing functions of genes and their encoded proteins is not even half finished in 2012 even for

*Arabidopsis*. One reason for our inability to put an unequivocal label onto every sequence is what has gradually been realized as “functional redundancy” and, to use a term from informatics, *network complexity*. Based on mutant screening and analysis and, more recently, advances in protein analysis and protein:protein interaction studies, we realize that function can be tissue- and/or cell-dependent, stimulus and condition-dependent, and time- and stage-dependent. In short, a protein may perform different functions depending on its context, that is, the environment defined by other proteins, metabolites, and signal molecules. A protein may have profoundly different functions depending on its presence or absence in a spatial or temporal context (Bossi and Lehner, 2009; Landry, 2011; Siefers et al., 2009; Stengel et al., 2010). Much of this recognition is owed to yet other advances in instrumentation and analytical capacity that gave rise to the disciplines of proteomics and metabolomics (Baker, 2012; Tohge and Fernie, 2010; Wienkoop et al., 2010).

The *Arabidopsis* genome sequencing was followed shortly by the sequences of the japonica and indica rice (*O. sativa*) genomes (Goff et al., 2002; Yu et al., 2002). Barely a decade later, as DNA sequencing has become more easily manageable, there are now dozens of plant genome sequences available (Table 9.1). Obtaining genome structures had previously been a complex logistical enterprise requiring partitioning of the genome into many small segments (BACs), assembling BAC-end scaffolds, filling in sequences of individual BACs after generating BAC-specific sublibraries, and finally carrying out extensive error control and assembly. That has been changed by the NGS platforms, which randomly generate extraordinarily large DNA sequence datasets, and emerging computational tools that have enabled management of such amounts of data (Miller et al., 2010; Sayers et al., 2012; Treangen and Salzberg, 2012). While currently existing platforms undergo incremental but significant improvements, fundamentally new ways to sequence DNA and RNA are also emerging (Niedringhaus et al., 2011; Thompson and Milos, 2011).

We will outline here a paradigm shift in plant biology originating from the relative ease of generating sequences. In addition, we point to tools that have enabled global transcript analyses (RNA-seq), replacing arduous microarray hybridization experiments and laborious quantitative RT-PCR platform designs. Soon, we can envision, one may replace individual transcript assays by measuring how the entire transcriptome responds globally to a manipulation or mutation. We advance a position here that the knowledge of genome content and structure and of conditional transcript expression characteristics will explain a plant’s growth and adaptation to the environment.

We highlight this point by comparing the genome and transcriptome of the abiotic stress-sensitive *Arabidopsis thaliana* with its close relatives, *Thellungiella parvula* and *Thellungiella salsuginea* (Dassanayake et al., 2011a; Wu et al., 2012). In contrast to *Arabidopsis*, the *Thellungiellas* are extremophiles that grow under abiotic conditions lethal for *Arabidopsis* and most other

**Table 9.1** Sequenced and *de novo* assembled plant genomes.

Organism	Sequencing Tools	Family	Sequenced Size (Mb)	Importance	Year Sequenced and Reference
<i>Chlamydomonas reinhardtii</i>	Sanger, plasmid-fosmid	Chlorophyceae	105*	Single cell green alga	2007 Science, 318(5848):245
<i>Volvox carteri</i>	Sanger, BAC end	Chlorophyceae	125*	Multicellular green alga	2010 Science, 329(5988):223
<i>Physcomitrella patens</i> (moss)	Sanger	Funariaceae	454*	Early land plant evolution	2008 Science, 319(5859):64
<i>Selaginella moellendorffii</i>	Sanger	Selaginellaceae (Tracheophyta)	208*	Early vascular plant evolution	2011 Science, 332(6032):960
<i>Aquilegia coerulea</i>	Sanger, 454	Ranunculaceae	302	Early eudicot evolution	Early release on <a href="http://www.phytozome.net">www.phytozome.net</a>
<i>Arabidopsis thaliana</i>	Sanger	Brassicaceae	120*	First sequenced model	<a href="http://www.arabidopsis.org/index.jsp">www.arabidopsis.org/index.jsp</a>
<i>Arabidopsis lyrata</i>	Sanger	Brassicaceae	183*	Genome size evolution	2011 Nature Genetics, 43:476
<i>Capsella rubella</i>	454, Illumina, Sanger	Brassicaceae	135	Genus closest to Arabidopsis	Early release on <a href="http://www.phytozome.net">http://www.phytozome.net</a>
** <i>Brassica rapa</i>					
<i>Thellungiella parvula</i> #	Illumina	Brassicaceae	284	Improvement of vegetable crops	2011 Nature Genetics, 43:1035
* <i>Thellungiella salsuginea</i> #	Illumina	Brassicaceae	135	First extremophile plant genome	2011 Nature Genetics, 43:913
** <i>Carica papaya</i>					
** <i>Citrus sinensis</i> (sweet orange)	Sanger, BAC ends	Caricaceae	234	Extreme salt and Freezing tolerance	2012 Proc Nat Acad Sci USA; doi:10.1073/pnas.1209954109
** <i>Citrus papaya</i>					
(Mandarin orange)	454	Rutaceae	271*	Improvement of fruit crops	2008 Nature 452:991
** <i>Citrus sinensis</i> (sweet orange)					
** <i>Citrus clementine</i> (Mandarin orange)					
<i>Eucalyptus camaldulensis</i>	Sanger, plasmid-BAC-fosmid	Myrtaceae	319	Improvement of fruit crops	Early release on <a href="http://www.citrusgenomedb.org">www.citrusgenomedb.org</a>
** <i>Manihot esculenta</i> (Cassava)					
(Cassava)	Sanger, 454	Euphorbiaceae	296	Improvement of fruit crops	Early release on <a href="http://www.citrusgenomedb.org">www.citrusgenomedb.org</a>
<i>Eucalyptus camaldulensis</i>					
Tropical food and energy crop					
2011 BMC Proceedings 5 (Suppl 7)					
2012 expected; Trop. Plant Biol 5:88					

(Continued)

Table 9.1 (Cont'd).

Organism	Sequencing Tools	Family	Sequenced Size (Mb)	Importance	Year Sequenced and Reference
** <i>Ricinus communis</i> (castor bean)	Sanger, plasmid-fosmid	Euphorbiaceae	352*	Oil seed crop	2010 Nat Biotechnol, 28(9):951
* <i>Jatropha curcas</i>	454, Illumina, Sanger, BAC ends	Euphorbiaceae	285*	Tree oil crop	2011 DNA Research, 18:65
<i>Populus trichocarpa</i>	Sanger	Salicaceae	427*	First tree genome; wood development	2006 Science, 313(5793):1596
** <i>Linum usitatissimum</i> (flax)	Sanger, BAC	Linaceae	282*	Fiber and oil crop	2011 BMC Genomics, 12:217
* <i>Glycine max</i> (soybean)	Sanger	Fabaceae	973	Food and oil crop	2010 Nature, 463:178
** <i>Medicago truncatula</i>	—	Fabaceae	314*	Model legume crop	Early release on www.medicago-hapmap.org
** <i>Phaseolus vulgaris</i> (common bean)	—	Fabaceae	487	Vegetable legume crop	Early release on www.phytozome.net
** <i>Cajanus cajan</i> (pigeonpea)	Illumina	Fabaceae	408*	Legume food crop	2012 Nature Biotech, 30:83
** <i>Solanum tuberosum</i> (potato)	Sanger, BAC-fosmid end, 454, Illumina	Solanaceae	663*	Tuber crop	2011 Nature, 475:189
** <i>Solanum lycopersicum</i> (tomato)	—	Solanaceae	759*	Crop domestication	Early release on www.solgenomics.net
<i>Solanum pimpinellifolium</i> (wild tomato)	Illumina	Solanaceae	422*	Tomato progenitor	Early release on www.solgenomics.net
<i>Nicotiana benthamiana</i>	Illumina	Solanaceae	2951	Model-plant microbe interactions	Early release on www.solgenomics.net
** <i>Cucumis sativus</i> (Cucumber)	Sanger, Illumina	Cucurbitaceae	225*	Vegetable crop	2009 Nature Genetics, 41:1275
** <i>Cucumis melo</i> (melon)	Sanger, BAC	Cucurbitaceae	450	Model vasculature development	2010 BMC Genomics, 11:339
** <i>Malus x Domestica</i> (apple)	Sanger, 454	Rosaceae	871*	Improvement of fruit crops	2010 Nature Genetics, 42:833
* <i>Prunus persica</i> (peach)	Sanger	Rosaceae	212*	Improvement of fruit crops	Early release on www.rosaceae.org
* <i>Fragaria vesca</i> (strawberry)	454, Illumina, SOLiD	Rosaceae	202*	Improvement of fruit crops	2011 Nature Genetics, 43:109

** <i>Theobroma cacao</i> (cocoa)	454, Illumina, Sanger, BAC Illumina	Malvaceae Cannabaceae	291* 534*	Tropical fruit crop Fiber and oil crop, medicinal and intoxicating properties
** <i>Vitis vinifera</i> (grape)	Sanger, 454 Sanger BAC end	Vitaceae Phrymaceae	471* 322	Improvement of fruit crops Model for ecological genetics
<i>Mimulus guttatus</i> (monkey flower)				Early release on <a href="http://www.mimulusrevolution.org">www.mimulusrevolution.org</a>
** <i>Oryza sativa</i> (japonica)	Sanger	Poaceae	410*	Cereal crop
** <i>Oryza sativa</i> (indica)	Sanger	Poaceae	466	Cereal crop
<i>Brachypodium distachyon</i>	Sanger	Poaceae	272*	Model monocot
<i>Setaria italica</i> (Foxtail millet)	—	Poaceae	406	C4 cereal crop
* <i>Zea mays</i> (corn)				Early release on <a href="http://www.phytozone.net">www.phytozone.net</a>
** <i>Sorghum bicolor</i>	Sanger	Poaceae	2046*	C4 cereal crop
** <i>Hordeum vulgare</i> (barley)	Illumina, 454, Sanger	Poaceae	698*	Cereal crop
** <i>Phoenix dactylifera</i> (date palm)	Illumina, Sanger, fosmid	Arecales	n.d. 366*	Cereal crop Fruit crop

Note: Species in which NGS was used exclusively for sequencing are shown in boldface.

+For *T. sativina* an early release by a different group exists that is posted on [www.phytozone.net](http://www.phytozone.net).

#*Thellungiella* species are listed in <http://www.ncbi.nlm.nih.gov/homologene> under the family name Eutrema.

\*These data are taken from <http://www.ncbi.nlm.nih.gov/homologene>.

\*\*Indicates a crop species character as the primary justification.

A brown alga genome is available at <http://www.nature.com/proxy2.library.illinois.edu/nature/journal/v465/n798/full/nature09016.html>.

plants. Comparing the molecular genetic machineries of closely related species that show different “lifestyles” provides insights into how the environment has shaped plant genomes and brought about the evolution of species fit for new habitats (Dassanayake et al., 2011a; Oh et al., 2012). Particularly unexpected has been the discovery of an extraordinarily large number of changes in gene structure and expression that have accumulated in the approximately 10 million years since these species separated, and, in particular, to realize how rapid such changes have been (Oh et al., 2010).

## 9.2 Plant genomes 2012—a transient account

Between 2000 and 2010 the number of plant genomes sequenced amounted to fewer than ten, while since 2010 some 30 have appeared or their appearance is imminent. Table 9.1 lists species whose genomes have become available. The table also includes the sequencing methods used and sizes of the sequenced genomes, often estimates, and the model character—other than being a crop species—by which the sequencing may have been justified. Without question, new genome sequences will be added in rapid succession over the next decade, because improvements to existing tools and new instruments will make the task progressively easier, faster and less costly.

Plant Biology will benefit from genome sequences of a large number, possibly including all economically important crop species. Once the genome blueprint for a species has been sufficiently curated and its overall quality has been verified, adding and anchoring genome sequences of individual breeding lines and regional variants will become fast and routine. Similarly fossil, ancestral and progenitor species can be merged with existing genome and chromosome structures. Multiple genome sequences will benefit many biological disciplines, from ecology to systematics, physiology and biochemistry. Importantly, evolutionary and comparative genetics will, we envisage, take center stage.

Foremost among possible concerns is the idea that now that we can, we should sequence all (plant) species. However, a quasi-industrial complex of sequencing technologists and companies should not determine policy, even if overall costs become progressively lower. There are at least 2 million species on earth, and there may well be more. The justification for sequencing all of them as a program is difficult to understand; more likely is that the immense amount of data will result in diminishing returns. Similarly, sequencing the genomes of all of the estimated 220,000 higher plant species should not be a purpose in itself; rather, species should be added based on understanding of a novel pathway or a distinct environmental adaptation.

However, we will benefit from additional genome sequences representing every family, clade or subclade of the plant branches in the tree of life. This is

also justifiable based on the recent recognition that, within families, chromosomes have been rearranged such that large numbers of genes are distributed as contiguous segments throughout the members of a clade. This had already been suggested in the pre-genomics era based on markers and gene mapping (Bennetzen and Ma, 2003; Feuillet and Keller, 2002). Confirmations of the significant co-linearity of genes in related species will facilitate genome and chromosome assemblies even when only very few or no markers are available. One example has been provided by the analysis of the grass model species, *Brachypodium distachyon* (International Brachypodium Initiative, 2010). In this example, the evolution of chromosomes could be reconstructed in how segments comprised of hundreds or thousands of genes changed location in a traceable fashion among species in the Poaceae.

An example with relevance for crucifers ascertains the power of this approach. Using fluorescently labeled BAC sequences from *A. thaliana* a “comparative chromosome painting” approach has been developed (Lysak et al., 2010). Such painting works through hybridizing individual BACs to condensed chromosomes in a variety of species (Mandáková and Lysak, 2008). This resulted in identification, delineation and placing of 24 distinct segments from the five *Arabidopsis* chromosomes in all crucifer species analyzed so far, irrespective of their chromosome numbers (Schubert and Lysak, 2011). At least since the last genome duplication event in the crucifer lineage approximately 40 MYa, these segments have been reshuffled more or less intact to form the chromosomes of species in different crucifer tribes.

The segmental distribution model established for crucifers (Schranz et al., 2006) has been confirmed by the assembly of the seven chromosomes of two genomes in the crucifer tribe *Eutremiaeae*, *Thellungiella parvula* and *Thellungiella salsuginea* (Dassanayake et al., 2011a; Wu et al., 2012). Centromeric regions revealed size differences and significant sequence divergence in comparisons among *A. thaliana*, *T. parvula* and *T. salsuginea*. In a more general sense, at least for crucifers, the applicability of the segmental model makes chromosome assemblies possible in the absence of genetic markers. Considering that a similar situation exists in the Poaceae, the segmental reshuffling of blocks of chromosomes during speciation may be a general feature that characterizes all plants.

### 9.3 Genomes, transcriptomes, and bioinformatics

Technologies have matured such that sequencing of genomes in the range 100–300 Mb poses little technical problems with “short read” sequences as the starting material for assembly. What is called a *read* is the result of one set of consecutive sequencing reactions out of millions per run. Depending on the platform, reads may range from 70 to 600 bases and in some single molecule sequencing technologies reads can exceed 1,000 bases. The two—presently—established

sequencing platforms, Roche 454 and Illumina, can also generate paired end sequences with variable insert sizes from 100 to 20k to facilitate establishing long-range connectivity in complex genome assemblies. All NGS reads can also be used in so-called “hybrid” assemblies. In this process, different read types and variable read sizes can be effectively merged to build a higher quality contiguous assembly than with reads generated from a single sequencing platform.

Likewise, NGS is useful for establishing global transcript profiles that reflect the true representation of transcribed protein-coding and RNA genes at any time (Martin and Wang, 2011). Illumina, due to its high yield in sequence numbers, is predominantly used for transcript profiling with the reads referred to as RNAseq. This aspect should give rise to rethinking how transcript expression and presence will best be measured in the future. Quantitative PCR and microarray platforms are complemented with, and may best be replaced with, RNA-seq approaches. They provide a genome-wide expression snapshot superior to techniques based on assumptions concerning the genes analyzed (e.g., qPCR) or the number of transcripts printed on a microarray chip. RNA-seq, in contrast, does not depend on prior-knowledge of the sequences captured. For example, RNA-seq can identify novel alleles, splice variants or RNA gene models, even in the absence of a reference genome. The unbiased approach reflects the state of a plant’s transcriptome more accurately and systematically, and at least partially enables studying gene expression of non-model plants lacking a reference genome or established gene models.

Recent progress in sequencing is very much a function of the capacity for generating large numbers of short reads per experiment, coupled with the number of reliably identified nucleotides in each read. The output of a single sequencing run can now be measured in several Gb of sequence information. Adding to that the rapidity in obtaining sequence information and the declining cost makes short read sequencing the only practical approach. This is particularly relevant as research moves toward economically or ecologically important non-model species whose genomes are often considerably larger than the ~410 Mb genome of, for example, rice. The importance placed on additional genome sequences can be seen from the launch of projects such as “The Genome 10K,” which aims to assemble 10,000 vertebrate genomes, or the initiative to sequence 5,000 arthropod genomes within the next 5 years (Haussler et al., 2009; Robinson et al., 2011).

Most efforts in assembling genomes to date have involved some quantity of Sanger sequencing based on BAC/fosmid libraries, and genetic or physical maps to organize scaffolds to achieve chromosome level resolution. Marker-free assemblies based on NGS tools alone are few (see below). Recently, however, plant (and several animal) genomes have been published that used predominantly short reads to obtain the data for assemblies (Table 9.1).

Invariably this recent progress was enabled by exploiting various aspects of hybrid sequencing and assembly tactics. The programs used have evolved over

the last two decades initially due to lessons learnt from the human and other key model genome assembly projects, and recently due to the need to keep up with the NGS technology development. The biggest hurdle in the process of making sense of the increasingly more rapidly generated DNA sequences is now bioinformatics. This is particularly relevant for *de-novo* assemblies of the genomes for non-model species for which no genetic data exist.

Genomes for non-model plants are now being sequenced in increasing numbers. However, reconstructing a high-quality genome model for plants from short reads remains a challenge. The first challenge, usually unavoidable when sequencing a heterogeneous non-model species, is the unavailability of DNA from inbred lines where allelic diversity is minimized. This becomes increasingly difficult as genome size and ploidy level increase. The second challenge is related to library preparation protocols. As the paired-end insert size increases, yield and quality of the paired-end reads declines significantly, leading to loss of pairs or the appearance of chimeric pairs. Also, small amounts of input genomic DNA lead to more artifacts in the libraries due to the high proportion of adapter molecules in the library preparation (Kircher et al., 2011). Finally, assembling gigabases of short reads into contiguous assemblies in higher eukaryotes is computationally challenging due to intrinsic genomic complexities in each genome that cannot be predicted before assembly. The most common difficulty in assembly is caused by repeat sequences that are often prevalent in eukaryotes (Treangen and Salzberg, 2012). Sequence assembly is based on overlaps between reads. However, the first generation of assemblers was designed to do pairwise alignment between all possible reads allowing overlaps greater than a threshold length. This approach became computationally impractical with the large output of reads. As a result, the de Bruign graph based assemblers were specifically developed (Compeau et al., 2011; Salzberg et al., 2012). The de Bruign graph assemblers also perform suboptimally with repeat rich sequences. However, high-density paired-end reads with variable insert sizes have been shown to reduce assembly fragmentation due to repeats. The challenge for bioinformatics does not end with sequence assembly. Equally or even more difficult is to identify and correct for assembly errors.

Ideally, a genome assembly will have contiguous sequence fragments equal to the haploid chromosome number, or rather the number of chromosome arms because assembling centromeric regions is typically impossible in the first try. In reality, genome assemblies have a common end-result: several thousand to tens of thousands of “scaffolds” that represent the genome as a collection of pieces of varying lengths—some possibly the length of chromosome arms—in addition to scaffolds of increasingly shorter length. These shorter scaffolds might be placed in a chromosomal context, based on co-linearity arguments or, if available, physical or genetic maps, but gaps will remain.

Genome assemblies asymptotically approach chromosomes by having long-range contiguity expressed in contig sizes and longer range connectivity with

scaffolds. The closer both values come up to the length of arms of individual chromosomes, the more complete and less fragmented the genome will be at this stage. The most highly curated higher organism model genomes with more than a decade of ongoing research are still being improved with changes amending mis-assemblies, mis-annotations, and gap filling (Church et al., 2011; Lai et al., 2011; Macas et al., 2010). Nevertheless, any collection of sequences representing a genome, or at least the gene-rich regions, will be sufficient for many purposes. Thus, a genome that includes all, or nearly all transcribed regions, is useful to explore questions asked in particular studies for which the genome has been sequenced. Clearly, however, the usefulness of a collection of fragments increases as the contiguous sequence space grows in size. Not all information that is encoded in a genome is information contained in the genes. Comparative genomics must rely on information that resides in non-translated regions close to protein coding regions as well as in more distant regions (for examples, see below). Striving for a genome sequence with chromosome-length resolution and with a minimum number of breakpoints in the chromosomes must be the goal.

#### 9.4 Genomes that inform about abiotic stress

Work is legion that described and experimentally analyzed reactions of plants to abiotic stresses such as drought, flooding, temperature extremes, xenobiotics, or salinity (ion imbalances). Transcripts, genes and pathways have been identified that certainly advanced understanding, but the apparent multigenicity of responses required to establish stress tolerance has not been probed to a degree that successfully deals with this complexity (Bohnert et al., 2006; Flowers, 2004). Moreover, during the last two decades, hundreds of experiments have described some stress protection under controlled conditions of growth, in many cases with good data, but exporting this knowledge into the real world has been less than stunning (Agarwal et al., 2012; Flowers, 2004; Qin et al., 2011). In fact, we and others have on occasion considered that there may not be any stress tolerance, that all that plants can accomplish may be stress “avoidance.”

A safer endeavor may be attempts at breeding for stress tolerance. Such approaches have generally come under these objectives: (1) finding new alleles by introgression (wide crosses) of distant lines or closely related germplasm sources that are better adapted to some stresses (Niroula et al., 2012; Pratta et al., 2011); (2) developing xerophytes or halophytes, or naturally stress-adapted relatives of existing crop species as new crops (Glenn et al., 1991); and (3) disregarding stress per se and breeding for yield under agronomically realistic conditions in the targeted environment (Richards et al., 2010).

Breeding for the last thousands of years has mostly used the last of these strategies and with much success indeed. It is arguable that, as a result, the present crop genomes may have been modified to an unhealthy degree. It seems appropriate to focus on other approaches that would bring never previously harnessed germplasms and alleles into play (Elshire et al., 2011; Kilian and Graner, 2012; Trebbi et al., 2011). Such superior alleles, we argue, are highlighted by the genomic and transcriptome characters seemingly underlying the superior tolerance shown by extremophiles. NGS technologies can contribute to such efforts by allowing searches for genome rearrangements based on the activation of movable elements after wide crosses or changes in copy number variations (CNV; e.g., Jiang et al., 2011).

Whatever the approach, a limiting factor has been the lack of models for abiotic stress in general, or for any particular stress condition. Certainly no established crop species provided such a model, because the incentive was lacking to engage in large scale breeding of crop abiotic stress adaptation. Ecophysiological descriptions and molecular characterizations of a variety of stress-tolerant plant species have been conducted. A few samples of species relevant for the study of abiotic stress tolerances may be sufficient to make a point (Adams et al., 1998; Bartels, 2001; Flowers, 2004; O'Leary, 1987; Yensen, 2008; Zentella et al., 1999). Invariably these plants attracted little attention, mainly because they offered no genetic system. This aspect will be of diminishing importance in the age of genome sequencing. We can now view entire genomes, can scrutinize chromosome structures and transcriptome complexity, and can easily determine how and which transcripts and proteins are engaged under a stress regime. Now, every species whose genome is sequenced also becomes a model species.

Over the last two decades, most information on stress-related genes and pathways has been gained through the analysis of *A. thaliana* mutants despite the fact that the species is relatively stress-sensitive. However, the removal of genes by insertion mutagenesis often generated even more sensitive lines. Increasingly more sophisticated methods and tools have revolutionized mutant generation and detection of a mutated gene (e.g., Papdi et al., 2010). Some 10 years ago, a concept emerged suggesting a way to find processes in genuinely stress-tolerant species, that is, Arabidopsis-Relative Model Species (ARMS). ARMS was successful in finding “stress genes,” because such *Arabidopsis* relatives with high stress tolerance existed (Amtmann et al., 2005; Bressan et al., 2001; Inan et al., 2004; Orsini et al., 2010; Taji et al., 2010). In fact, accounts of stress-tolerant species indicate that ca. 5–10% of all higher plant species—at least 10,000 species—show abiotic stress tolerance, although some clades and families contain significantly larger numbers than others (Flowers, 2004; Flowers et al., 1977; Flowers and Colmer, 2008; Yensen, 2008).

A number of laboratories have focused on an *Arabidopsis* relative in the genus *TheLLungiella*. *T. salsuginea*, initially misidentified as *T. halophila* (Amtmann, 2009), provided a large number of genes, transcripts and pathways

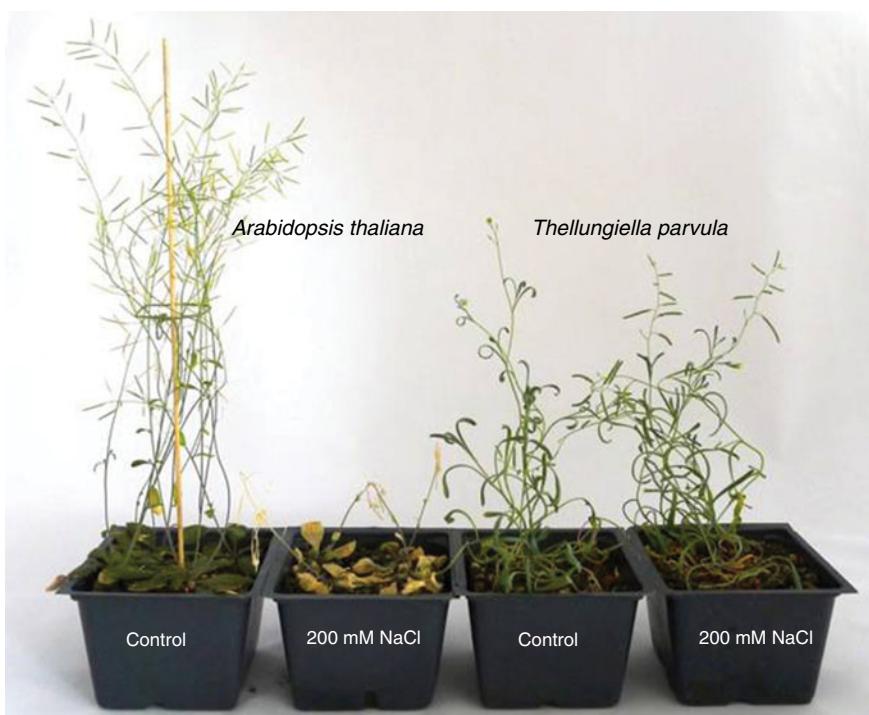
that are relevant to its salinity tolerance phenotype, but the species is also extremely cold and freezing tolerant and tolerates poor and degraded soil conditions (Gong et al., 2005; Inan et al., 2004; Taji et al., 2010; Wong et al., 2006). Another species, *T. parvula* has recently been introduced as a model because it shows an exceptionally high salt tolerance, higher than *T. salsuginea*, tolerates poor soils as well but shows less cold and freezing tolerance (Orsini et al., 2010). The genome sequences for both *TheLLungiella* species have been determined, with a resolution of seven chromosomes in each species (Dassanayake et al., 2011a; Wu et al., 2012). Their gene complements are known. As extremophiles, the *TheLLungiellas* provide an excellent platform for studying plant abiotic stress responses and adaptation mechanisms using comparative genomics approaches (Dassanayake et al., 2011b; Oh et al., 2012; Oh et al., 2010).

### 9.5 Plants evolved for salinity tolerance

The *TheLLungiella parvula* genome, with a sequenced size of 135 Mb is only slightly larger than the genome of *A. thaliana* (119Mb sequenced). In contrast, *T. salsuginea* has an estimated genome size of ~250 Mb, of which ~234 Mb have been arranged into scaffolds. The remaining sequence information in the latter case cannot presently be assembled into contigs because it represents the ~55% of repetitive DNA that characterizes this genome. In contrast, in the genomes of *A. thaliana* and *T. parvula* repetitive DNA is approximately 16% and 8%, respectively (*Arabidopsis* Genome Initiative. 2000; Dassanayake et al., 2011a; Wu et al., 2012). The contigs that are not included in the chromosome models consist, in all three species, of repetitive sequences in gene-poor regions.

In the following, we will focus on the *TheLLungiella* genomes with emphasis on genes, gene expression, and chromosome structures that seem to support the plant's extreme tolerance to ionic stress: *T. salsuginea*, the Shandong ecotype, collected from the estuaries in the Shandong province of northern China; another ecotype, Yukon, collected in northern Canada; and *T. parvula* from the shores of the land-locked, highly saline Lake Tuz in central Anatolia. Lake Tuz is particularly interesting as a source environment as it has particularly high concentrations of Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>2+</sup> as well as Cl<sup>-</sup> and SO<sub>4</sub><sup>2-</sup>, and a high pH (ca. 8; Helvaci et al., 2004; Nilhan et al., 2008). In satellite images, it usually appears white due to precipitated salt. Figure 9.1 shows phenotypes of *T. parvula*, in comparison with *A. thaliana*, for greenhouse-grown plants grown under control and salinity stress conditions.

*T. parvula* is a diploid species with 2N=14. The genome sequence, based exclusively on NGS (Roche 454 and Illumina reads) was assembled to large contigs, the 30 largest amounting to more than 100 Mb of sequence, that is, more than 75% of the genome. The lack of any markers or other genetic information made it initially impossible to assemble chromosomes, although the



**Figure 9.1** *Thellungiella parvula* compared with its relative *Arabidopsis thaliana*. Seeds were germinated and plants grown on root wash mix (<http://pcf.aces.illinois.edu/services/soil.html>) in a growth room with 14 hours light (120  $\mu\text{mol}/\text{m}^2/\text{sec}$ ) and 10 hours dark, for 4 weeks before treatment. Plants were irrigated with 1/20 Hoagland solution once a week. Treatment was done by adding of 200 mM NaCl to the irrigation solution. For color details, please see color plate section.

largest contigs appeared to be the size of chromosome arms (as judged by comparison with *Arabidopsis*). The seven haploid chromosomes could then be assembled based on work using Comparative Chromosome Painting (Mandáková and Lysák, 2008; Schranz et al., 2006). CCP carried out extensive hybridizations of fluorescently labeled BACs from *Arabidopsis* to chromosomes of a variety of crucifer species. The result showed clearly the presence of syntenic regions, 24 in total, that have been distributed largely intact in *T. parvula* (TP) and *T. salsuginea* (TS).

The gene complements of the two *Thellungiella* species compared against two *Arabidopsis* species (*A. thaliana* [AT] and *A. lyrata* [AL]) seem at first glance relatively similar (Dassanayake et al., 2011a; Hu et al., 2011; Wu et al., 2012). The numbers of protein-coding nucleic gene models range from 27,107 (AT) to 26,814 (TP), 28,457 (TS) and 32,670 (AL; <http://www.arabidopsis.org>). More interesting is the distribution of genes in various GO categories.

Statistically significant differences exist in categories that are commonly associated with abiotic stress defenses. Genes in the GO “biological processes” categories transport, response to abiotic stimulus, transcription, ATPase activity, and response to other stresses are present in higher copy numbers in the *Thellungiellas*. In contrast, the *Arabidopsis* species show gene enrichment in other categories, that is, receptor binding activity and signal transduction. The discrepancies are largely due to tandem duplications of existing genes in the respective categories. Such CNV has been proposed on theoretical accounts in the past (Haldane, 1932; Ohno, 1970), and has now very frequently observed in animal systems (Malhotra and Sebat, 2012).

The *Thellungiellas* also have inversions of parts of chromosome segments defined by CCP, relative to the *Arabidopsis* genome (Wu et al., 2012). In addition, we found typically small translocations of individual genes to small groups of genes, which then ended up in a different part of the genome. Such inversions and disruptions in segment structure are often associated with transposons and/or retrotransposons that disrupt the local chromatin structure and often alter the expression of genes in the vicinity of the translocation (Tillier and Collins, 2000; Oh et al., 2012; Oh et al., in preparation).

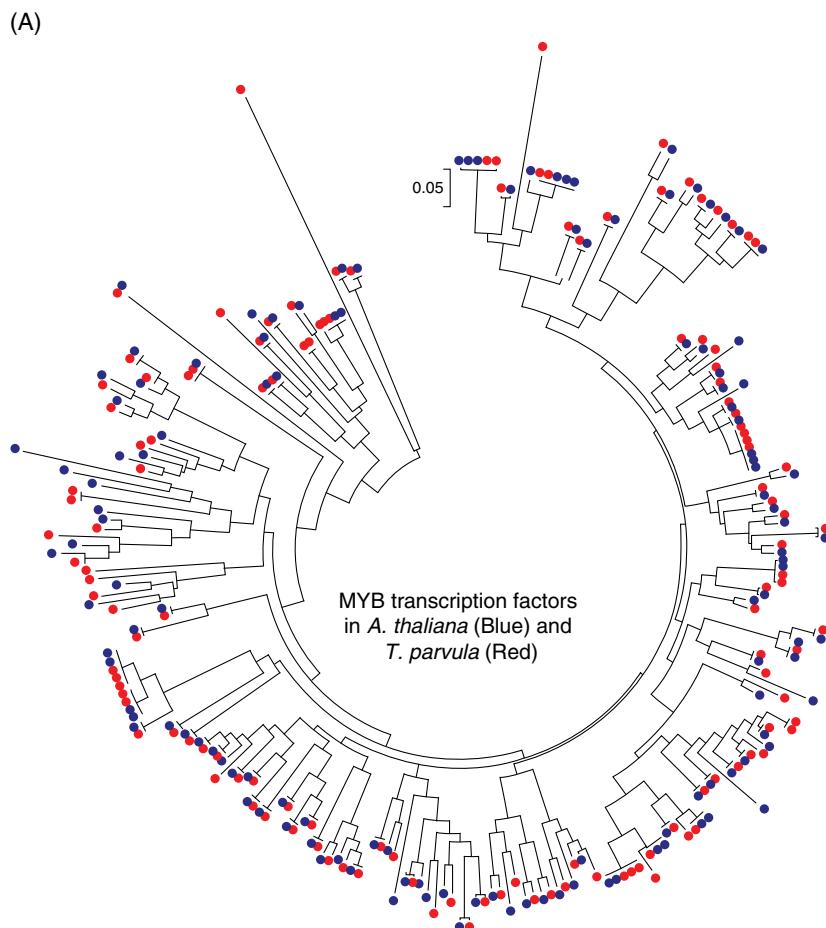
## 9.6 ARMS genomes—*Thellungiella* genome sequences

Results that emerged from the assembled genome sequences of *Thellungiella parvula* and *T. salsuginea* shed surprising insights about the extent of changes in their genomes and transcriptomes that accompanied their adaptation to stressful habitats. Juxtaposing the *Thellungiella* and *Arabidopsis* genomes can inspire new approaches to plant breeding for higher tolerance in crop species. The trajectory of changes that accumulated during a, possibly less than 10 MY separation between the genera identifies three fundamental mechanisms.

### 9.6.1 Lineage-specific gene duplications

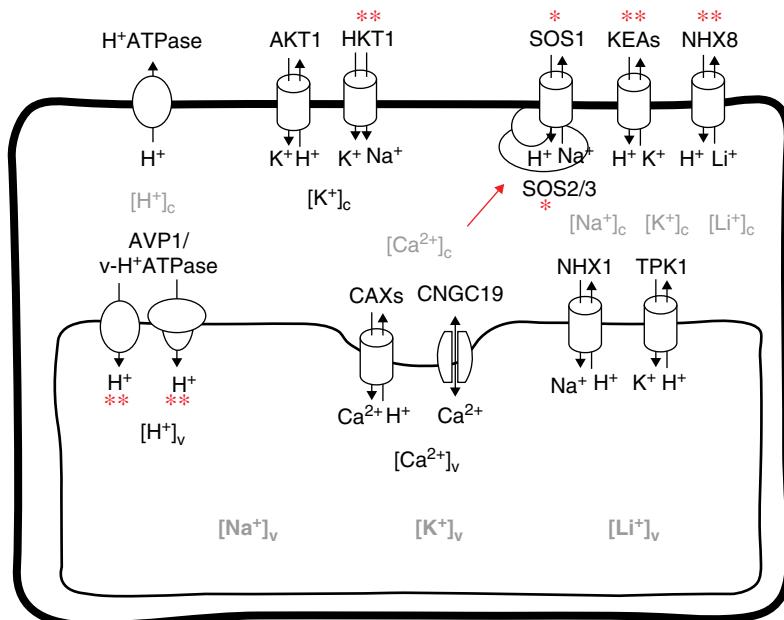
Anticipated based on theoretical considerations and other examples are gene duplications. *Thellungiella* and *Arabidopsis* genomes carry about 10–20% of all genes as tandemly duplicated copies. However, only half of those tandem duplication events are shared between AT and TP, for example, giving rise to the expansion of gene families and enrichment of functional classes that are unique to each genus. When comparing *T. parvula* to *A. thaliana*, approximately 5% of all genes have been translocated, or appearing in different places in the genome compared to their orthologs, breaking the co-linearity of the genome segments. Many translocations are also translocation-duplications, leading to an increase of copy numbers for particular genes. Copy number variation of orthologs, resultant from tandem duplication or translocation-duplication, are observed in comparable frequencies in *Arabidopsis* and *Thellungiella* species.

An example is shown in Figure 9.2A, where the copy numbers of genes encoding MYB transcription factor subfamilies are compared between *A. thaliana* and *T. parvula*. What may be termed the flavor of the duplications is, however, very different in each family. Gene duplications in the *Thellungiellas* show a preference with respect to functional categories that identify abiotic stress defenses, as far as we know these from work with *Arabidopsis* mutants.

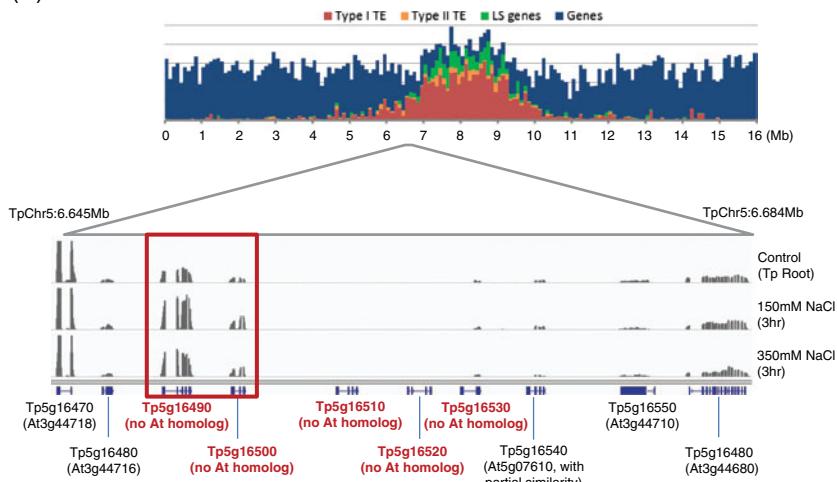


**Figure 9.2** Possible mechanisms underlying the divergence of *T. parvula* and *A. thaliana* genomes and lifestyles. (A) Copy number variation of orthologs. Evolutionary relationships exemplified by a phylogenetic tree including all MYB family genes in *T. parvula* (red) and *A. thaliana* (blue) for 126 *Arabidopsis* and 130 *Thellungiella* R2R3 MYB proteins. The phylogenetic tree was inferred by the Neighbour-Joining method, using the pairwise deletion option and 1000 bootstraps (Mega5; <http://www.megasoftware.net/>). For color details, please see color plate section.

(B)



(C)



**Figure 9.2** (continued). (B) Different expression of orthologs between *T. parvula* and *A. thaliana*. Shown is a selection of ion transport-related proteins crucial for ion movement in the plant defense against excess salinity. \*Genes with significantly higher expression in *T. parvula* compared with *A. thaliana*. These genes have undergone either translocation or severe sequence divergence in their promoter regions. \*\*Duplicated genes in *T. parvula*. (C) Expression of lineage-specific genes in *T. parvula* using a *T. parvula* chromosome segment harboring lineage-specific genes. The upper panel shows *T. parvula* chromosome 5, with the proportion of genes, lineage-specific genes (LS genes), and transposable elements (TE) plotted as a histogram. The lower panel represents a magnification of a 29 Kbp segment of *T. parvula* chromosome 5. The bar graph shows the RNA-seq coverage on gene models. The *A. thaliana* homolog for each gene model is shown in parentheses.

Examples of stress-related genes duplicated specifically in *Thellungiella parvula* and *T. salsuginea* include HKT1, AVP1, KEA1, CBL10, MAH1/CYP96A15, NHX8 (TP), MYB47(TP), ZEP(TS), AAO2(TS), CYP707A3(TS) and SAT32(TS; Dassanayake et al., 2011a; Wu et al., 2012). These genes are known to be related to ion transport, abscisic acid (ABA) synthesis, leaf wax biosynthesis, and salt stress signaling and response, based on studies with *Arabidopsis*. *Arabidopsis*-specific tandem duplications include defensin genes, PR1 and membrane receptor kinase family genes. When the total gene complement of *T. parvula* is compared to that of *A. thaliana*, duplications result in enriched GO terms for response to abiotic stimulus, response to stresses, and transport functions. The GO term signal transduction is enriched in *A. thaliana* (Chi-square test,  $p < 0.05$ ; Dassanayake et al., 2011a). Gene duplications are often followed by gene loss that reestablishes gene dosage. Therefore, remaining duplications are either under positive selection due to their contribution to the organism's fitness or are in the process of being replaced.

Tandem duplication events often fail to duplicate regulatory regions of the parent gene such that duplicated copies gain a novel regulatory context. This may lead to an altered phenotypic range subject to rapid selection (Cannon et al., 2004; Goffeau, 2004; Hanada et al., 2008). The unique tandemly duplicated genes in the *Thellungiella* and *Arabidopsis* genomes support the view that the present majority of unique tandem duplicates have been preserved in each genome as a result of positive selection.

Duplications that are retained by selection often undergo sub- or neo-functionalization. An example is the tandem duplicated copies of HKT1 orthologs in *Thellungiella*. While both *A. thaliana* and *A. lyrata* contain single HKT1 genes, *T. parvula* and *T. salsuginea* contain two and three copies, respectively. The additional HKT1 orthologs in *Thellungiella*, TsHKT1;2 and TpHKT1;2, show significantly higher expression than the single copy ortholog in *Arabidopsis*. The *Thellungiella* HKT1 coding regions include amino acid residues in the second pore domain that differ from most other HKT1 genes. These changes imbue the ability to complement yeast potassium transporter mutants in the presence of sodium, suggesting a selective affinity toward potassium ions that is absent in *Arabidopsis* HKT1 (Ali et al., 2012). Promoters of the tandemly duplicated HKT1 copies in the two *Thellungiella* species do not show any significant sequence similarity. Indeed, the *T. parvula* and *T. salsuginea* HKT1 copies show different expression patterns especially in root tissues, indicating a divergence within the genus (Hong et al., in preparation). Obviously, gene duplication resulted in functional diversification of stress-related genes, in addition to changes in gene dosage.

#### 9.6.2 Divergence of transcriptome profiles and responses

While *Thellungiella* and *Arabidopsis* share extensive genome colinearity and overall gene complements, variation in gene copy numbers and regulatory sequences will affect their expression, shaping distinct transcriptomes for

each species. Even before the completion of the draft genome sequences, we observed fundamental differences in the expression of orthologous genes in the *Thellungiellas* relative to *Arabidopsis*, often remarkable with respect to the abiotic stress-relevant genes that are typically more highly expressed in the *Thellungiellas* (Ali et al., 2012; Dassanayake et al., 2011b; Kumar and Purty, 2009; Oh et al., 2010). Stress-related genes that show significantly higher expression in *T. parvula* include genes that are known to play major roles in cellular monovalent ion transport such as *SOS1*, *HKT1*, *NHX8*, and *KEA1* (Figure 9.2B).

Draft genome sequences and annotations identified all orthologs between the species, enabling systematic comparison of their expression strength as well as the responses to stresses. Among 27,132 *T. parvula* gene models, 20,163 (~75%) have an *A. thaliana* ortholog with sequence similarity over more than half of the gene length. Comparison through RNA-seq has revealed that about a fifth of all orthologs (i.e., 4,063 orthologs) show significantly different expression strength between *A. thaliana* and *T. parvula* in either root or shoot samples, even without any stress treatment (binomial test with three biological repeats, adjusted p-value <0.1). Tandem duplication and translocation events were enriched among those genes with significantly different expression levels; they accounted for 24.9% of all *T. parvula* genes with different expression levels compared to their *A. thaliana* orthologs (Hong et al., in preparation).

PCA-based analyses such as Fuzzy K-means clustering have been useful for finding genes that are differently regulated between two species (Gong et al., 2005), as well as shared and unique components of transcriptome responses to different abiotic stresses. In a pilot experiment, for example, when plants of similar developmental stage were subjected to a change of soil solution from 0mM to 150mM or 350mM NaCl for relatively short time (3 hours and 24 hours), *T. parvula* root and shoot transcriptomes showed responses distinct from those of *A. thaliana*. *T. parvula* responded more actively to the salt treatment; with genes induced that are known as responding to ABA and water deprivation signals and many transcription factors of yet unknown functions, especially in the root. In contrast, genes related to defense and abiotic stimuli other than ABA and water deprivation tended to be downregulated in *T. parvula*, suggesting a focused salt response. *A. thaliana* shoots accumulated mRNAs related to synthesis of secondary metabolites such as flavonoids and phenylpropanoids, and reduced transcription of genes responding to auxin upon salt treatment. These responses were not observed in *T. parvula* (Hong et al., in preparation). We also point to differences in the ionic stress responses between *T. parvula* and *T. salsuginea*. The adjustment of *T. parvula* to the soil ion composition of its natural habitat lets the plant grow, not simply survive, in the presence of high concentrations of Li<sup>+</sup>, K<sup>+</sup> or Mg<sup>2+</sup>, in which neither *A. thaliana* nor *T. salsuginea* can survive (Hong et al., in preparation). Comparison between *T. parvula* and *T. salsuginea* will reveal the shared and unique strategies and pathways for their ionic stress tolerance. Eventually,

accumulated RNA-seq data need to be viewed as networks based on gene expression data (Lee et al., 2010; Ma et al., 2007). Comparison of network structures between species will facilitate annotation of yet unknown functions of genes, identification of abiotic stress-related pathways that have diverged among species, as well as sequence elements that are responsible for such different gene regulations.

#### 9.6.3 Lineage-specific genes

While comparison of transcriptomes can identify different regulations of orthologs, additional source of divergence between species derives from the presence of lineage-specific or “orphan” genes. These genes lack orthologs in another species. When the total gene models are compared between newly annotated genomes orthologs are typically defined based on their amino acid sequence similarity. That is, the gene with the highest sequence similarity in the other species being compared is considered the ortholog of the query gene if the similarity is observed over more than 50% of the entire gene length, based on BlastP e-value  $< 10^{-5}$  (Li et al., 2003). When *T. parvula*, *T. salsuginea* and *A. thaliana* were compared, the three species contained 18–24% of all genes that show only partial similarity (i.e., similarity over less than 50% of gene length) or no detectable similarity at all, with genes in other species (Wu et al., 2012). Both the *Arabidopsis* and *Thellungiella* species contain up to 10% of all genes, defined as coding regions with deduced amino acid sequences, that lack any detectable similarity with a gene in the other species (Dassanayake et al., 2011a; Donoghue et al., 2011). In *T. parvula* and *T. salsuginea*, genes without any similarity to an *A. thaliana* gene are enriched in transposable element-rich regions. In *T. parvula*, of 3,597 such gene models, at least 773 showed expression in either root or shoot tissues, and a high percentage of these presumptive coding regions showed regulation by salt treatment. One such example is presented in Figure 9.2C. Further studies employing transgenic plants would be required to reveal putative functions and contributions of these lineage-specific genes in the abiotic stress response of *Thellungiella* species.

### 9.7 A breeding strategy for abiotic stress avoidance

With respect to salinity tolerance, a series of events at different developmental phases requires intervention to avoid deleterious effects. The genes underlying these responses are highlighted in the *T. parvula* genome and transcriptome by the CNV concept: we observe more genes and also higher expression of genes even when the gene copy number is not increased.

A classical breeding procedure can be imagined. Non-invasive monitoring of thousands of seedlings can not only be used to evaluate phenotypes (Fiorani

et al., 2012), such growth facilities can also be used to harvest individual leaves, process RNA and carry out quantitative PCR analyses to screen for a variety of CNV events. Even more desirable will be to carry out RNA-seq on (some of) these samples. The samples themselves would come from the progeny of wide crosses, or the introgression of foreign germplasm, or from lines of crop species that have been reproductively separated for extended periods of time.

Individual plants displaying interesting CNV events, identified by either technique, would be followed by growing them to maturity and with further crosses, the goal being to pyramid CNV alleles for desired genes and pathways. If, in a first round, 10 to  $10^2$  individuals with CNVs for 10 different genes could be detected, and repeating this process should lead to progress. Variations on this theme can easily imagined, e.g., by the introduction of non-lethal but growth-reducing stresses, or by adding physiological screens. Studies reported that under such stress condition, CNVs of various transposable elements and genes accumulated in the genome within several generations (DeBolt, 2010; Hilbricht et al., 2008).

## 9.8 Conclusion

Genome sequences and RNA-seq data provide unparalleled views into the genetic basis that underlies plant phenotypes. Until quite recently, such views have not been possible. The new tools also open new possibilities to merge molecular genetic data with independently generated protein and metabolite data and their dynamics. Finally, availability of genome and transcriptome overviews from different species and plant families makes for powerful advances in not only comparative genomics but in our understanding of the distribution of species, the physiological challenges posed by different environments, and the potentials and prospects of new species to become agriculturally useful.

At the same time, the emerging information from genomes has opened a series of new questions. New inquiries must be directed toward the understanding of lineage-specific coding regions that appear to be generated in centromeric regions in association with transposon activity. Other specific queries emerge about the fine-tuning of signaling and response pathways with apparently different thresholds in species differing in stress tolerance. Also, results emerging about epigenetic effects on stress response competence, modification, and magnitude (Kapazoglou et al., 2010; Karan et al., 2012; Kou et al., 2011; Lira-Medeiros et al., 2010; Sharma et al., 2009; Urano et al., 2010; Yaish et al., 2011) demonstrate the necessity of adding information on the dynamic structure of the epigenome to the primary sequence-based genome.

Another question, presented by the comparative studies of *Thellungiella* species with their relative model species, concerns the selection of strategies to

explore the relation between the genotype and phenotype spaces. Established strategies such as genome-wide association studies (GWAS) and quantitative trait loci (QTL) mapping can be used if species or subspecies being compared can be crossed. However, as exemplified by *Thellungiella* and *Arabidopsis* species, dramatic phenotypic differences in abiotic stress response often appear between species that cannot be crossed. Required would be a clever mixture of forward and reverse genetics. NGS can assist the systematic comparison of stress and tissue-specific transcriptome responses with RNA-seq, mining potentially interesting alleles. In forward genetics approaches, mapping of mutated loci can now be achieved by whole-genome resequencing. Comparisons of the transcriptome and interactome network structures will reveal novel pathways leading to the observed phenotypic divergence. Implanting such pathways, rather than single or a few genes, for crop improvement might require another level of technical advance.

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## **10 QTL and association mapping for plant abiotic stress tolerance: trait characterization and introgression for crop improvement**

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### **10.1 Introduction**

Nutrient deficiencies (particularly phosphorus and nitrogen, but also micro-nutrients), toxicities (particularly Na, Al, Bo, HCO<sub>3</sub>), cold, drought, and heat stress are widespread problems in agriculture areas. Land degradation has been extensive in agricultural systems, and there has been a direct correlation between population density and land degradation in the developing world (Bot et al., 2000). The global loss of productive land has been estimated at between 5 and 7 million hectares annually from both degradation and urbanization (Bot et al., 2000). In irrigated areas, salinity and boron toxicity have become serious issues. Around 1.5 million hectares of arable land is being lost annually to salinization (Foley et al., 2005). Water and wind erosion remain major problems in many parts of the world, with an estimated 25 billion tons of topsoil lost each year. Table 10.1 summarizes the three major soil constraints by region. The table also shows the small proportion of the world where there are no significant soil constraints (note the potential of Central Asia).

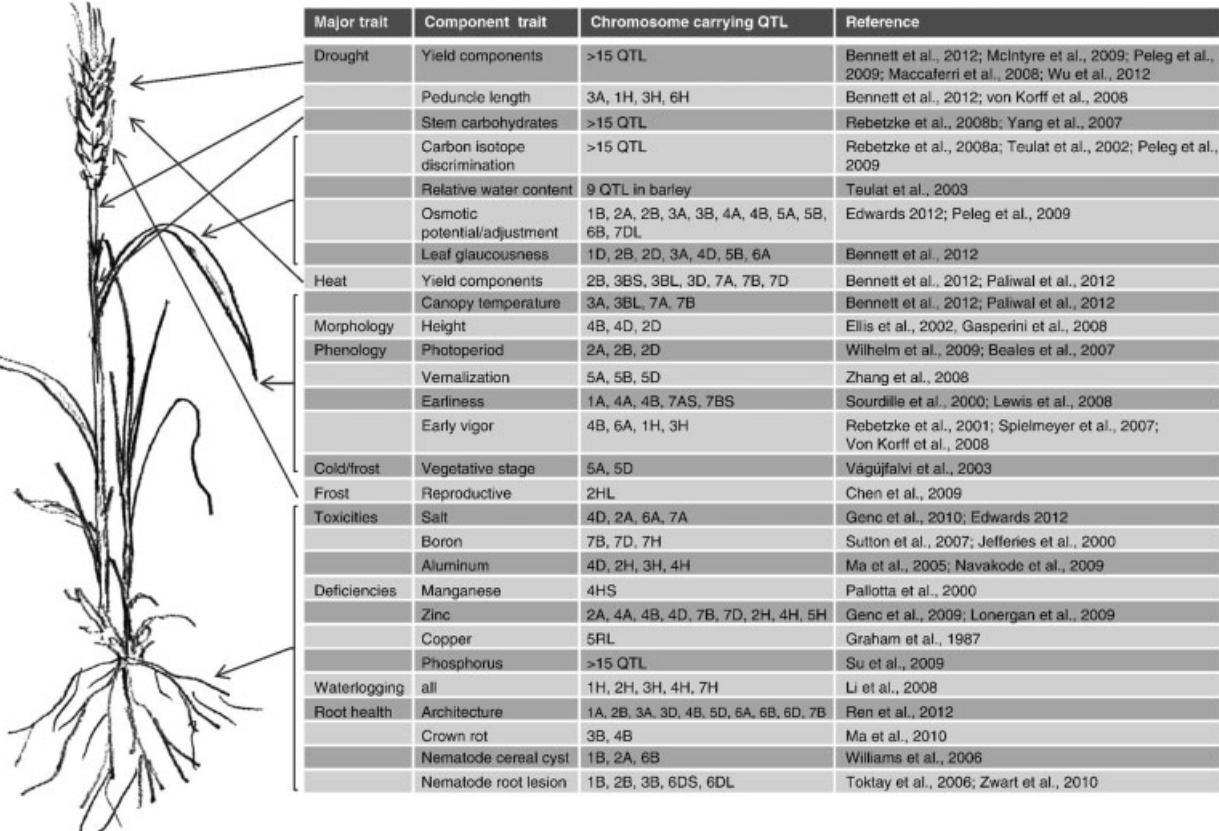
In order to survive and produce under highly variable environments, plants have developed multiple strategies and mechanisms of tolerance. The majority of traits associated with abiotic stress tolerance in plants are quantitative with complex phenotype and genetic control. These traits are usually controlled by multiple genes and show low heritability and high genotype by environment (G×E) interactions (Blum, 1988; McWilliam, 1989). Although quantitative genetics approaches are best suited to discover new genes, the success of such approaches depends largely on the complexity of the trait. Yield is the end product that integrates all variables of plant physiology over time and in fluctuating environmental conditions, and it is consequently a highly complex trait to target for improvement. By contrast a trait such as sodium concentration in leaf

**Table 10.1** Percentage of arable land by region suffering from major soil constraints, caused by aluminium and salt toxicities and erosion (data from Bot et al., 2000).

	Area (million km <sup>2</sup> )	Aluminium Toxicity	Salinity and Sodicity	Erosion Hazard	Soils without Major Constraints
Sub-Saharan Africa	24	18%	4%	15%	18%
North Africa and Near East	12	0%	6%	10%	9%
Asia and Pacific	29	14%	11%	16%	23%
North and Central Asia	21	4%	10%	16%	40%
South America	20	39%	5%	19%	19%
North America	21	10%	1%	18%	27%
Europe	7	8%	3%	20%	31%
World	135	15%	6%	16%	24%

tissue targets a specific mechanism that will contribute to the overall yield under salt stress. Genetic control of a specific trait can also vary between species. While aluminum tolerance is a monogenic trait in wheat and sorghum (one locus controlling 80–85% of the phenotypic variation; Magalhaes et al., 2007; Sasaki et al., 2004), Al tolerance is a complex trait in maize involving five distinct genomic regions and several physiological mechanisms (Maron et al., 2010). Therefore, the approach used to discover new genes and alleles for tolerance to a specific stress scenario should be designed for the species and target trait in well-defined environments.

There have now been many studies of the genetics of abiotic stress tolerance using biparental crosses and association mapping. In 2012 alone there were reports of quantitative trait loci (QTL) mapping of drought tolerance loci in wheat (Bennett et al., 2012a; Kumar et al., 2012; Nezhad et al., 2012), rice (Dixit et al., 2012), bentgrass (Merewitz et al., 2012), maize (Lu et al., 2012; Nikolic et al., 2012; Wang et al., 2012a; Yuan et al., 2012), barley (Chen et al., 2012; Naz et al., 2012), sorghum (Sabadin et al., 2012), pearl millet (Sehgal et al., 2012), chickpea (Vadez et al., 2012), common bean (Asfaw et al., 2012), tomato (Kazmi et al., 2012), soybean (Jiang et al., 2012), grapevine (Marguerit et al., 2012), lettuce (Uwimana et al., 2012) and sunflower (Abdi et al., 2012). Our level of understanding of the genetics of abiotic stress tolerance has grown substantially in recent years. Figure 10.1 shows examples of recent mapping work in wheat, barley, and rye. For these closely related species many of the major abiotic stress tolerance loci have been mapped, and it is now becoming a significant challenge to work out the best approach to utilize this large pool of information. Lessons can be learned from these and other examples on how to choose methods and avoid pitfalls in using quantitative genetics to study the genetic control of abiotic stress tolerance in plants.



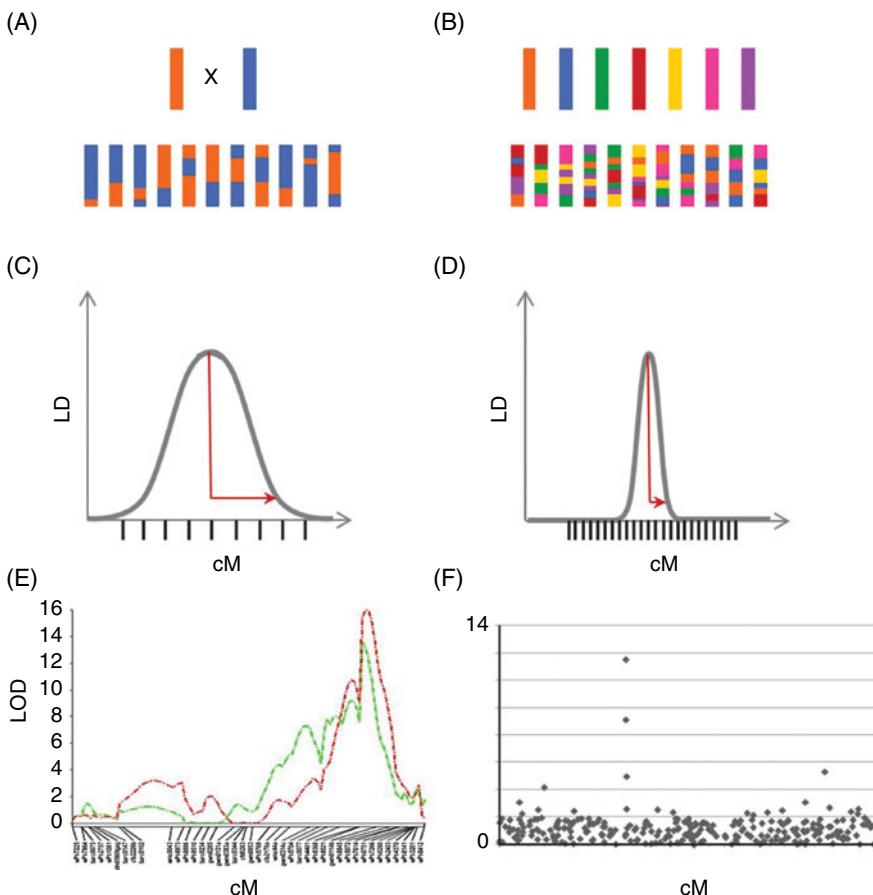
**Figure 10.1** QTL identified in wheat, barley, and rye for abiotic stress tolerance per se and plant traits affecting the overall tolerance (morphology, phenology, and root health). A, B, and D refer to each genome of hexaploid wheat, H to the diploid barley genome, and R to the rye genome. Wheat and barley chromosomes are syntenic to one another.

## 10.2 Genetic mapping of abiotic stress tolerance traits

### 10.2.1 Quantitative trait loci

The complexity of the inheritance of abiotic stress tolerance varies between stresses and species. Where the inheritance of abiotic stress tolerance is complex, the most common approach to identify genome regions or QTL associated with tolerance is based on linkage or genetic mapping. QTL detection is the observation of a significant relationship or genetic linkage between the alleles at a specific locus or genomic region and the variation in a quantitative trait. QTL analysis enables dissection of the genetic architecture of a trait by identifying the number of loci controlling the trait and the importance of additivity, epistasis and QTL-by-environment effects on the genetic control of trait expression. Biparental populations (F<sub>2</sub>:3, backcross, double-haploid [DH], recombinant inbred lines [RIL]) are most commonly used to establish the relationship between specific regions and the trait. Segregating populations are extensively genotyped to construct a genetic map, and phenotyped for the traits of interest. Statistical methods enable the measurement of the effect and significance of alleles at each locus on the trait and therefore detect and map QTL (Lynch and Walsh, 1998). Because the number of meioses will influence the number of recombination events generated, the mapping resolution of these populations for QTL analysis can be quite low, ranging from 2 to 30 cM (Figure 10.2). Additionally only the alleles of the chosen parents are detected, limiting the number of QTL that can be identified in a specific population. New or additional QTL are often identified when other biparental populations are screened for the same trait. Ideally one would hope to identify QTL that are common across multiple populations and environments. QTL that appear consistently are likely to be of greatest practical value and are regarded as “stable QTL”.

QTL define regions of the genome associated with the expression of the target trait. The region underlying a QTL can represent a single gene (due to a change in gene expression or an alteration in a gene coding sequence), multiple genes of similar or related function, or can result from a structural change in the genomic regions that alters the expression of one or more genes. Isolation and characterisation of the DNA sequence underlying the QTL is important in defining the biochemical and physiological basis of the QTL and in seeking alternative alleles. The classic procedure for isolating a gene underlying a QTL is long and costly involving: validation of the QTL in additional populations and repeated phenotyping assays; large segregating populations of RIL or the creation of Near-Isogenic Lines (NIL) to identify meiotic recombination events in the target region; large genomic resources to increase the density of markers at the target region aiming to achieve a genetic resolution of less than 1 cM; anchoring of the QTL position onto a physical map and identification of candidate genes by BAC or genome sequencing. Expression QTL (eQTL) and



**Figure 10.2** Basis of linkage analysis and association study. Linkage analysis relies on biparental population with low recombination (A) resulting in low resolution mapping of QTL but high power of detecting a significant linkage between trait and loci (C and E). By contrast association study uses historical recombination accumulated over time in a diversity panel (B), which results in high-resolution mapping (D and F). For color details, please see color plate section.

metabolite QTL (mQTL) can assist in the identification of candidate genes in the defined region by revealing specific pathways that may be regulated by the same locus (Gilad et al., 2008). eQTL and mQTL are identified by large scale mapping of gene expression profiles (eQTL) or metabolite profiles (mQTL) across the segregating population. Functional analysis of the candidate genes within the target region is then required to determine which gene or genes are responsible for the phenotype. Functional confirmation can be achieved through the use of mutants (generated by T-DNA or transposon insertion or through screening for point mutations [TILLING]) or by reverse genetics approach

of complementation, overexpression or suppressed expression in transgenic plants. Although many QTL have been identified for abiotic stress tolerance in plants, only few have been cloned and studied at the molecular level.

#### 10.2.2 QTL for abiotic stress tolerance

Most QTL cloned for abiotic stress tolerance have been found in species where a full genome sequence is available such as rice, maize and sorghum. For example, a major rice QTL that increases tolerance of phosphorus deficiency, Phosphate uptake 1 (*Pup1*) was cloned from rice based on positional cloning. NIL with the favorable *Pup1* allele showed higher grain weight per plant under low P than the intolerant lines (Chin et al., 2010). The region was fine-mapped to 278 kb with a dirigent-like gene *OsPupK20*, and a protein kinase gene *OsPupK46* identified as candidates. Neither gene encoded a known structural P uptake gene, suggesting a new mode of action of *Pup1* (Chin et al., 2011).

Crosses between the *japonica* and *indica* subspecies of rice have frequently been used to increase polymorphism and therefore the number of molecular markers in the target regions (Miura et al., 2011). Such crosses successfully revealed a QTL, *SKC1*, which was cloned to identify a member of the HKT-type transporter family that maintained K<sup>+</sup> homeostasis in the tolerant variety under salinity (Ren et al., 2005). The QTL *qLTG3-1* for low-temperature germinability was identified in a cross between two *japonica* rice varieties from Japan and Italy. The QTL was found to encode a protein of unknown function, which is strongly expressed in the embryo during seed germination (Fujino et al., 2008). A major QTL conferring tolerance to submergence stress due to flash flooding, *Sub1*, was narrowed down to a cluster of three putative ethylene response factor genes named *SUB1A*, *SUB1B* and *SUB1C*, using NIL between *japonica* and *indica* (Fukao et al., 2006). The cluster restricts plant elongation when seedlings are submerged and this allows the plants to conserve energy and restart growth after the floodwater recedes.

In several cases there are similarities between species for the mechanism of stress tolerance and candidate gene sequences can be used to accelerate the QTL cloning. For example, the main mechanism for Al tolerance is based on exudation of organic acid anions such as malate and citrate, which chelate Al<sup>3+</sup> in the rhizosphere. The complexes formed are stable and non-toxic, preventing Al from entering the root. The two first Al tolerance genes isolated in plants encode an Al-activated malate transporter (*TaALMT1*) in wheat and a plasma membrane citrate transporter (*SbMATE*) in sorghum (Raman et al., 2005; Sasaki et al., 2004). Genetic mapping and gene expression data identified *ZmMATE1* and *ZmMATE2* genes that co-localize two major Al tolerance QTL on chromosomes 5 and 6 in maize (Maron et al., 2010).

In species where a reference genome sequence is not available, gene-based markers are used to identify syntenic regions in the closest reference species.

New markers in the region can be designed based on genes from the syntenic region and it may be possible to identify candidate genes in the region. Classically, rice and Brachypodium are model genomes for the grasses (International Brachypodium Initiative, 2010), Medicago for legumes (Young et al., 2009), and Arabidopsis for Brassicas (Schranz et al., 2007). Using rice as a model, additional markers were designed in the major QTL on chromosome 4H, which is one of four QTL for tolerance to boron in barley. High-resolution mapping using 6,720 meioses and markers generated from the collinear genes of rice delineated the tolerance locus to a 0.15 cM interval. No candidates were identified in the collinear rice region but sequencing of the barley BAC clones spanning the region identified the barley *Bot1* gene, similar to Arabidopsis BOR1 efflux transporter. The barley tolerance allele was due to multiple copies of the *Bot1* gene in the tolerant landrace Sahara 3771 but the gene was completely missing from the collinear region in rice (Sutton et al., 2007).

### 10.3 Association mapping of abiotic stress tolerance traits

#### 10.3.1 Linkage disequilibrium and population structure

Association mapping (AM) measures the non-random association of alleles with specific traits. The aim is to associate QTL for the target trait with nearby loci based on historical recombination events in germplasm collections (Rafalski, 2010; Figure 10.2). The power of association analysis depends on the degree of linkage disequilibrium (LD) between genotyped markers and functional loci. The resolution depends therefore of the decay of LD with genetic distance that varies with species, population type (cultivars versus landraces) and region of the genome (Comadran et al., 2009). Outbreeding species like maize and many tree species have limited LD, which means that only polymorphisms separated by a few hundred bases are likely to be significantly associated with the trait variation (Chu et al., 2009; Gonzalez-Martinez et al., 2006). Therefore genome wide association studies (GWAS) can require a very large number of markers. However, low LD also means that the resolution of GWAS can be very high. Self-fertilizing or inbreeding species usually show a narrow genetic base and extensive LD, resulting in low resolution and high frequency of spurious associations.

LD is highly variable along the chromosomes. It has been shown at the sequence level of transcription factors that some closely linked markers are in complete linkage equilibrium while some distant markers show high LD (Haseneyer et al., 2010). An increase in marker coverage seems to increase the chance of detecting additional associations or improves the significance of known QTL (Pasam et al., 2012).

LD mapping enables the exploitation of all recombination events in the population being studied and therefore it can give increased mapping resolution

compared to the biparental mapping method. By integrating GWAS into a breeding program, it is possible to use pre-existing phenotypic data collected during a selection and cultivar registration program. A study of heading date in barley showed that phenotypic data from breeding programs enable the detection of QTL by GWAS if the appropriate population size and experimental design were used (Wang et al., 2012b).

Since genetic relatedness between lines can lead to spurious associations, the choice of germplasm for an association study is critical. The confounding effect of population structure in an association panel increases the chance of detecting false positive and negative associations compared to biparental populations. Inbreeding crops such as wheat and barley are characterized by a high level of population structure due to non-random mating and subsequent selection history. Growth habit, spike morphology and geographical origin are the major factors affecting population structure in barley (Pasam et al., 2012). This effect can be removed by analysing the population structure based on principal component analysis or mixed linear model to estimate relatedness between individuals. Several programs are available to help do this, such as STRUCTURE. Although false positive associations can be reduced, these methods do not overcome all structure effects (Yu and Buckler, 2006).

#### 10.3.2 Association study of abiotic stress tolerance

The genetic resolution of AM is usually much higher than mapping based on segregating populations. Consequently, GWAS can accelerate the positional cloning by directly “landing” on functional polymorphisms. GWAS has been particularly successful in the detection of loci underpinning highly heritable traits controlled by a small number of loci. For example, using only a small set of DArT markers across 1,055 wheat accessions, it was possible to identify the previously known loci for aluminum tolerance, including the major locus *TaALMT1* (Raman et al., 2010). Although a number of gene or marker-trait associations have been found for disease resistance and flour and milling quality, GWAS has been less widely applied to the dissection of complex traits underlying abiotic stress tolerance.

As the cost of genotyping has declined, a candidate gene approach based on gene markers chosen for their putative or known function has been used for the study of abiotic stress tolerances. A custom Illumina GoldenGate assay with 1,536 SNP markers was developed from candidate genes associated with drought tolerance and used to study 95 maize inbred lines grown under well-watered and water-stress conditions (Hao et al., 2011). Significant associations were found between 28 SNP and grain yield under drought and the stress tolerance index, which compares phenotype in well-watered versus drought conditions. Another candidate gene study using 1,229 SNP targeted to 540 genes identified SNPs associated with metabolite accumulation in developing floral

tissues in response to drought in maize (Setter et al., 2011). Interestingly, allelic variation in the putative aldehyde oxidase *ZmAO3* gene affected abscisic acid (ABA) level in silks of water-stressed plants. Aldehyde oxidases catalyse the oxidation of abscisic aldehyde to ABA in *Arabidopsis* (Seo et al., 2004). The association in maize suggests a causal link between the *ZmAO3* SNP and ABA response to drought.

GWAS has been less successful in targeting traits of high complexity such as yield under drought. A GWAS of 189 elite durum wheat accessions evaluated in 15 environments for drought stress tolerance showed the limitations of the association method where the inheritance of the trait is complex and shows strong genotype  $\times$  environment interactions. Most of the consistent marker-trait associations across environments were identified in medium- and high-yielding environments. The number of markers significantly associated with grain yield under drought was much lower than under well-watered conditions (Maccaferri et al., 2011). Biparental mapping is generally a more effective method for studying adaptation to stresses such as drought.

Association studies in animals use family based populations in contrast with “natural” populations used in plant studies (Laird and Lange, 2006). Family based designs incorporate the advantages of both linkage-based and LD-based approaches, using for example, a transmission disequilibrium test (TDT). Controlled crosses among diverse unrelated individuals will shuffle alleles across backgrounds to enhance the level of LD. The families generated are then used as association populations. Family based methods are robust in dealing with population admixture and structure. However, TDT requires individuals to have heterozygous ancestors in the pedigree. This method is particularly suited to tree species. TDT has been used on an association population of 961 clones from 61 families of loblolly pine (Gonzalez-Martinez et al., 2008) using 46 SNPs tested for association with carbon isotope discrimination, a trait related to water-use efficiency. However, the significant associations explained less than 1% of the phenotypic variance for this trait. Similar observations have been made in other species where only low QTL effect (percentage of genetic trait variation explained at the locus) were found by association mapping compared to the same QTL detected in biparental populations (Pasam et al., 2012).

#### 10.4 Transfer of QTL findings to breeding programs

Genotypic selection is now routinely used by many breeding programs for tracking major effect genes in their selection programs. A recent survey of wheat breeding programs identified over 50 loci that were being tracked with molecular markers; several markers were closely linked to QTL including markers for quantitative resistance to rusts and fusarium head scab, bread making quality and resistance to sprouting (Gupta et al., 2010). Where the gene

underlying the QTL is known, highly reliable or diagnostic marker can be produced and used for direct screening within the breeding population or to look for new variation or novel alleles in germplasm collections. Although many QTL have been identified for abiotic stress tolerance (see Figure 10.1) and some have been validated over years of field trials and across populations, very few loci have actually been deployed in breeding (Gupta et al., 2010). This is partly due to the fact that few QTL for abiotic stress tolerance have been validated in multiple populations and under a range of both favorable and adverse environmental conditions. In addition, many of these QTL account for only a small proportion of the phenotypic variation due to the large environment component. For breeders, the value of the QTL screen over phenotypic screening is often not sufficiently well established to justify marker based screening.

If the gene or genes underlying the QTL have been isolated, the molecular, biochemical and physiological basis for the QTL can often be determined. In these cases, additional options are open for practical application. Diagnostic markers can be developed to track specific allelic differences such as presence/absence of the gene, gene copy variation, promoter and intron variation that can modify gene expression levels, amino acid and protein length changes (Beales et al., 2007, Sutton et al., 2007, Ellis et al., 2002). Allele mining can be performed by screening germplasm collections to explore for novel sequence variants at target genes. If the QTL is shown to be involved in a pathway or process, it may be feasible to screen for variation in other genes within the pathway.

In crop improvement programs variation can come from three major sources: cultivated varieties, land races or wild relatives. The cultivated gene pool is generally the easiest to evaluate and deploy since this material is usually fully domesticated and adapted to modern agricultural practices. In most cases these lines will have appropriate height, flowering time and a spectrum of disease resistances. Traits associated with wild germplasm such as seed shattering, will have been removed. These lines can be directly evaluated in cropping environments and can usually be directly crossed into breeding lines. After a QTL study, the frequency of desirable alleles within the advanced lines of the breeding program can be assessed. If the desirable allele is present at only a low frequency, significant improvement in the target trait can probably be achieved by increasing the allele frequency within the pool of advanced breeders' lines. For example, a germplasm survey revealed that *Pup1*, a QTL conferring tolerance to phosphorus deficiency, is present in most modern irrigated rice varieties and highly conserved in drought-tolerant breeding lines and upland varieties, suggesting that *Pup1* has been selected in breeding-program in drought-prone environments (Chin et al., 2011). Increasing the frequency of this allele in irrigated rice breeding programs is likely to be of considerable benefit.

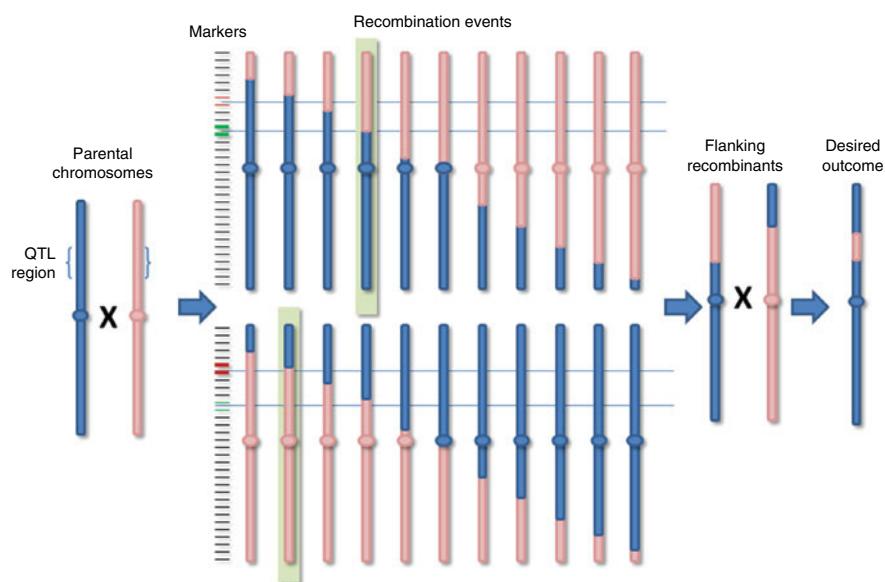
Detection of useful alleles sourced from land races or wild relatives can present significant problems. QTL studies with these classes of germplasm are complicated by the generally poor performance of such lines in modern

production environments. For example, a major component of salinity tolerance is salt exclusion from the roots (Munns and Tester, 2008). Therefore, wild germplasm can be directly screened for the ability to keep Na<sup>+</sup> out of the leaves when the plants are grown under high salt conditions (e.g., Shavrukov et al., 2010). In addition, several genes underlying QTL for salinity tolerance have been cloned, such as Na<sup>+</sup> transporters (Ren et al., 2005). This provides the opportunity to screen land races and wild germplasm for novel variants in both the coding and regulatory sequences of these genes. New alleles can be back-crossed into adapted germplasm or introduced by transgenesis for evaluation.

In cases where the QTL has not been cloned, it is possible to introgress new alleles based on flanking markers. However, introgression can be difficult if the markers are not tightly linked to the QTL, if there is a negative interaction between the QTL and the genetic background, or if linkage drag results in the transfer of unfavorable alleles at loci linked to the QTL. For example, the introgression of three QTL for boron tolerance from a barley landrace, Sahara 3771, led to a yield decrease in the resultant lines (Jefferies, 2000). In another example, a relatively short fragment of 273 kb surrounding the rice *Pup1* QTL was tracked in five rice breeding populations of BC2F3 sister lines (*Pup1+/Pup1-*). The lines were analyzed under field conditions for yield under contrasted P application. The largest positive effect of *Pup1* on grain yield was obtained in P-fertilized conditions, while significant effect were observed only in some genetic backgrounds under P-deficiency (Chin et al., 2011). In these cases careful reduction of the introgressed segment could help alleviate the introgression problems and the use of flanking markers, as illustrated in Figure 10.3, has proved a useful technique.

Most QTL studies for grain yield under drought have used susceptible varieties that are low yielding under drought as parental lines. The identified QTL may not show similar effects in high-yielding varieties and this may limit their use in crop improvement. Ideally, the effect of a QTL should be measured in several populations prior introgression. For example, the major QTL *qDTY1.1* for grain yield under rainfed conditions was identified in rice using populations from crosses between a drought-tolerant cultivar with three high-yielding varieties (Vikram et al., 2011). Its consistent effect across different genetic backgrounds meant that this locus was considered suitable for marker-assisted breeding.

Other considerations such as adoption of the new germplasm by farmers may also be important in choosing the most appropriate recipient background. A strategy adopted by the International Rice Research Institute in The Philippines (IRRI) for delivery of novel rice alleles involves backcrossing potentially useful tolerance QTL into locally well-adapted varieties that are widely used by growers from target countries. The varieties developed should have retained grain quality and local adaptation traits and therefore are more likely to be adopted by farmers (Chin et al., 2011).



**Figure 10.3** Introgression of favorable QTL allele from a donor line (pink) into a recipient line (blue) with desirable genetic background. A recombinant population is screened with markers flanking the QTL region. Lines showing a recombination event on each side of the QTL (boxed) are crossed to generate progeny containing the QTL allele of the donor line in the genetic background of the elite recipient line. The strategy is effective if there is no recombination between the actual QTL and its flanking markers, and therefore relies on markers tightly linked to the QTL. For color details, please see color plate section.

## 10.5 Issues in genetic analysis of abiotic stress tolerance

### 10.5.1 Phenotyping methods

Phenotyping methods and resources have become a rate-limiting step in the genetic analysis of abiotic stress tolerance. The use of physiologically relevant stress is essential if results generated under controlled conditions are to be related to field performance and relevant for breeding programs. The phenotypic data can also be critical for the identification of genes underlying a QTL. If the molecular and quantitative genetic data are unrelated because they were collected on different genetic material or under different experimental conditions, it will be difficult to differentiate between candidate genes in the QTL region.

Controlled growth conditions are often appropriate to test interactions of the plant with specific factors that can be readily controlled (such as temperature, humidity, light intensity, nutrient availability). However, mapping of QTL associated with traits such as yield, drought tolerance or nutritional quality is

best done under field conditions where the soil, climate and biotic stress factors are similar to the complex environments where the final varieties will be grown.

Appropriate protocols for drought phenotyping have been proposed and should be consistently applied across a gene mapping and discovery program (Salekdeh et al., 2009). Some molecular studies have used methods that have little relationship to the development of drought stress under field conditions, including desiccation of detached leaves or growth of plants in hydroponic culture with polyethylene glycol used to induce osmotic stress (Salekdeh et al., 2009). Such methods have little relevance when compared to QTL studies, which evaluate yield and yield components in the field. They serve largely as tools for studying biochemical pathways and process.

The reductionist approach of studying isolated stress has considerably increased our knowledge of tolerance mechanisms. However, interaction between multiple stresses and stress combinations should be studied to make progress relevant to field conditions. Some physiological mechanisms for stress tolerance may have opposing effects under different stresses. Plants can avoid heat stress by increasing stomatal conductance and using evaporative cooling to stay cool (Reynolds et al., 2009). Closing the stomata helps to decrease the loss of water and maintain turgor under conditions of low soil water potential (Salekdeh et al., 2009). The two mechanisms will conflict when high temperature and drought occur simultaneously, which is frequently the case. Some biochemical mechanisms might also collide under stress combination. For example, the amino acid proline is accumulated as an osmo-protectant under water stress but has a toxic effect under heat stress (Rizhsky et al., 2004; Salekdeh et al., 2009).

In devising the most suitable phenotyping procedure for assessing tolerance to a complex stress such as drought, it is important to remember that the specific physiological mechanisms of water management will vary depending on the growth conditions. For example, a plant growing in a field needs to deal not only with the climatic and edaphic factors but it must also successfully compete with its genetically identical or similar neighbors. A key adaptive trait for crop plants is their ability to grow and survive as a large community. In maize, it has been suggested that the key factor underlying the large yield increases achieved over the past few decades has come through improvements in planting density (Duvick, 2001). The key stress imposed under these conditions is competition with other plants for resources. Under water or nutrient limited conditions the ability of the whole plant community to tap water or nutrients becomes critical. Clearly, pot and glasshouse based experiments are unable to simulate these types of stresses and are unlikely to yield meaningful QTL for stress tolerance under cropping conditions.

Key to the interpretation of QTL identified using field data is the detailed record of environmental conditions in which experiments are conducted. For example, canopy temperature can be used as a selection trait in improving

drought tolerance since it provides a measure of plant transpiration rate and indicates the ability of the plants to tap water. Genotypes with cooler canopy extract more water from deep in the soil profile, which represents a drought tolerance trait in environments where there is a deep soil and moisture disappears down the soil profile as the season progresses (Reynolds et al., 2009). Plants can be selected using non-destructive methods such as infrared thermometry to increase genetic gain for yield under drought (Reynolds et al., 2009). However, this trait is only relevant in conditions where water is available at depth. A warmer canopy, associated with lower transpiration, might be more relevant for yield improvement in soils with limited moisture in the soil profile. In a study conducted in loblolly pine, significant associations between carbon isotope discrimination and drought-inducible genes could reflect adaptation to water-limiting conditions (Gonzales-Martinez et al., 2009). However, the water table at the phenotyping sites indicated that water was not limiting for growth during the experiment. The detected associations might actually have been assessing differences in photosynthetic activity during transient periods of water deficit that occurred during peaks of temperature.

#### *10.5.2 Selection of germplasm for genetic analysis*

The species and traits to be assessed clearly impact on the genetic approach to be taken. The choice of genetic material depends first on the type of analysis to be conducted, standard QTL mapping versus association or LD mapping. Association mapping is sensitive to the structure of the populations of lines used in the analysis. As noted above this is particularly significant for inbreeding species such as rice, wheat and barley, where the populations tend to be highly structured around heading date, which will impact yield related traits (Huang et al., 2010; Pasam et al., 2012). The problem can be partially alleviated by assessing the size and structure of population used for genetic analysis. LD and structure can be first measured in large association panels of several thousand accessions. Then a subset of germplasm might be chosen with a structure level that can be more easily controlled for association study. The resolution of the association mapping study will also be influenced by the type of germplasm used. The extent of LD can be very extensive in inbred crops meaning that only very crude mapping is possible within the cultivated gene pool. However, far more recombination events are likely to have separated land races and these will show a far narrower LD range than cultivars. Still higher resolution is possible if wild relatives of crops plants are used in the association mapping panels. The approach of using different germplasm pools at different stages of an association mapping study was proposed by Caldwell et al. (2006). The complexity level of the targeted trait is also important. The relatively low power of association analysis might be sufficient for a trait under simple genetic control such as leaf Na content under salt stress. In contrast, genetic mapping with

biparental populations is generally seen as more appropriate for complex trait like yield under stressed conditions (Maccaferri et al., 2011).

Biparental crosses often use genetically distant parents in order to maximise the polymorphism in the descent and therefore the ease of map construction. The use of highly diverse parents will also often expand the phenotypic variation between the parents and the individual progeny lines and this will facilitate phenotyping. However, there are significant disadvantages in such an approach when the aim is to map complex traits under variable conditions and to produce information that will be relevant to practical breeding programs. The agronomic and phenological characteristics of the genetic material will often influence the performance of the lines under stress. In most cases these and related traits will have already been optimized in breeding lines and consequently, a QTL or LD analysis will probably only identify loci that have already been identified. Genes of strong effect have usually been optimized during domestication or during the breeding process and they will hide the effect of novel loci, which are the real target of the QTL mapping exercise. This problem has arisen in several studies of the genetics of drought tolerance where loci controlling phenology, largely maturity, height and tillering, have been identified as major components of the drought response. For example, most QTL in yield in wheat under drought collocate on chromosomes 2, 4, and 5 with main loci controlling plant height (Reduced height *Rht*) and maturity (Vernalization *Vrn* and Photoperiod *Ppd* genes; Bennett et al., 2012a; Peleg et al., 2009; Wu et al., 2010). Early flowering is a mechanism of avoidance particularly in environments with terminal drought stress (Turner, 1979). Small plants with few tillers can show higher water-use efficiency (WUE) than tall mult\tillered plants. Since the genotypic variation of WUE is mainly driven by variations in water use rather than by variations in plant assimilation, WUE traits will often map to plant height loci instead of drought tolerance per se (Blum, 2005). In well-developed agricultural regions, crop flowering time and plant morphology have already been optimized by breeders to matches the environment (Passioura, 2007). Consequently, the chances of discovering new loci are low when one uses genetic material unadapted to local environmental conditions. It is therefore recommended to use several large populations based on parents adapted to targeted environment but differing in stress responses.

One approach to tackling the phenology problems is to fit the maturity time as a co-variate in the analysis of the phenotypic trait prior to QTL mapping (Bennett et al., 2012b) or by using the QTL controlling flowering time as co-factors in the QTL analyses (Sabadin et al., 2012). However, adjustment of data for flowering time cannot account for the frequent drought scenario where late flowering lines experience stronger stress than earlier flowering lines (Bennett et al., 2012b). Selecting parents that do not differ in their flowering dates may not be sufficient to deal with this problem. As flowering is a multi-gene trait, various combinations of alleles at different loci may lead to the

same flowering time in parental lines but show segregation in the progeny. The best approach is to design populations with a short range of flowering time by eliminating lines in the early or late flowering tail (Pinto et al., 2010). However, this will fix lines at specific regions of the genome with the consequence that QTL in these regions will be missed. For example, some QTL for WUE in wheat were actually associated with variation in heading date and plant height (Rebetzke et al., 2008a). Fixing parental lines for known traits can be applied to both maturity and plant morphology by eliminating plants that are outside an acceptable height range. Where the genes controlling the developmental phenotype are already known, lines can be chosen based on their genotype for traits such as flowering time and plant height.

#### *10.5.3 Stability of QTL across environments*

Pathways and gene networks involved in controlling responses to abiotic stresses overlap. For example, about 40% of drought or salt stress inducible genes are also activated by cold treatment in rice (Shinozaki and Yamaguchi-Shinozaki, 2007). It is therefore expected that some QTL will show significant effects across environments. However, many QTL identified for tolerance to abiotic stress are not “stable” in different growth conditions. Due to the strong G×E interactions, a QTL can have positive, null or negative additive effects depending on the stress conditions (Collins et al., 2008). In wheat a substantial proportion (22.8%) of total genotype-by-environment interaction could be explained by the interactions between QTL for grain yield with specific climatic co-variables such as latitude, rainfall and temperature (Kuchel et al., 2007). Even simple biochemical traits can show strong G×E interactions. For example, 7 QTL out of 10 for stem water-soluble carbohydrate content interacted with the environment in wheat (Yang et al., 2007). These interactions greatly complicate genetic analysis since the residual variance can account for most of the phenotypic variation and hide the effect of QTL. It is not unusual for abiotic stress mapping work to find that only a small proportion of the total variation for the trait is due to genetic effects with the remainder due to the environment. In consequence, the trait will show low heritability across environments. Instead of searching for stable QTL across environments, a detailed understanding of QTL-by-environment interaction is necessary to map or clone QTL and deploy them in breeding.

Many abiotic stress response phenotypes are determined by the interactions of genes and environment and these interactions can have a major effect on expression of QTL. There are two main methods for dealing with this problem. One method is to fix the environment using growth room, glasshouse or other systems. If the environment can be fixed then the observed variation between lines should be largely due to genetic differences. The second method is to screen the germplasm across a large number of environments and then include components of the environment in the genetic analysis.

The most obvious strategy to increase the heritability and control some of the G×E effects is to minimize the effects of environmental variability. Environmental variation can be decreased by growing plants under controlled conditions in a glasshouse, growth room, or hydroponics system to reproduce experiments under similar conditions, as discussed above. Construction of high-throughput phenotyping platforms facilitates the study of large genetic populations (Berger et al., 2010). Controlled conditions also allow single stresses to be produced rather than the multiple stresses that often occur under field conditions. This reductionist approach enables dissection of specific mechanisms of tolerance. For example, nutrient toxicities in the soil can restrict root growth and impair water uptake leading to drought stress and deficiencies for other nutrients. This makes soil or field-based screening difficult. In some cases hydroponics provides an alternative method for inducing a specific stress. Hydroponics systems have been used effectively for mapping QTL for tolerance to salinity, boron and aluminum and for studying the genetics of nutrient and micronutrient efficiency (Genc et al., 2007; Ma et al., 2005; Sutton et al., 2007). However, there are examples where phenotypic differences could not be observed in controlled conditions. For example, no contrasting phenotype was observed for *Pup1* NIL in hydroponic system and lines had to be screened in a soil assay (Chin et al., 2011). Moreover, plants of large stature and natural populations of trees may not be amenable to evaluation in controlled environments and their genetic study will rely on field trials.

Under field conditions it may be feasible to control specific aspects of the environment. For example, rainout shelters or irrigation can be used to simulate various levels of water availability in field and polytunnels with closed sides can increase the air temperature to simulate heat stress. In many cases, the only option is to measure accurately the environmental variables in the field over the crop cycle using weather stations and soil analysis. Careful experimental design and statistical analysis of phenotyping data can alleviate some issues related to residual variation (van Eeuwijk et al., 2010). Models of genetic dissection of quantitative traits should take into account as many environmental parameters as possible (Cooper et al., 2009). The G×E effect can be regressed on the environmental characterization to produce stress indices and create eco-physiological QTL models.

The choice of parameters used to assess stress can have a major impact on the stability QTL across studies. Integrated traits, such as grain yield and weight, measure the impact of the stress over the full life cycle of the plant (Reynolds et al., 2009). It might be difficult to observe the same QTL in other trials under regimes where the stress level can vary over time, for example a cyclic drought stress. Time or treatment can be replaced by dynamic variable like fraction of soil water or transpiration. These variables can be used in the QTL analysis to identify regions of the genome associated with plant responses along gradients of stress levels and developmental stages (Salekdeh et al., 2009).

## 10.6 Current directions of quantitative genetics for abiotic stress tolerance

### 10.6.1 Physiological components of abiotic stress tolerance QTL

For crop plants, the key trait or phenotype of interest is yield. Yield can be a direct reflection of biomass or the proportion of biomass that is converted to the harvestable commodity; for grain this is the harvest index. As noted above, yield is the integration of many physiological processes over time and in environments that may be fluctuating or highly variable. Therefore a QTL mapping study based around the assessment of yield or yield components under abiotic stress will actually be based around an array of different responses. The analysis can be simplified by breaking down the stressed environment to look into different aspects as described earlier. Alternatively, the response to stress can be broken down into physiological components.

There are several advantages in dissecting a complex trait into its components for QTL cloning. By mapping the subtraits as QTL, colocation with QTL for the major traits can be sought. If QTL overlap, it might be easier to phenotype large recombinant populations by tracking the subtrait. For example, drought stress affects development of male floral organs in maize, delaying the silk growth that can be measured as anthesis-silking interval (ASI). ASI often shows a strong negative correlation with yield in segregating lines and has been used successfully as a selection criterion in breeding programmes and as a drought tolerance trait for association mapping (Bolaños and Edmeades, 1996; Setter et al., 2011). If the subtrait can be measured in high-throughput phenotyping facilities or at early developmental stages, it can considerably accelerate the map-based cloning of the genes underlying the QTL. This strategy still presents the risk of tracking a gene that might co-segregate with the target without being responsible of the trait variation. It is therefore recommended to phenotype representative recombinant lines with the original trait in targeted environment.

A detailed analysis of the components of traits can also support the identification of candidate genes underlying the QTL by pointing out specific physiological mechanisms where genes may already be known in other species. The *qFSR4* QTL for drought tolerance in rice was found to co-segregate with flag leaf width and spikelet number per panicle (Ding et al., 2011). The 38-kb QTL region contains the *NARROW LEAF 1* gene known to control leaf width, vein patterning and polar auxin transport. This is a strong candidate for the *qFSR4* gene.

Reducing a complex trait into its elements for genetic mapping can be combined with the development of mathematical models to explain the relation between the components and the main trait (Reymond et al., 2003; Tardieu, 2003). This can help describe a complex trait independently of environmental

conditions by calculating the dynamic variables (Chenu et al., 2009). For example, total leaf area can be measured by automatic imaging during plant growth under different abiotic stress conditions. Leaf growth rate over time can be calculated in both stressed and non-stressed conditions for all lines. The response of leaf growth to stress can then be represented as a linear variable and a stable trait for the QTL search. The method has been successfully used to study maize under water-limited conditions. The response of leaf size to temperature and water deficit in maize has been broken down into duration of development and leaf growth rate. The slope of leaf elongation rate response in 100 RIL enabled identification of environmentally independent QTL (Reymond et al., 2004).

#### *10.6.2 Integration of physiological components into abiotic stress tolerance QTL*

Modeling can also be used for *de novo* QTL searches by integrating data for as many environmental and traits parameters as possible on a mapping population in multitrait and multienvironment QTL analyses (reviewed by van Eeuwijk et al., 2010). The basic quantitative genetic model states that phenotype = genotype + error. The aim here is to extract as much information as possible from the experiment to increase the phenotypic variance explained by genetics and decrease the error.

Multienvironment trials are used to assess performance of genotypes across a range of sites and years. There are different options for the analysis of multienvironment trials. The two-step analysis first identifies clusters of environments, each with a distinctive growing season pattern. The second step averages the genotype performance across sites within each cluster for QTL mapping (Mathews et al., 2008). The approach has been used in wheat to identify QTL in clusters of environments with different rainfall patterns (Bennett et al., 2012a). Another approach uses mixed models to evaluate QTL-by-environment interaction (Mathews et al., 2008). The model is chosen to recognise that stress may have decreased the genetic variance and that pairs of environments with significant genetic correlation show similar growing conditions.

The mixed model approach can be extended to QTL mapping of multiple traits (van Eeuwijk et al., 2010). Instead of searching QTL for yield only, each yield component, such as grain size, number of seed per spike and number of tillers per plant, can be integrated into a single model that is used for QTL identification. This method increases the statistical power of the analysis by increasing the number of phenotypic measurements used and decreasing the residual error. This type of analysis increases the likelihood of detecting significant new QTL, which would be hard to reliably identify, due to the large unexplained variance, if integrated data, such as yield, is used.

### 10.6.3 *Meta QTL*

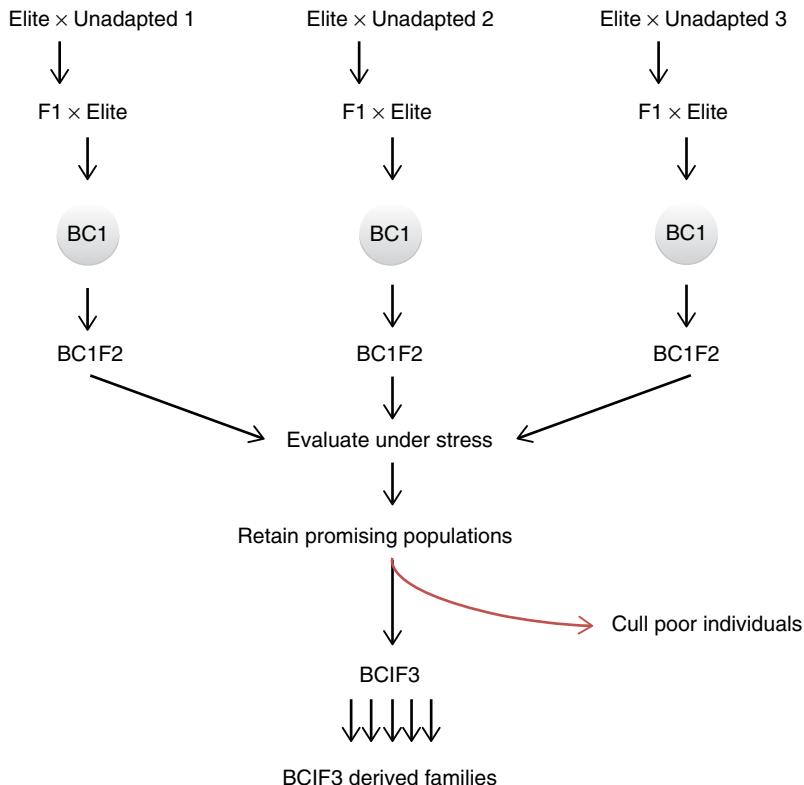
Extensive QTL studies have been conducted for a wide range of abiotic stress tolerance traits in many of our major crops. This information represents a substantial resource. As the number of studies grows it becomes feasible to reanalyze both the new and old results in combination to explore commonality and identify QTL or genomic regions that reappear in different studies, with different populations and often with different phenotyping strategies. Interest is growing in combining information from various sources into a single database that will permit data mining. These approaches allow researchers to increase marker density surrounding key QTL and build models around the relationship between traits, environments and germplasm. The reliability of models around putative mechanisms and candidate genes underlying a QTL can also be supported through these approaches. For example, the QlicRice database is a web-interface tool that supports the search for abiotic stress responsive QTL and identifies the related genome sequence in rice (Smita et al., 2011).

The underlying concept is to use meta-QTL analysis to compare separate QTL studies and combine them in a single genetic map (Swamy et al., 2011). Several new software packages have been developed to support these analyses. Biomercator software permits comparison and integration of genetic maps into a consensus map based on common molecular markers and then superimposes QTL from different studies onto the map (Arcade et al., 2004). Where QTL co-located, it is probable that they represent the same locus. The concept of meta-QTL is attractive since it makes use of multiple populations and trials and gives an indication of the total number of loci controlling a trait and also permits estimation of the stability of a QTL across studies. However, the environmental conditions in which abiotic stress tolerance QTL were identified in each study must be considered in interpreting the overall relevance of these types of studies.

### 10.6.4 *New population designs for QTL mapping*

The degree of genetic diversity captured within a breeding program varies between programs and species. As noted above, mapping in highly diverse or unadapted germplasm is problematic for complex traits and often only reveals known loci such as those associated with plant phenology. Consequently, the genetic diversity available in most QTL studies is narrow. Nevertheless markers developed for such regions can be deployed in breeding programs to increase the frequency of key desirable QTL alleles in advanced lines.

Allelic diversity present in a mapping population can be expanded by using complex crosses involving multiple parents. Multiparental populations combine the advantages of high genetic variability of LD studies with the power of QTL analysis. Multiparent Advanced Generation Inter-Cross (MAGIC) populations are created by intercrossing multiple parental lines to recombine the genetic background and then self-crossing the progeny to fix the recombinant loci in RIL



**Figure 10.4** Strategy for constructing Nested Associated Mapping populations incorporating exotic germplasm into elite reference lines. The elite line is intercrossed with diverse unadapted accessions. Two or three elite lines can be selected to represent different favorable genetic backgrounds. The diversity set can consist of 30–50 accessions that might be chosen from environments subject to the relevant climate for the target stress tolerance. The progeny are backcrossed once to reduce the exotic background. BC1F2 lines showing undesirable phenotypes, such as aberrant plant morphology or phenology that would confound the phenotyping of stress tolerance, are eliminated. One or two rounds of selfing will generate a set of backcross families that can be genotyped and phenotyped for genetic studies.

(Kover et al., 2009). However, MAGIC populations are still quite limited in the extent of diversity that can be included, usually six to eight parents. The problems raised above concerning diverse plant phenology are likely to be exacerbated in MAGIC populations and this can seriously mitigate against the use of such populations for the assessment of abiotic stress tolerance QTL. An alternative approach uses Nested Association Mapping (NAM) populations that are made by crossing diverse lines with the same reference line to develop sets of related mapping progeny (RIL; Yu et al., 2008). NAM has the advantages that some RIL subsets can be chosen for phenotyping to help deal with the phenology problem (illustrated in Figure 10.4). The study of maize kernel composition

in NAM and inbred association panels showed that NAM populations are also valuable for GWAS (Cook et al., 2012). Inter- and intrachromosomal LD among SNP in the NAM founders was reduced during population development through random chromosome assortment and recombination, enabling the identification of some causal genes. The majority of the associations located within annotated genes were elements that regulate complex molecular pathways such as transcription factors, zinc finger binding proteins, kinases and the histone H1 variant H1.2.

Another approach involves combining genetic mapping and association studies by merging linkage and LD datasets of independent populations (Lu et al., 2010). The application of integrating linkage and LD data in maize was shown for the ASI trait under drought where several novel QTLs were identified. The use of haplotype-based analysis can also increase the significance of QTL detected and the phenotypic variation explained by the QTL. Two candidate genes encoding a SET domain protein and an aldo/keto reductase showed significant haplotype associated to ASI under drought (Lu et al., 2010).

The high genetic diversity that exists in germplasm collections is currently underused for abiotic stress tolerance. Geographic information systems can help identifying target environments and local germplasm that may contain novel alleles for tolerance to abiotic stress (Salekdeh et al., 2009). Although there are several issues in using such exotic germplasm, which are often genetically unstable (landraces are often genetic mixtures) and unadapted to modern production system, there have been several studies where highly diverse parents have been successfully used in QTL mapping (Peleg et al., 2009; Miura et al., 2011; Kazmi et al., 2012). As commented above, phenology and morphology have a large effect on yield under stress and therefore may hide the abiotic stress response. The problems of screening unadapted germplasm for key adaptive traits can be addressed by diluting the genomic contribution of the unadapted line through several cycles of backcrossing into elite germplasm prior to population development. These populations, known as advanced backcross populations, have been successful used to map quite complex traits such as yield (von Korff et al., 2006) and leaf wilting under drought (Sayed et al., 2012). An advantage of this approach is that lines that may have inappropriate phenology can be removed from the population during the backcrossing. The lines generated can also be used to prepare a series of recombinant chromosome substitution lines (RCSL), where only defined regions of the unadapted genome are present in an elite or adapted background (del Pozo et al., 2012; Mullan et al., 2009; Yasui et al., 2010). RCSLs allow evaluation of single regions and can be directly fed into breeding and selection programs to accelerate the utilisation of the novel germplasm (Mengistu et al., 2012). New NAM populations are also being constructed to introduce exotic genetic background into cultivars. For example, the Triticeae-CAP project in USA is creating wheat and barley NAM populations from crosses between modern lines and landraces

(<http://www.triticeaecap.org/about/>). At F2, plants are screened for dwarfing genes and lines carrying tall alleles are culled. F4 plants are then screened for flowering under a 12 h photoperiod. Families that are photoperiod insensitive and semi-dwarf are then selfed to create RIL subpopulations. This strategy increases the genetic diversity of the population for gene discovery without compromising the minimum agronomic performance required in modern agriculture.

### 10.7 Conclusion

Crop improvement is dependent upon genetic variation to achieve genetic gain through selective breeding. The rate of genetic gain in breeding programs can be increased by either extending the amount or nature of variation available for selection or by accelerating the selection process to produce varieties more rapidly.

Traditional plant breeding has been based on phenotypic selection. This has been very effective for yield in adverse environments but has suffered from several limitations. The selection programs have frequently focused on genes of major effects since these usually give a clear phenotype, such as race-specific or single gene disease resistances. For additive gene effects, large populations can be used to give a reasonable probability of combining desirable alleles at multiple loci into a single line. For complex traits with little or no clear genetic understanding, breeders can treat the germplasm in their breeding program as a population and endeavor to shift the mean population response in a particular direction, usually toward higher yield in stressed environments. Tolerance to abiotic stresses such as drought, heat, and cold stress have proved difficult to analyze at the genetic level due to both the complexity of the genetic control and the poor reliability of phenotyping techniques.

The precision of phenotyping for the trait of interest plays a major role in QTL mapping and gene isolation. The whole area of high-throughput phenotyping, or phenomics, has developed into a highly active research field. For intensively bred species, such as the major cereal crops, many key traits have already been optimized, and future gains may depend on minor shifts in pathways or processes. We know that a key characteristic of our crops is their adaptation to growth in mono-culture and in a tightly packed community. Consequently, tolerance to abiotic stress shown by plants grown as a crop can be completely different to tolerance when grown in isolation in a pot.

Currently, the main application of genetic analysis is through a range of marker-assisted selection strategies. These strategies have been highly successful and molecular markers are now used routinely in most well-resourced breeding programs. Molecular plant breeding is built around predictions of phenotype based on genotypes. The reliability of these predictions is derived from measurements of phenotypic performance in large segregating populations and

then application of statistical procedures based on quantitative genetic theory. Analysis of complex abiotic stress tolerance traits has been supported by developments in statistical and modeling techniques for phenotypic data that are generated from field and controlled environmental studies.

As our knowledge of genome structure, function, and behavior grows, and the technologies for genotyping continue to be more accessible, a whole new series of screening techniques is likely to develop. By coupling these capabilities with improved statistical and mathematical models and modern computing power, further genes and alleles controlling abiotic stress tolerance will be available as diagnostic markers. The efficiency of selection and the design of new population structures will also expand. This is already happening with genome-wide selection. The next major challenge will be the integration of the full omics datasets into genetic mapping, modeling, population structure, and selection strategies.

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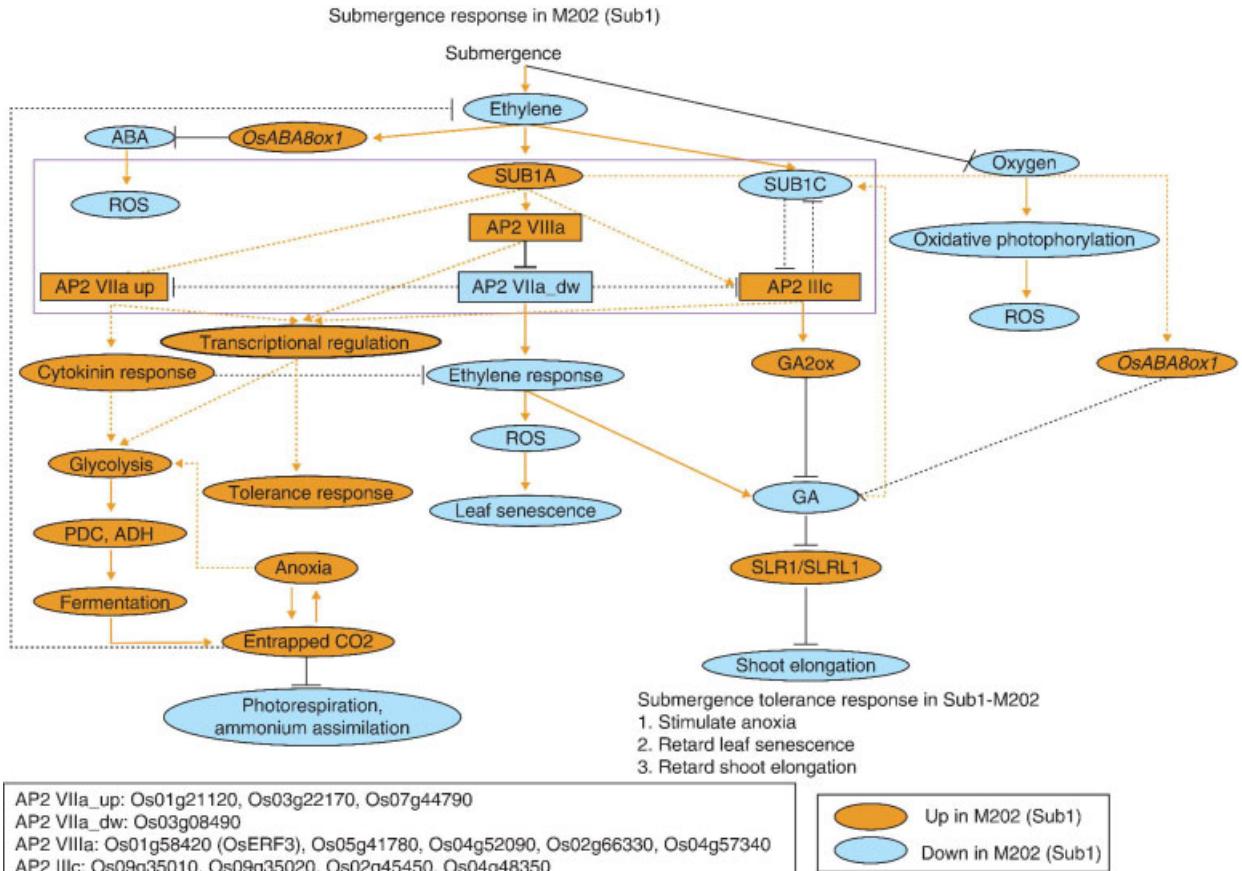
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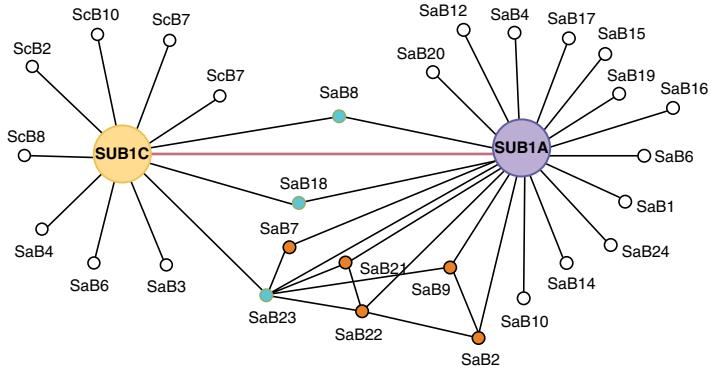
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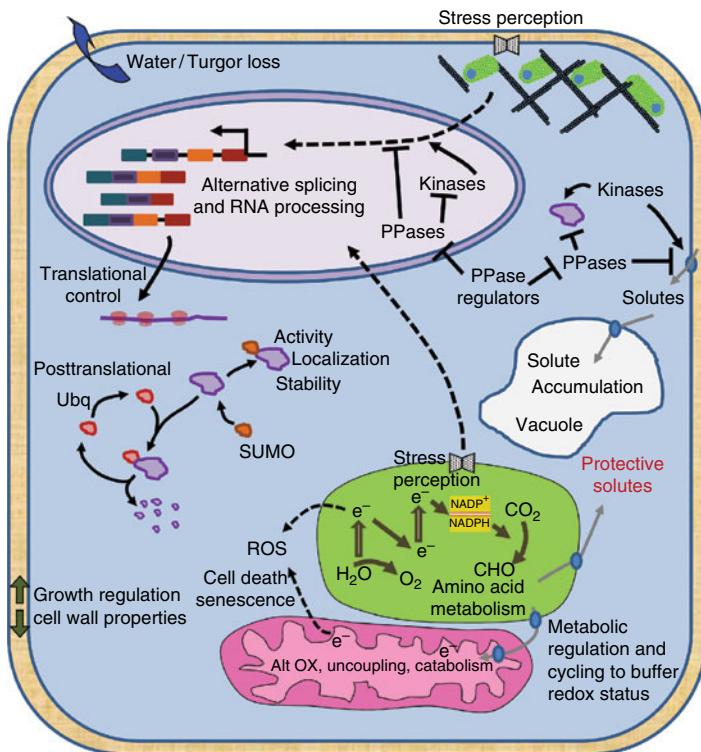
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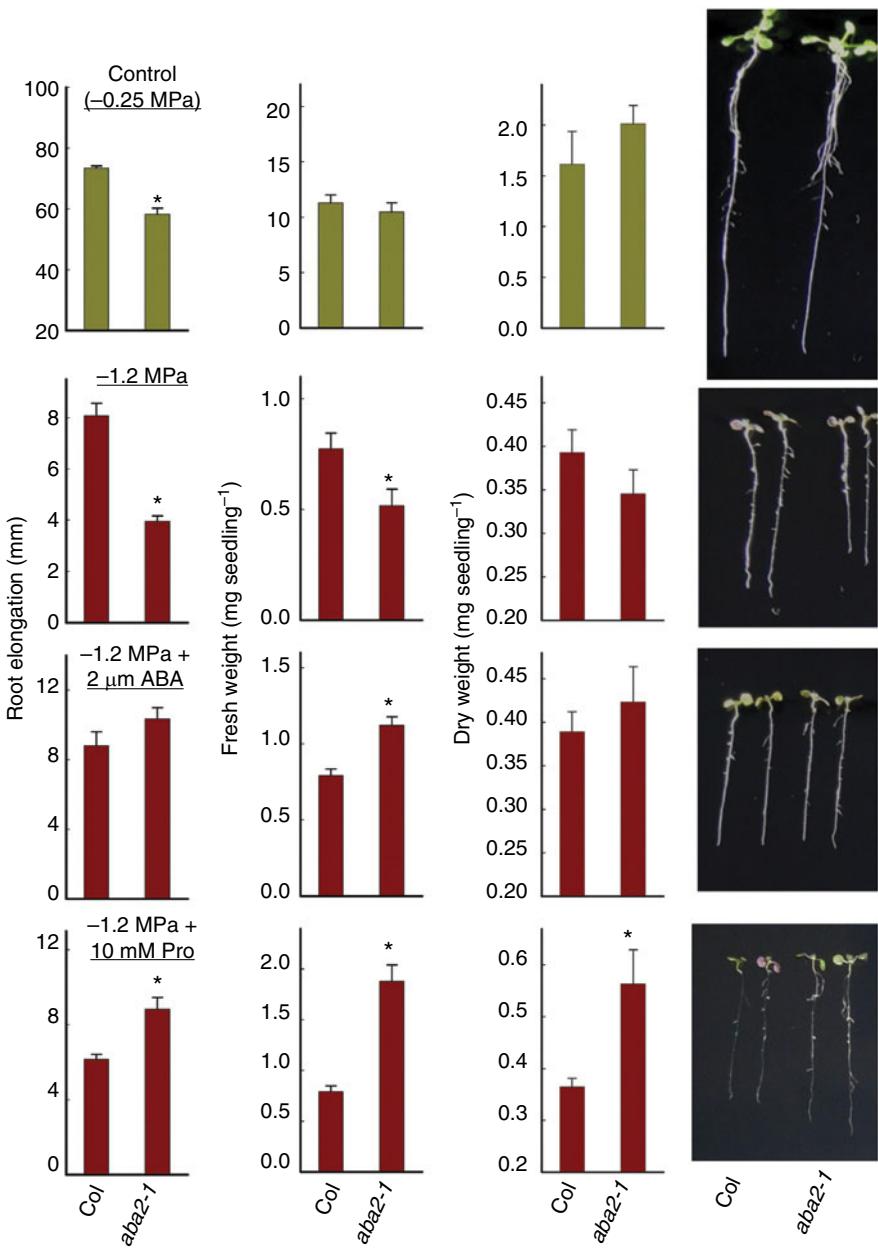
**Figure 1.1** SUB1A-mediated submergence tolerance responses revealed by integrating omics tools (Jung et al., 2010). Orange boxes indicate events upregulated in M202(Sub1) after submergence, and blue boxes indicate events downregulated in M202(Sub1) after submergence. Several of the AP2/ERF TFs are associated with submergence tolerance response.



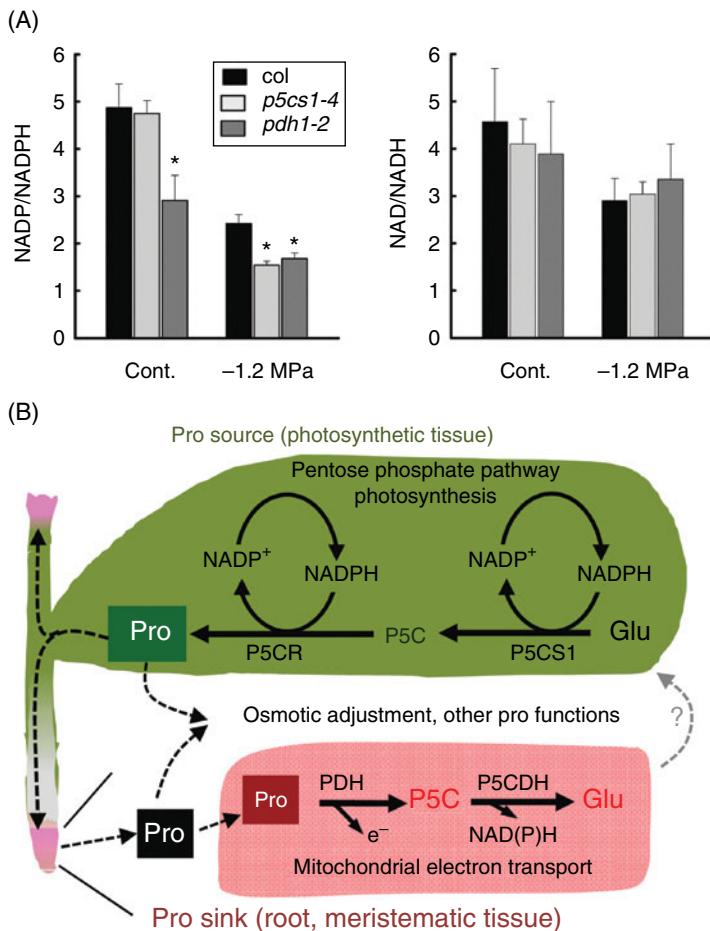
**Figure 1.2** The rice SUB1A/SUB1C interactome. The interactome map represents 28 proteins identified from high-throughput Y2H screening using SUB1A and SUB1C as baits. Proteins in blue represent interactors with both SUB1A and SUB1C (Seo et al., 2011).



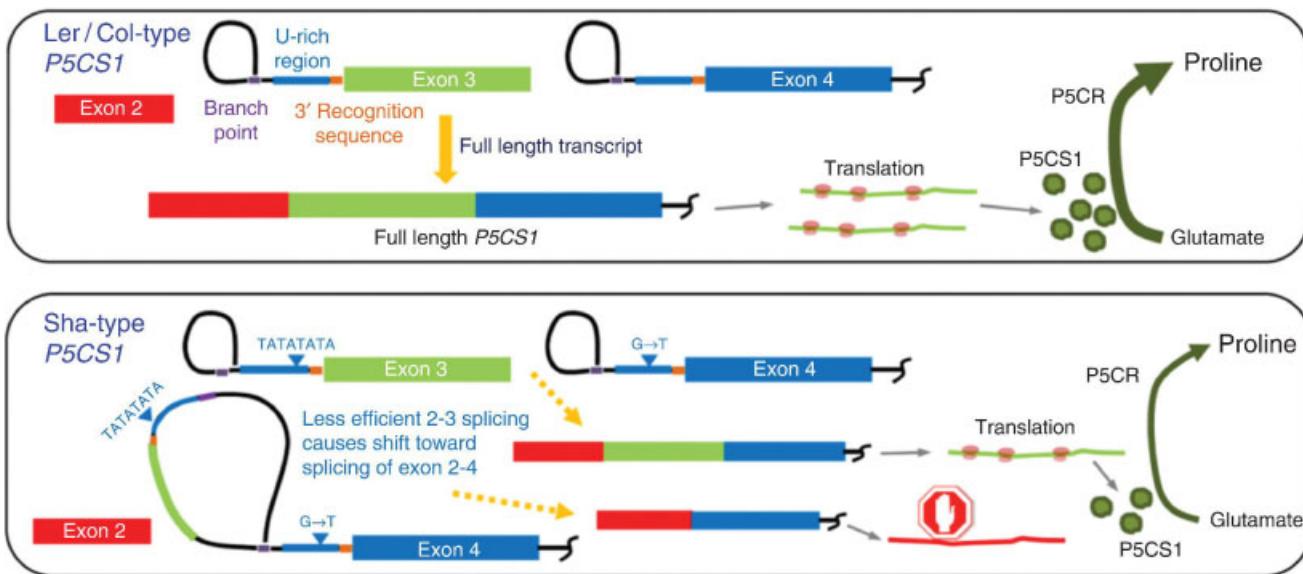
**Figure 2.1** Summary diagram of regulatory mechanisms and drought tolerance related cellular changes discussed in this chapter. Sensing and signaling of drought stress (top half of diagram) begins with an initial perception of water loss or loss of turgor that occurs via unknown mechanisms but may involve plasma membrane or organelle localized sensors and cytoskeleton changes. Downstream signaling involves the action of kinases and phosphatases (PPases), which can have opposing effects on a range of targets including transporters, transcription factors, and other proteins. Alternative splicing, selective translation, and protein modification by attachment of ubiquitin (Uqb) or small ubiquitin-like modifier proteins (SUMO) pathways generate additional changes in protein content and activity important for drought response. The regulator events lead to specific cellular changes related to drought tolerance (bottom half of diagram) including solute uptake and synthesis of protective solutes, changes in chloroplast and mitochondrial metabolism to buffer cellular redox status, and control of solute synthesis, as well as changes in cell wall properties. Adjustment to photosynthetic metabolism as well as mitochondrial alternative oxidases and uncoupling proteins act to dissipate reducing potential when necessary and prevent reactive oxygen species (ROS) formation. Even with this, the mitochondria and chloroplast are also sources of ROS, which, along with other specific signals, determine the time and extent of cell death and senescence during drought.



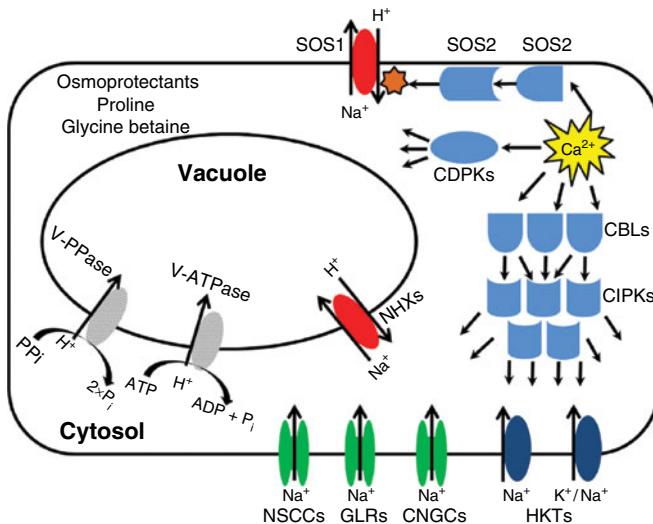
**Figure 2.2** Regulated changes in growth at low water potential: stimulation of *aba2-1* by proline. Seedlings of Arabidopsis Columbia wild type or the ABA-deficient mutant *aba2-1* were transferred from control media to low water potential (-1.2 MPa) on polyethylene glycol-infused agar plates. Measurements of seedling growth (root elongation, fresh weight and dry weight) were performed 10 days after transfer. Transpiration is low in this experimental system, thus dehydration avoidance through stomatal closure has a relatively minor role. Because of this, *aba2-1* differed little from wild type in the high water potential control (top panels). At low water potential *aba2-1* was inhibited in root elongation, consistent with previous observations that ABA is required to promote root elongation at low water potential. Fresh weight and dry weight, which indicate shoot growth as well as root growth, were slightly decreased or unchanged. Adding a low level of ABA complemented the reduced growth *aba2-1*. However, adding the compatible solute proline had a more dramatic effect of increasing fresh weight and dry weight by 1.5 to nearly 2-fold (bottom panels). Thus, when the growth restraining effect of ABA was removed, added proline could greatly stimulate shoot growth at low water potential. Asterisks (\*) indicate significant differences between wild type and *aba2-1*. Pictures show representative seedlings from a series of replicated experiments. Figure is modified from Sharma et al., 2011.



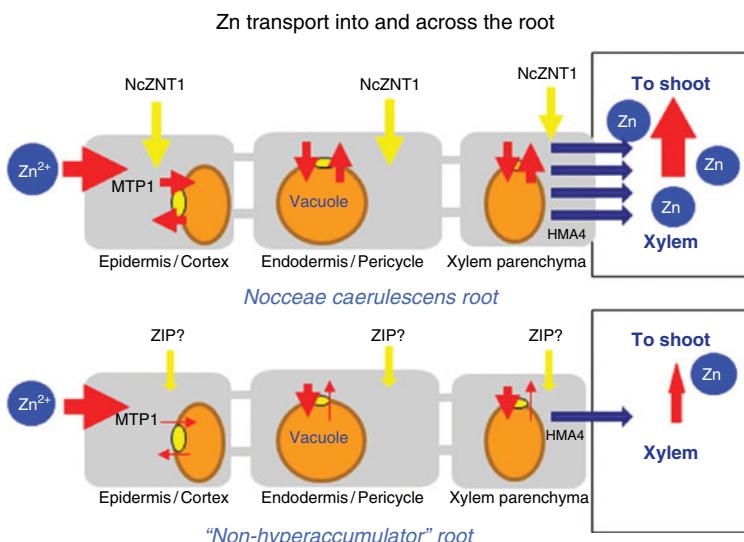
**Figure 2.3** NADP/NADPH ratio as an indicator of redox status of plants at low water potential and the role of proline metabolism in buffering NADP/NADPH. (A) Seven-day-old *Arabidopsis* seedlings of Columbia wild type, *p5cs1-4* (lacking expression of *D<sup>1</sup>-pyrroline-carboxylate synthetase1*, which encodes a stress-induced enzyme of proline synthesis) and *pdh1-2* (lacking expression of the proline catabolism enzyme *proline dehydrogenase1*), were transferred to control (-0.25 MPa) or low water potential (-1.2 MPa) media and pyridine nucleotide levels measured 96 hours later. Low water potential caused a decline in NADP/NADPH, indicating that a reduced supply of NADP in the chloroplast may increase the potential for reactive oxygen production or inhibition of photosynthesis. In contrast, NAD/NADH was less affected by low water potential. *p5cs1-4* and *pdh1-2* both had a greater decline in NADP/NADPH than wild type at low water potential. *pdh1-2* also had decreased NADP/NADPH in the unstressed control treatment. These data indicated a role of proline metabolism in controlling NADP/NADPH in addition to other protective roles of proline as a compatible solute that accumulates during drought. (B) Model of proline metabolism and its roles in regenerating NADP in photosynthetic tissue and supplying energy and reductant to meristematic and growing tissue to support continued growth during low water potential. In photosynthetic shoot tissue, synthesis of proline is relatively high, indicated by induced expression of *P5CS1* and repressed expression of *PDH1*. Because of its probable location in the chloroplast (reviewed in Verslues and Sharma, 2010; Szabadó and Savoure, 2010), proline synthesis can serve to regenerate NADP as an electron acceptor to avoid reactive oxygen production and inhibition of photosynthesis. In growing tissue, especially the root apex, *PDH1* expression is induced rather than repressed by low water potential, and proline serves as an alternative respiratory substrate to sustain growth. Additional investigation of the localization and regulation of *P5CS1* and *PDH1* and mechanisms of proline transport are needed for further understanding of how proline metabolism promotes drought tolerance. Both (A) and (B) are modified from Sharma et al., 2011.



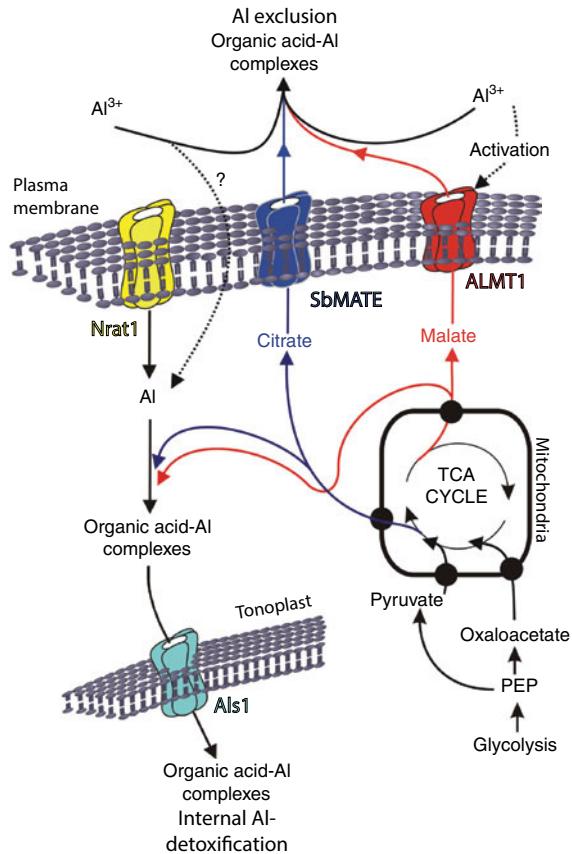
**Figure 2.4** Intron sequence polymorphisms lead to varying rates of *P5CS1* alternative splicing and varying capacity for proline accumulation among *Arabidopsis* accessions. The *Arabidopsis P5CS1* transcript can be alternatively spliced into two transcripts: a full-length transcript that encodes *P5CS1* protein and a transcript missing exon 3 (exon 3-skip *P5CS1*) that cannot be translated to *P5CS1* protein. Most accessions produce only a low level of exon 3-skip *P5CS1* and can accumulate high levels of proline in response to low water potential stress. However, in some accessions up to half of the *P5CS1* transcript is the non-functional exon 3-skip *P5CS1*. These accessions have reduced levels of proline accumulation. Accessions having high levels of exon 3-skip *P5CS1* have extra TA repeats in intron 2 and a specific G to T transversion in intron 3; these are sufficient to drive high levels of alternative splicing. The percentage of exon 3-skip *P5CS1* is correlated with temperature and rainfall conditions from the accessions sites of origin, indicating the *P5CS1* and proline synthesis are under selection as part of local adaptation of *Arabidopsis* accession to climates differing in rainfall and temperature patterns. Figure is modified from Kesari et al., 2012.



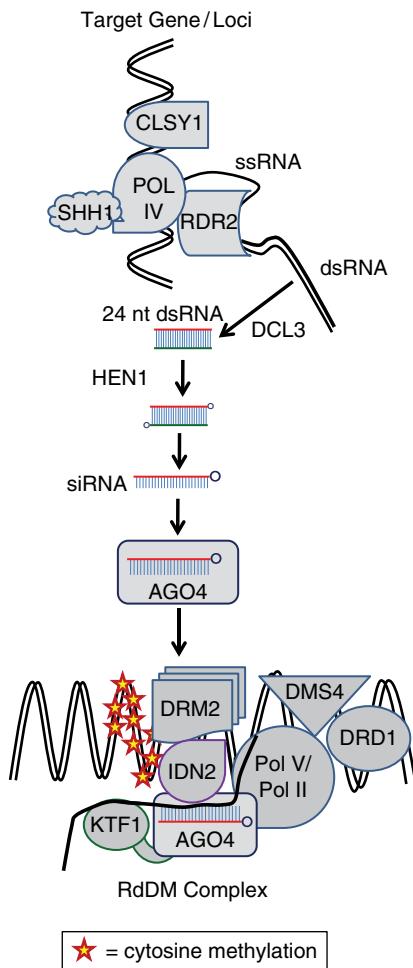
**Figure 6.3** A selection of well-characterized cellular processes involved in salt tolerance.  $\text{Na}^+$  can passively enter into plant cells through ion channels, such as non-selective cation channels (NSCCs), glutamate receptor-like channels (GLRs), cyclic-nucleotide gated channels (CNGCs), and the  $\text{Na}^+$  or  $\text{K}^+/\text{Na}^+$  HKT transporters.  $\text{Na}^+/\text{H}^+$  antiporters like SOS1 or NHXs transport  $\text{Na}^+$  out of the cell or into the vacuole and require the establishment of an  $\text{H}^+$  gradient by membrane bound PPases and ATPases. SOS2 and SOS3 regulate the activity of SOS1, ensuring the transporter is active during salt stress—SOS3 and SOS2 are members of the CBL/CIPK calcium signalling pathway. Both CDPKs and CBLs/CIPKs are involved in the  $\text{Ca}^{2+}$ -dependent salt stress signalling pathways and regulate the cell response to salt stress by post-translationally modifying a variety of proteins, such as transporters. Finally, salt stressed cells accumulating high concentrations of  $\text{Na}^+$  produce proline and glycine betaine as osmoprotectants.



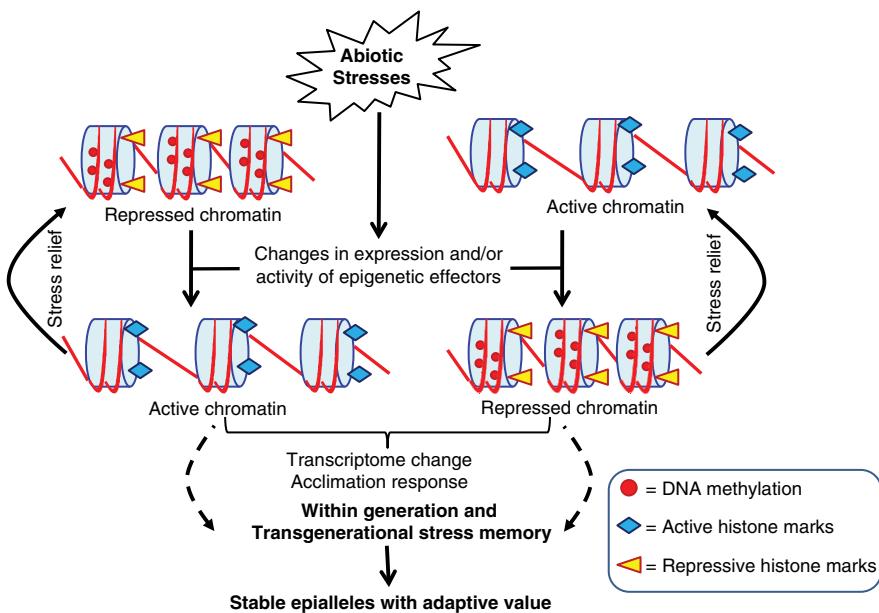
**Figure 7.1** Model of Zn transport into and across the root of the Zn/Cd hyperaccumulator, *Noccea caeruleescens*. This is a speculative model for  $\text{Zn}^{2+}$  transport from the soil into the root, radial Zn transport to the center of the root, and Zn loading into the xylem. The model depicts differences between the Zn hyperaccumulator, *Noccea caeruleescens*, compared with a “typical” non-accumulating plant species. Elevated root  $\text{Zn}^{2+}$  influx into the root from the soil is depicted for *N. caeruleescens* (larger green arrows compared with root  $\text{Zn}^{2+}$  influx in the non-hyperaccumulator). It has been suggested that NcZNT1 may be the transporter facilitating this uptake, but recent localization of the Arabidopsis homolog of the *TcZNT1* gene suggests it may also be involved in metal loading into the stele. Hence we also show NcZNT1 facilitating Zn influx from the apoplast into cells of the pericycle and other cells within the stele. The model also indicates there is less vacuolar sequestration of Zn in roots of *N. caeruleescens*. Thus in the hyperaccumulator, there would be a larger pool of mobile Zn in the root that can more readily move through the endodermis and pericycle to the xylem parenchyma. We also show the elevated Zn loading into the xylem in *N. caeruleescens*, via the Zn/Cd ATPase, HMA4, for subsequent transport to the shoots.



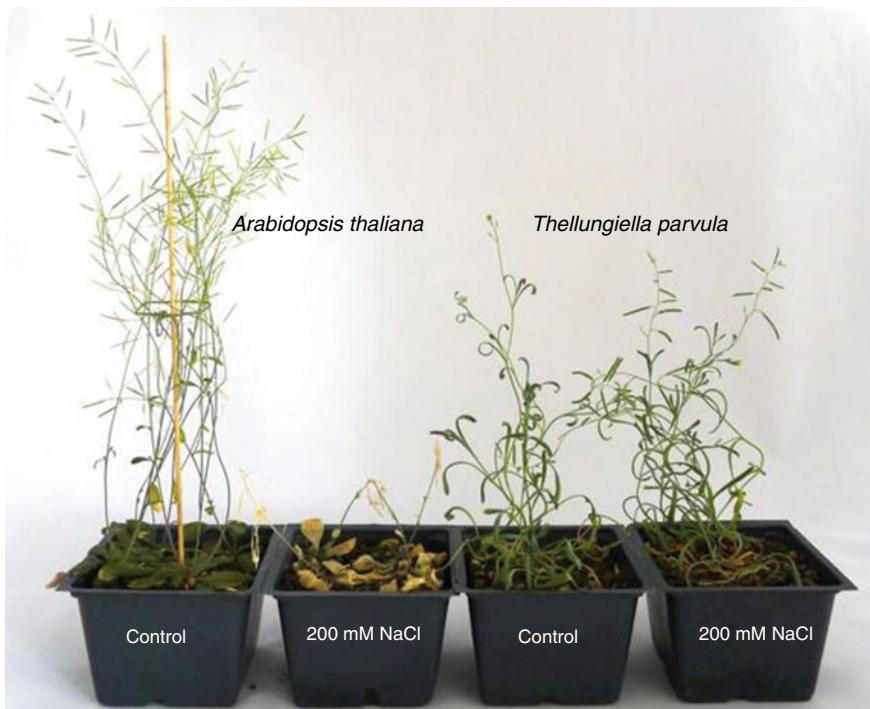
**Figure 7.2** Physiological mechanisms of aluminum (Al) tolerance. The model depicts the well-characterized root tip Al exclusion and less well-studied internal Al detoxification mechanisms of Al tolerance. The Al exclusion mechanism involves the transport of organic acids (OA) across the root-cell plasma membrane into the rhizosphere via an Al-gated anion channel for malate (ALMT1) or an Al-activated citrate efflux transporter (MATE). Activation of the ALMT channels appears to be due to direct activation of the transport protein by Al. Al activation of the MATE may be more indirect and could involve Al interacting with a second membrane-bound receptor protein that associates with the MATE protein, or by Al entering the cytosol and triggering MATE activation. It is known that Al also triggers changes in expression of genes involved in Al tolerance. The internal Al detoxification involves the entry of Al via an Al transporter that is known to be OsNrat1 in the rice root but has not been identified in Al accumulators such as buckwheat and *Hydrangea*, where the Al is sequestered in the leaf vacuole. For these shoot Al accumulators, the tolerance mechanism involves chelation of cytoplasmic Al by organic acids with the subsequent sequestration into the vacuole. Here we also suggest that in the rice root, possibly Al transported into the root cell by OsNrat1 might be transported into the vacuole by OsALS1 mediated by the transport of Al complexed with organic acids.



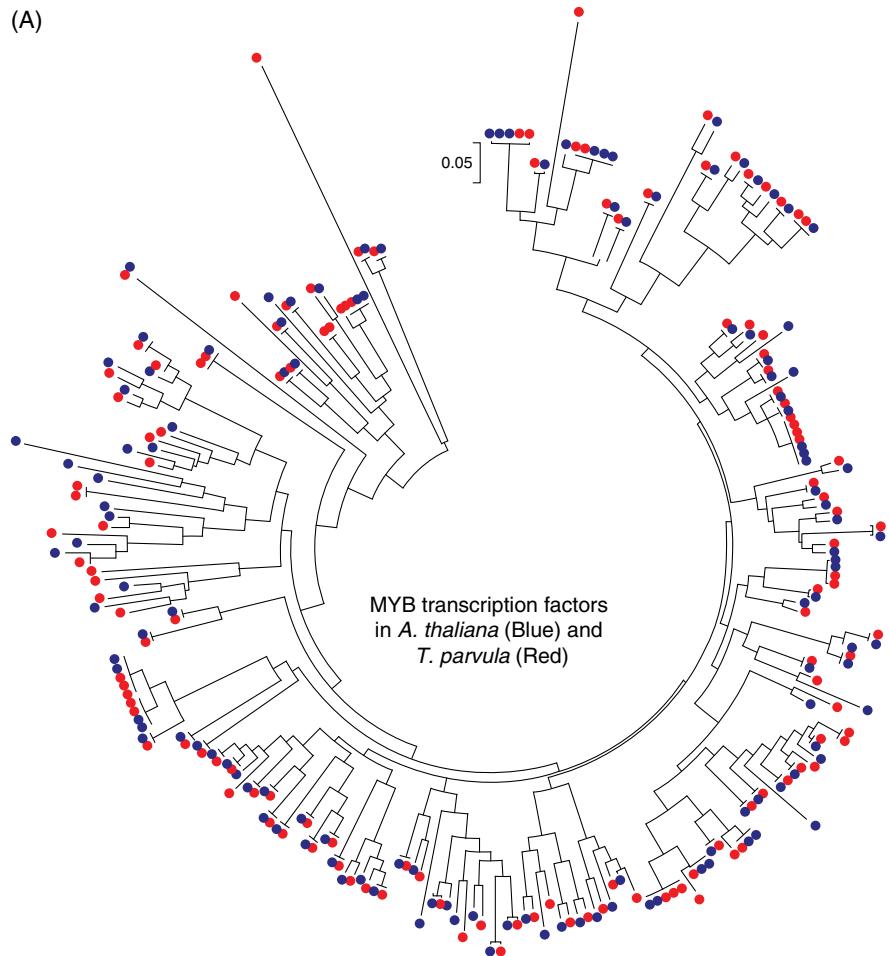
**Figure 8.2** RNA-directed DNA methylation in plants. The SNF2 chromatin remodeling protein CLASSY 1 (CLSY1) facilitates chromatin decondensation and access of RNA Pol IV to the target loci. Pol IV transcripts are converted into dsRNA by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). These dsRNAs serve as substrate for DICER-LIKE 3 (DCL3) catalyzed production of 24-nt small RNA duplex. HUA ENHANCER-1 (HEN1), an RNA methyltransferase, catalyses the methylation of the 2'-OH group of three-terminal nucleotides in the small-RNA duplex. SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) is required for Pol IV dependent siRNA production. The 24nt siRNAs are loaded onto Argonaute 4 (AGO4) protein. DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1, chromatin-remodelling factor) and DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) facilitate Pol V/II access to the target loci. Transcription factors such as RNA-DIRECTED DNA METHYLATION4 (RDM4)/DMS4 help recruit RNA polymerase V. Pol V transcribes target loci to produce ssRNAs. These transcripts serve as scaffolding for recruitment of complementary siRNA bound AGO4. The KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1)/SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L) binds to nascent scaffold transcript RNA and recruits AGO4-bound siRNAs to the RdDM effector complex. INVOLVED IN DE NOVO 2 (IDN2)/RNA-dependent DNA methylation 12 (RDM12) binds to the hybrid siRNA-nascent scaffold transcripts and aids recruitment or retention of *de novo* DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). DRM2 catalyzes cytosine methylation in DNA sequences complementary to siRNAs.



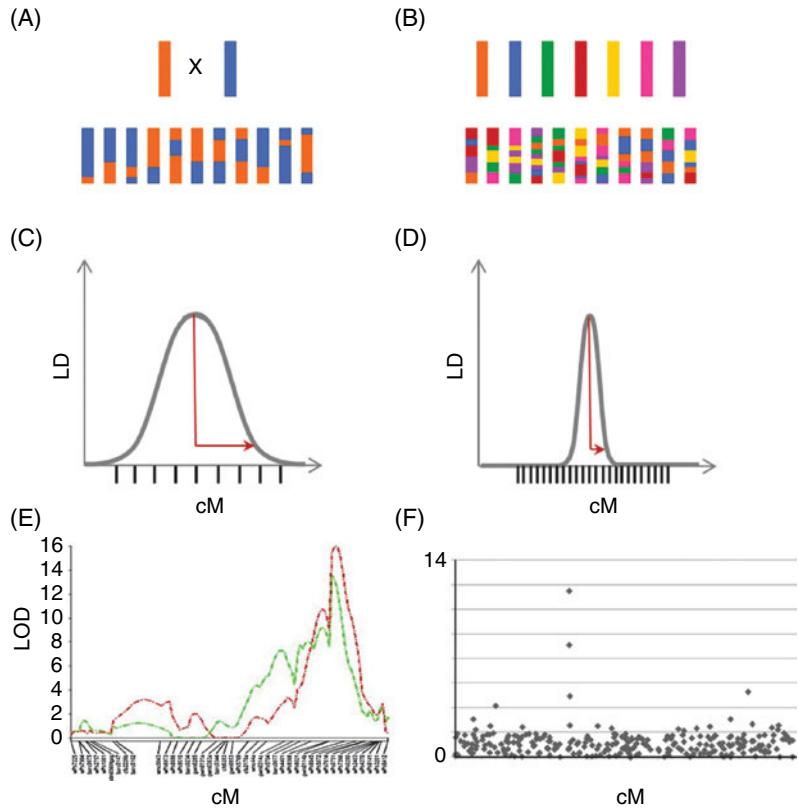
**Figure 8.4** Epigenetic regulation of stress tolerance. Abiotic stresses alter epigenetic state, which determines stress responsive gene expression. Transient chromatin modifications mediate acclimation response. Heritable epigenetic modifications provide within generation and transgenerational stress memory.



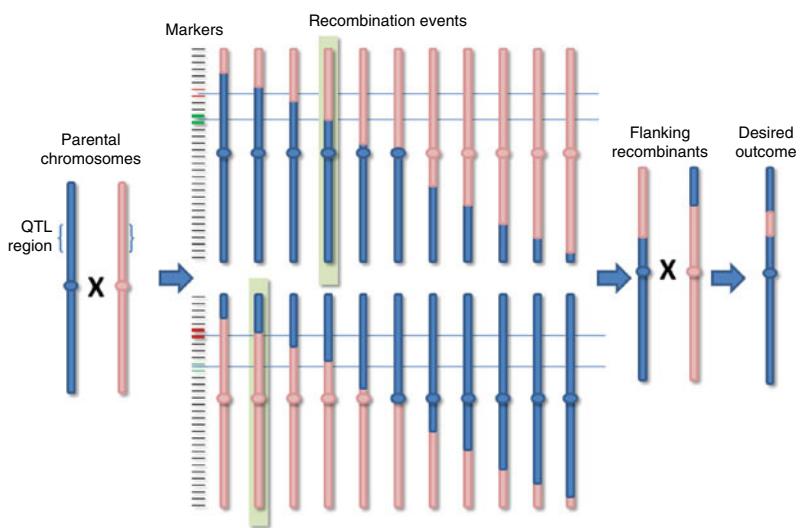
**Figure 9.1** *Thellungiella parvula* compared with its relative *Arabidopsis thaliana*. Seeds were germinated and plants grown on root wash mix (<http://pcf.aces.illinois.edu/services/soil.html>) in a growth room with 14 hours light (120  $\mu\text{mol}/\text{m}^2/\text{sec}$ ) and 10 hours dark, for 4 weeks before treatment. Plants were irrigated with 1/20 Hoagland solution once a week. Treatment was done by adding of 200 mM NaCl to the irrigation solution.



**Figure 9.2** Possible mechanisms underlying the divergence of *T. parvula* and *A. thaliana* genomes and lifestyles. (A) Copy number variation of orthologs. Evolutionary relationships exemplified by a phylogenetic tree including all MYB family genes in *T. parvula* (red) and *A. thaliana* (blue) for 126 *Arabidopsis* and 130 *Thellungiella* R2R3 MYB proteins. The phylogenetic tree was inferred by the Neighbour-Joining method, using the pairwise deletion option and 1000 bootstraps (Mega5; <http://www.megasoftware.net/>).



**Figure 10.2** Basis of linkage analysis and association study. Linkage analysis relies on biparental population with low recombination (A) resulting in low resolution mapping of QTL but high power of detecting a significant linkage between trait and loci (C and E). By contrast association study uses historical recombination accumulated over time in a diversity panel (B), which results in high-resolution mapping (D and F).



**Figure 10.3** Introgression of favorable QTL allele from a donor line (pink) into a recipient line (blue) with desirable genetic background. A recombinant population is screened with markers flanking the QTL region. Lines showing a recombination event on each side of the QTL (boxed) are crossed to generate progeny containing the QTL allele of the donor line in the genetic background of the elite recipient line. The strategy is effective if there is no recombination between the actual QTL and its flanking markers, and therefore relies on markers tightly linked to the QTL.