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ARABIDOPSIS: A RICH HARVEST 10 YEARS AFTER COMPLETION OF THE GENOME SEQUENCE

Research on plant abiotic stress responses in the post-genome era: past, present and future

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SUMMARY

Understanding abiotic stress responses in plants is an important and challenging topic in plant research. Physiological and molecular biological analyses have allowed us to draw a picture of abiotic stress responses in various plants, and determination of the Arabidopsis genome sequence has had a great impact on this research field. The availability of the complete genome sequence has facilitated access to essential information for all genes, e.g. gene products and their function, transcript levels, putative *cis*-regulatory elements, and alternative splicing patterns. These data have been obtained from comprehensive transcriptome analyses and studies using full-length cDNA collections and T-DNA- or transposon-tagged mutant lines, which were also enhanced by genome sequence information. Moreover, studies on novel regulatory mechanisms involving use of small RNA molecules, chromatin modulation and genomic DNA modification have enabled us to recognize that plants have evolved complicated and sophisticated systems in response to complex abiotic stresses. Integrated data obtained with various 'omics' approaches have provided a more comprehensive picture of abiotic stress responses. In addition, research on stress responses in various plant species other than Arabidopsis has increased our knowledge regarding the mechanisms of plant stress tolerance in nature. Based on this progress, improvements in crop stress tolerance have been attempted by means of gene transfer and marker-assisted breeding. In this review, we summarize recent progress in abiotic stress studies, especially in the post-genomic era, and offer new perspectives on research directions for the next decade.

Keywords: abiotic stress, *Arabidopsis thaliana*, genome information, transcription factor, signal transduction, crop design.

INTRODUCTION

Abiotic stress responses are important for sessile organisms such as plants because this type of organism cannot survive unless they are able to cope with environmental changes. The term 'abiotic stress' includes numerous stresses caused by complex environmental conditions, e.g. strong light, UV, high and low temperatures, freezing, drought, salinity, heavy metals and hypoxia. These stresses will increase in the near future because of global climate change, according to reports from the Intergovernmental Panel of Climate Change (http://www.ipcc.ch). In the European heatwave of 2003, crop production was reduced by around 30% (Ciais *et al.*, 2005). Therefore, understanding abiotic stress responses is now thought to be one of the most important topics in plant science. Major progress in this research field has come from the application of molecular biology. After this methodology was employed in plant science, many abiotic stress-inducible genes were isolated and their functions were precisely characterized in transgenic plants. The availability of these data broadened and deepened our view of abiotic stress responses and tolerance in plants.

Another major progress in this area followed completion of determination of the genome sequence of *Arabidopsis thaliana* in 2000. This genome information has allowed monitoring of expression profiles for all predicted genes at a single time by means of microarrays. The availability of the genome data has also enabled identification of potential

cis-regulatory elements and *trans*-factors. In addition, the availability of whole-genome tiling arrays, based on the complete genome sequence data, has opened the ways to access any transcribed unit, alternative splicing variation or chromatin and/or chromosome modification using chromatin immunoprecipitation (ChIP) technology (Lister *et al.*, 2009). Information from all-gene data has allowed examination of all the metabolic pathways in plant cells, and has provided a way to assign polypeptides detected in mass spectrometry-based protein analyses. Combination of these 'omics' data is now necessary to elucidate cellular and whole-plant processes.

Genome information has been used in genetic analysis to determine the sites of many gene knockout mutants produced by T-DNA insertion via *Agrobacterium* transformation or transposon movement (Kuromori *et al.*, 2009). The obtained insertion mutants have been used in functional analyses of the corresponding genes. Such mutations are now available for almost all Arabidopsis genes, and have enhanced studies on abiotic stress responses and in other research fields.

Simultaneous progress in other newly developing research fields has further enhanced understanding of abiotic stress responses in plants. For example, the discovery of functional small RNAs, such as micro RNA (miRNA) and small interference RNA (siRNA), has facilitated elucidation of the complex regulatory network in response to abiotic stresses (Sunkar *et al.*, 2007). In this review, we summarize recent progress in abiotic stress studies fueled by availability of the Arabidopsis genome sequence and associated 'omics' studies, and offer a new prospective on the research directions for the next decade. Because of limited space, we will consider only a few select abiotic stresses, namely drought, salinity and cold stresses, and discuss future perspectives in these research fields.

OVERVIEW OF ABIOTIC STRESS RESPONSES: FROM THE PRE-GENOMIC ERA TO DATE

Abiotic stresses have been shown to cause accumulation of many intracellular substances, including nucleic acids, proteins, carbohydrates and amino acids. After the introduction of molecular biological techniques into plant biology, a great deal of effort went into the identification of stress-inducible genes, such as *RD29A*, using differential screening or differential display techniques for various plant species, including Arabidopsis. These studies succeeded in isolating genes that are presumed to function in stress responses and tolerance. Over-expression of some of these genes *in planta* confers some abiotic stress tolerance (Bartels and Sunkar, 2005; Umezawa *et al.*, 2006b). More importantly, using the expression of such inducible genes as markers, an overall scheme of transcriptional regulation was developed. In the emerging picture, transcriptional activation occurs at distinct time points in response to stress stimuli. The various induction phases for stress-inducible genes are due to their varying dependency on *de novo* synthesis of proteins or signaling molecules, such as abscisic acid (ABA) (Yamaguchi-Shinozaki and Shinozaki, 2006). These findings suggest that abiotic stress responses are never simple, and that each induction phase may be controlled by a different signaling mechanism and different transcription factors. Identification of the relevant factors in each pathway has been addressed using responsive genes as markers. For example, an abiotic stress-responsive *cis*-element, dehydration responsive element (CRE)/C-repeat (CRT) (A/GCCGAC), was identified, which in turn has triggered important studies to identify the transcriptional regulating factors, DRE-binding protein (DREB)/C-repeat binding factor (CBF), and their post-translational regulatory mechanisms (Thomashow, 2001; Shinozaki and Yamaguchi-Shinozaki, 2007). In addition, genetic screens for mutations that affect the expression of stress-inducible genes have allowed the identification of novel components in the regulatory system (Chinnusamy *et al.*, 2002). These results have provided a basic picture of gene regulatory networks in abiotic stress responses in plants. However, the stress-inducible genes isolated in this way were largely restricted to those with higher expression levels, and thus our knowledge on gene expression profiles was limited.

Complete determination of the genomes of Arabidopsis, *Oryza sativa* spp. *japonica* cv. Nipponbare, and other plants has changed the situation dramatically. The complete genome sequence has enabled genome-wide gene expression profiling in response to various abiotic stresses (e.g. using AtGenExpress; Kilian *et al.*, 2007). Using microarray technology, genes responding to abiotic stresses have now been identified more comprehensively than ever before. Comprehensive transcriptome analysis revealed the relationships among stress-regulated transcripts, and enabled the prediction of their *cis*-regulatory elements (Kilian *et al.*, 2007; Weston *et al.*, 2008). Ma and Bohnert (2007) showed a clear correlation between expression profiles and the 5′ regulatory motifs of stress-regulated genes. These analyses indicated that stress-regulated genes are controlled by a complicated regulatory network. This type of network has been proposed based on transcriptome data using various theoretical approaches (Chen and Zhu, 2004; Ma *et al.*, 2007; Long *et al.*, 2008).

Determination of the function of stress-inducible genes has been addressed by the reverse genetic approach, aided by the use of insertional mutation lines. The products of stress-inducible genes identified in vast microarray experiments can be classified into two groups (Shinozaki *et al.*, 2003): one containing mainly proteins functioning in direct abiotic tolerance [e.g. late embryogenesis abundant (LEA) proteins], and the other consisting of regulators for intracellular signaling and stress-inducible gene expression (e.g. protein kinases such as MAP kinases, phosphatases,

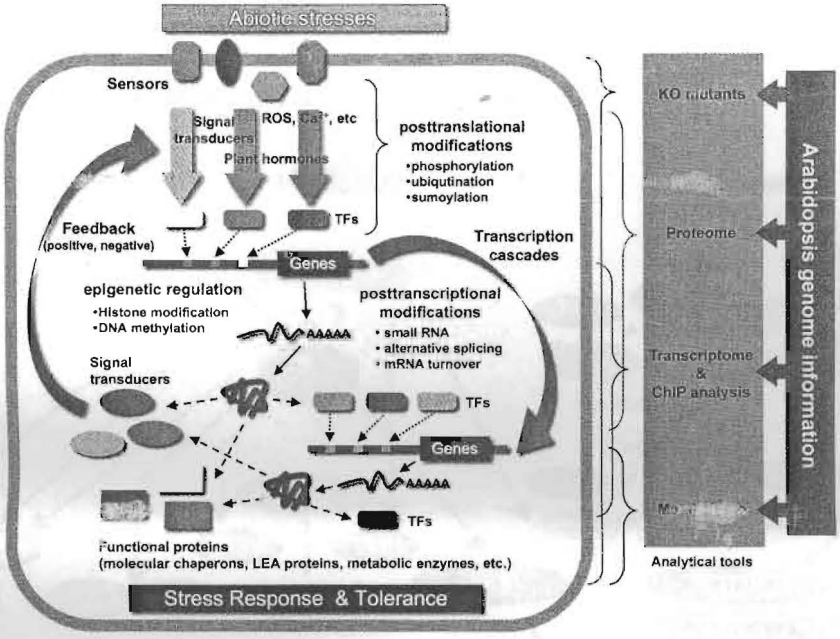

Figure 1. Overall model of an abiotic stress response.
Plant cells receive stress signals through various sensors (not yet known), and the signals are transduced by various signaling pathways in which many second messengers, plant hormones, signal transducers and transcriptional regulators function. Stress-inducible genes are regulated by multiple stress signals, and some of them are regulated by transcription factors (TFs) that are induced by stress stimuli, e.g. a transcriptional cascade. Some stress-inducible genes encode functional proteins that are directly involved in stress tolerance. Other stress-inducible genes encode regulatory proteins, such as signal transducers, that presumably form positive and negative feedback loops to regulate stress responses. The availability of Arabidopsis genome information has made countless contributions in clarifying this system, not only in terms of transcriptional regulation but also in terms of post-transcriptional and post-translational modifications and epigenetic regulation.

phospholipid metabolic enzymes, and various types of transcription factors). The identification of stress-inducible signal transducers gave rise to the idea that plants have developed flexible cellular response mechanisms to efficiently respond to various abiotic stresses. Figure 1 shows the current understanding of abiotic stress responses in conjunction with the contribution of Arabidopsis genome information.

REGULATION OF TRANSCRIPTION IN ABIOTIC STRESS RESPONSES

As described above, many transcription factors involved in stress responses have been identified. As an example, we describe here the transcriptional regulatory system for cold and drought (or osmotic) stresses (Figure 2). The DREB1/CBF family comprises APETALA2 (AP2) type transcription factors that recognize DRE/CRT and function in cold stress responses. Expression of *DREB1A/CBF3* or *DREB1C/CBF2* is regulated at the transcriptional level by INDUCER OF CBF EXPRESSION 1 (ICE1) (Chinnusamy *et al.*, 2003) or calmodulin binding transcription activator (CAMTA) (Doherty *et al.*, 2009), respectively. ICE1 is a MYC-type transcription factor that has also been shown to regulate stomata formation (Kanaoka *et al.*, 2008). CAMTA transcription factors recognize Conserved Motif (CM) sequences providing a link with the Ca^{2+} signaling that is activated in the abiotic stress response, as CAMTA proteins have a calmodulin-binding domain. ZAT12, a zinc finger protein, is also implicated in the regulation of *DREB/CBF* expression (Vogel *et al.*, 2005). DREB2, another AP2-type transcription factor that recognizes DRE/CRT, is involved in drought or salinity stress responses. Osmotic stress activates several other transcription factors, including zinc finger homeodomain (ZFHD) proteins and NAM ATAF CUC2 (NAC). ZFHD1 binds the CACTAAATTGTCAC motif, named ZFHDR, in the promoter region of *EARLY RESPONSE TO DEHYDRATION 1* (*ERD1*). NAC proteins recognize a MYC-like target sequence and activate *ERD1* (Tran *et al.*, 2004, 2006). Osmotic stress increases the ABA level, which in turn activates sets of genes. ABA-responsive transcription factors (AREB/ABF), with a bZIP type DNA-binding domain that binds the ABA-responsive element (T/CACGTGGC), have a pivotal role in ABA-dependent gene activation (Choi *et al.*, 2000; Uno *et al.*, 2000). MYB and MYC transcription factors are synthesized *de novo* under osmotic stress conditions, and cooperatively activate stress-inducible genes such as *RD22* (Abe *et al.*, 2003).

Some stress-associated transcription factors are themselves regulated at the transcriptional level, constituting a transcriptional cascade (Figures 1 and 2). Other transcription factors are regulated by post-translational modifications. Such regulation of transcription factors may be

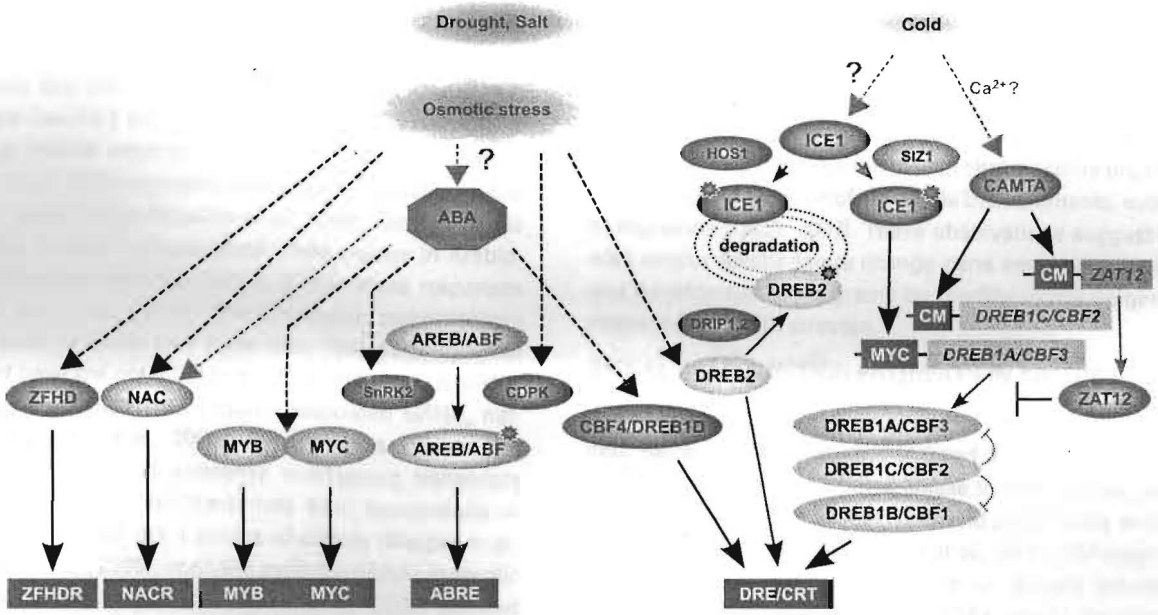

Figure 2. Transcriptional regulatory network functioning in drought, salinity and cold stress responses.
Elliptical objects and gray boxes indicate functional proteins and *cis*-elements, respectively. Solid lines and dotted lines are direct or indirect links, respectively. Colored lines indicate modifications. Gray dotted lines show uncertain connections. For details, see text.

required for rapid and fine-tuned regulation under abiotic stress conditions. There is much evidence showing that ubiquitination, which usually induces degradation of the target protein, plays a pivotal role in abiotic stress responses (Vierstra, 2009). DREB2 was shown to be regulated by DREB-INTERACTING PROTEIN 1 and 2 (DRIP1 and DRIP2), which are RING finger E3 ligases, through ubiquitination (Qin *et al.*, 2008). ICE1 was demonstrated to be under the control of HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), another RING finger protein (Dong *et al.*, 2006). Sumoylation is also involved in the regulation of transcription factors in abiotic stress responses. In contrast to ubiquitination, sumoylation regulates the activity of target proteins. A recent study showed that a SUMO E3 ligase, SIZ1, sumoylated ICE1 and enhanced its activation of DREB1A/CBF3 (Miura *et al.*, 2007).

As in other organisms, transcription factors in plant systems are regulated by phosphorylation. The ABA-responsive transcription factors ABI5 and AREB/ABF of Arabidopsis and TRAB1 of rice are regulated by the phosphorylation of multiple Ser/Thr residues. SNF1-related kinase 2 (SnRK2)-type protein kinases (Kobayashi *et al.*, 2005; Furihata *et al.*, 2006) and Ca^{2+}-dependent protein kinase (CDPK) are good candidates for regulators of AREB/ABFs (Choi *et al.*, 2005; Kaplan *et al.*, 2006; Zhu *et al.*, 2007). SnRK2s are activated by osmotic stress or ABA (Boudsocq *et al.*, 2004), while CDPKs are activated by increased intracellular Ca^{2+} levels induced by various stimuli (Harper *et al.*, 2004) (see below). Presumably, such transcription factors function as a hub component that integrates multiple signal inputs under abiotic stress conditions.

POST-TRANSCRIPTIONAL REGULATION OF ABIOTIC STRESS-INDUCIBLE TRANSCRIPTS

Recent studies have indicated that post-transcriptional regulation contributes to stress responses more than was previously thought. Transcribed RNA goes through various modifications: addition of a 5′ cap structure, splicing, and 3′ polyA addition. After these processes, mRNA is exported actively from the nucleus to the cytoplasm, where translation occurs and unnecessary or abnormal mRNA is promptly degraded. Each step is regulated coordinately (Houseley and Tollervey, 2009). Genetic studies on Arabidopsis mutants exhibiting an abnormal response to abiotic stress or ABA revealed that mRNA processing and metabolism have a close link with stress responses (Fedoroff, 2002a; Kuhn and Schroeder, 2003; Hirayama and Shinozaki, 2007). For example, RNA helicases are implicated in abiotic stress responses in various organisms including plants (Owttrim, 2006). In addition, Iida *et al.* (2004) showed that alternative splicing, which enables production of diverse polypeptides from one gene, is regulated by various abiotic stresses, such as cold stress. Moreover, cold stress changed the alternative splicing profiles of splicing factors, suggesting that complex multi-step regulation controls the splicing profiles in abiotic stress responses (Iida *et al.*, 2004; Reddy, 2007). Alternative splicing events are considerably conserved between Arabidopsis and rice, indicating their importance (Wang and Brendel, 2006).

SMALL RNA-DEPENDENT GENE REGULATION IN ABIOTIC STRESS

An emerging topic in gene regulatory systems in abiotic stress responses is the regulation of gene expression by small RNAs. The evidence shows that the basic mechanisms of small RNA synthesis and function are common to many eukaryotes but that numerous organism-specific mechanisms are involved (Siomi and Siomi, 2009). Presumably, plants, as sessile organisms, have developed unique features of small RNA regulatory mechanisms (Brodersen and Voinnet, 2006; Jones-Rhoades *et al.*, 2006). During abiotic stress, the levels of several small RNAs change in Arabidopsis, indicating their function in abiotic stress responses (Sunkar and Zhu, 2004). Comprehensive transcriptome studies have revealed that more than 7000 transcriptional units that have the potential to produce endogenous siRNA (called natural antisense transcript-associated siRNA, nat-siRNA) (Yamada *et al.*, 2003). Abiotic stress induces the accumulation of novel antisense overlapping transcripts (Matsui *et al.*, 2008) and transcripts from transposons or pseudogenes, which are a source of siRNAs (Matzke *et al.*, 2007; Zeller *et al.*, 2009), implying a role for siRNAs in abiotic stress responses in plants. Borsani *et al.* (2005) showed that nat-siRNA produced from overlapping mRNAs for Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) and SIMILAR TO RCD ONE 5 (SRO5) regulated the expression of these genes. The authors showed that, under salt stress conditions, *SRO5* is induced and siRNAs are produced, which in turn down-regulates *P5CDH* and leads to accumulation of the osmoprotectant proline. This *P5CDH–SRO5* regulatory system appears to function only in Arabidopsis and not in other plants.

LONG-TERM REGULATION OF GENE EXPRESSION: EPIGENETIC REGULATION

Plant cells constituting the whole body are constantly derived from a few stem cells, the meristematic cells. In such a system, the information that meristematic cells have obtained from the environment can be transferred to all lineages of cells, including gametes. Recent studies have shown that abiotic stresses cause long-term regulation of gene expression, mostly conferred by epigenetic gene regulatory mechanisms, e.g. chromatin remodeling through various histone modification or DNA methylation processes (Berger, 2007; Chinnusamy and Zhu, 2009). A combination of the availability of genome information and progress in techniques for the detection of chromatin conditions (e.g. ChIP) or modified bases in DNA (e.g. bisulfite method) has allowed studies on not only genes of interest but also the whole genome. Using these techniques, stress-induced chromatin modifications have been demonstrated (Tsuji *et al.*, 2006; Kim *et al.*, 2008). There is much evidence to support the link between chromatin modification and abiotic stress responses. Zhu *et al.* (2008) showed that a defect in the deacetylase-like protein HOS15 caused an abnormal response to ABA and abiotic stress. AtCHR12, a chromatin remodeling protein, is required for growth arrest under stress conditions (Mlynárová *et al.*, 2007). SWI3B has been identified as one of the targets of an ABA-related PP2C, HAB1 (Saez *et al.*, 2008). DNA methylation has also been implicated in abiotic stress responses (Matzke *et al.*, 2007; Boyko and Kovalchuk, 2008). A change in DNA methylation status causes alterations not only in gene expression levels but also in chromosome organization through re-location of moveable DNA elements, such as transposons (Lisch, 2009). These observations suggest that ABA and/or abiotic stress change gene expression profiles and developmental programs by modifying the epigenetic status to cope with stresses.

SIGNAL TRANSDUCTION PATHWAYS IN ABIOTIC STRESS

Information on the sensors for abiotic stresses is very limited. Reverse genetic studies showed that AHK1/ATHK1, a membrane-spanning histidine kinase in Arabidopsis, could act as an osmosensor in yeast cells and presumably in plant cells (Tran *et al.*, 2007; Wohlbach *et al.*, 2008). Although the downstream signaling mechanisms are largely unknown, response regulator-like proteins (ARRs) might function in signaling processes (Wohlbach *et al.*, 2008).

MAP kinase cascades function as major cellular signaling components in eukaryotes. Therefore, plant genes for MAP kinase cascades have been examined to determine whether they are involved in various stress responses. Arabidopsis genome information allowed identification of 20 MAP kinases, 10 MAP kinase kinases, and 60 MAP kinase kinase kinases (Ichimura *et al.*, 2002). These MAP kinase components appear to function in several different signaling processes, so they might not constitute simple cascades but rather networks, making it difficult to identify the function of each component. Many genetic and biochemical studies have clarified MAP kinase cascades (Nakagami *et al.*, 2005), among which the Arabidopsis MEKK1–MKK2–MPK4/6 cascade has been clearly demonstrated to transduce salinity and cold stress signals (Teige *et al.*, 2004). Currently, however, the direct upstream factors of MAP kinase cascades have not been identified.

Sucrose non-fermentation 1 (SNF1)-related kinases (SnRK1, SnRK2 and SnRK3) have been demonstrated to function in various stresses and ABA responses. Yeast SNF1 is implicated in the starvation response. Similarly, in Arabidopsis, two SnRK1-type kinases (KIN10/SnRK1.1 and KIN11/SnRK1.1) are presumed to act in the regulation of metabolic pathways (Baena-González *et al.*, 2007). In addition to SRK2J/SnRK2.9, nine Arabidopsis SnRK2s are activated under osmotic stress conditions (Boudsocq *et al.*, 2004). Among them, SRK2D/SnRK2.2, SRK2I/SnRK2.3 and SRK2E/OST1/SnRK2.6 are activated strongly by ABA. Recent studies showed that these three ABA-activated SnRK2s are

essential for ABA signaling (Fujii and Zhu, 2009; Nakashima *et al.*, 2009; Umezawa *et al.*, 2009) (see below). This result suggests that other SnRK2-type kinases play pivotal roles in transducing the signals which activate them.

Ca^{2+} is one of the most important second messengers in response to extracellular stimuli in plants (Harper *et al.*, 2004; Ludwig *et al.*, 2004). Increased levels of intracellular Ca^{2+} are presumably induced under stress conditions by signal molecules such as inositol trisphosphate (IP_3), diacylglycerol, inositol hexaphosphate (IP_6), cADP-ribose or reactive oxygen species, although the molecular identities of receptors for these molecules are largely unknown in plants. A Ca^{2+}-permeable stretch-activated channel of Arabidopsis also contributes to Ca^{2+} release upon stimulation (Nakagawa *et al.*, 2007). Two protein kinases are postulated to be the targets of the Ca^{2+} signal in plants. One is SnRK3, whose activity is dependent on the Ca^{2+}-binding calcineurin B-like (CBL) proteins (Hrabak *et al.*, 2003). Arabidopsis has 25 SnRK3-type kinases, according to genome information, of which the best characterized is SALT OVERLY SENSITIVE 2 (SOS2)/CIPK24/SnRK3.11, which was identified as an essential factor in the salinity stress response. In conjunction with SOS3/ScaBP8/CBL10 Ca^{2+}-binding protein, SOS2 activates the plasma membrane Na^+/H^+ antiporter (SOS1) required for salinity tolerance (Mahajan *et al.*, 2008; Luan, 2009). The other protein kinase is Ca^{2+}-dependent kinase (CDPK). According to the annotation, Arabidopsis has more than 30 CDPK genes (Hrabak *et al.*, 2003), and several of them have been shown to function in abiotic stress and ABA responses. CPK3 and CPK6 regulate the ABA response in guard cells (Mori *et al.*, 2006), and CPK4, CPK11 and CPK32 positively regulate the ABA response (Choi *et al.*, 2005; Zhu *et al.*, 2007). In addition, CPK4 and CPK11 phosphorylate AREB/ABF transcription factors in an ABA-dependent manner (Zhu *et al.*, 2007).

HORMONE RESPONSE IN ABIOTIC STRESS RESPONSES

Under salinity or drought stress, accumulated ABA plays pivotal roles in stress responses in plants through a drastic change in the gene expression profile and cellular processes. Our knowledge on the synthetic and catabolic pathways of ABA has increased in the last decade (Nambara and Marion-Poll, 2005). In particular, the identification of rate-limiting enzymes, such as 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) in the ABA biosynthetic pathway and P450 CYP707As in the ABA catabolic pathway, has greatly enhanced our understanding of how plants control the level of this phytohormone. The genes for these two enzymes are activated by various stress treatments; *NCED3* is down-regulated strongly by rehydration, while *CYP707A3* is further up-regulated, suggesting fine-tuning of the ABA level by the coordinated regulation of ABA production and breakdown systems (Umezawa *et al.*, 2006a). In addition, ABA is stored in an inactivated form, ABA glucosyl ester, in vacuoles and apoplasts. Under dehydration conditions, ABA is released from the glucosyl ester form by β-glucosidase (Lee *et al.*, 2006).

The action of ABA is the one of the most studied topics in abiotic stress response research (Hirayama and Shinozaki, 2007; Wasilewska *et al.*, 2008). To understand ABA action, identification of receptors is necessary. Two types of ABA receptors, soluble and membrane-anchored, have been reported. Pyrabactin Resistance1 (PYR1)/PYR1-like proteins (PYLs)/Regulatory Component of ABA Receptor1 (RCAR1)-type or START-type soluble ABA receptors were identified very recently using chemical genetic and biochemical approaches (Ma *et al.*, 2009; Park *et al.*, 2009). In-depth biochemical and genetic analyses revealed that these proteins directly bind PP2Cs, such as ABI1, and inhibit their activity in an ABA-dependent manner (Ma *et al.*, 2009; Park *et al.*, 2009). This indicates that ABA regulates cellular processes by modulating PP2C activity, presenting a unique system among plant hormone signaling pathways (Santner and Estelle, 2009). In addition, a recent biochemical analysis succeeded in showing that clade A PP2Cs interact with ABA-activated SnRK2s and inactivate them efficiently through dephosphorylation at specific amino acid residues (Umezawa *et al.*, 2009). Because SnRK2s can phosphorylate and activate some ABA-dependent transcription factors (see above), the details of one of the ABA signaling pathways have now been established from perception to gene expression, namely PYR1/PYLs/RCAR1-type receptor → clade A PP2C → ABA-activated SnRK2 → AREB/ABF bZIP transcription factors (Figure 3) (Umezawa *et al.*, 2009). Because some PP2Cs have been demonstrated to regulate other proteins, such as AKT2 and SWI3, several branched pathways are expected. As the triple knockout mutant of three ABA-activated SnRK2s was extremely insensitive to ABA (Fujii and Zhu, 2009; Nakashima *et al.*, 2009; Umezawa *et al.*, 2009), this soluble ABA receptor–PP2C–SnRK2 pathway seems to be the major pathway. Establishment of the major ABA signaling pathway will enhance our understanding of abiotic stress responses dramatically. The membrane-anchored G-protein-coupled receptor-type proteins, GTG1 and GTG2, are other potential ABA receptors (Pandey *et al.*, 2009). The downstream signaling pathway has not been fully elucidated, but will hopefully be clarified soon and provide information on the physiological relevance of multiple types of ABA receptor.

Other plant hormones play substantial roles in abiotic stress, either directly or indirectly. In particular, salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) have been shown to affect abiotic stress responses through interplay with ABA in a complex manner (Fedoroff, 2002b; Fujita *et al.*, 2006; Grant and Jones, 2009; Pieterse *et al.*, 2009). The transcription factors AtMYC2 and ERF1 have been shown to be the convergent points of JA/ABA or JA/ET signals, respectively (Lorenzo and Solano, 2005). The interaction between ABA

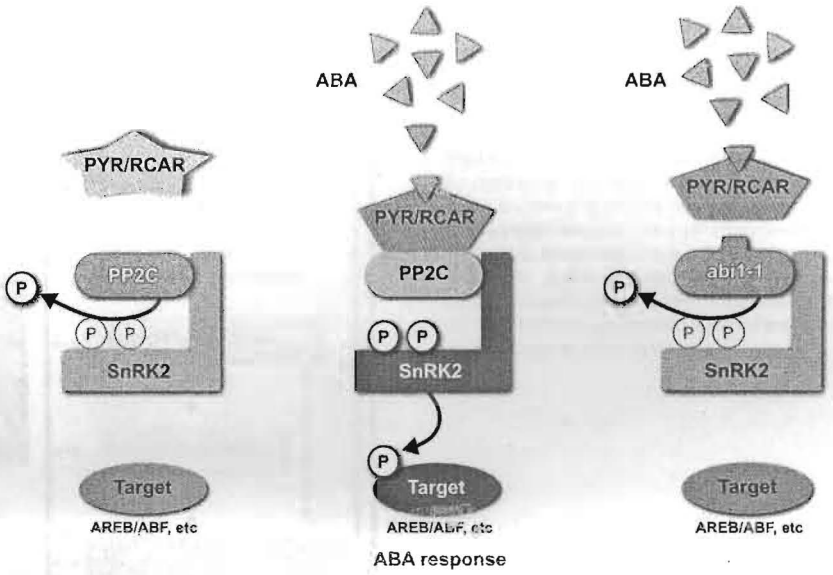

Figure 3. ABA signaling pathway.
Schematic representation of the major ABA signaling pathway. Clade A PP2C interacts with ABA-dependent SnRK2 and represses its kinase activity through dephosphorylation at specific residues. A PYR1/PYLs/RCAR1-type ABA receptor binds PP2C and inhibits its phosphatase activity, thereby allowing activation of SnRK2. SnRK2 phosphorylates targets and evokes the ABA response. In the case of the dominant ABA-insensitive *abi1-1* mutant, the ABA receptor cannot bind the mutant PP2C, and the mutant PP2C has enough phosphatase activity to inhibit SnRK2, consequently conferring dominant ABA insensitivity.

and ET has been well documented in several reviews (Gazzarrini and McCourt, 2003; Fujita *et al.*, 2006). These interactions are presumably necessary for integrating various stress signals and maintaining a balance among the response reactions at both the cell and whole-body levels.

METABOLIC PROFILE CHANGES UNDER ABIOTIC STRESS

Metabolism reflects biological activities and conditions, and is very closely related to crop production. Study of metabolic regulation under stressful conditions has been facilitated in the last decade as the detection and identification of metabolites has improved, in particular through mass spectrometry-based analytical methods (Sawada *et al.*, 2009). To obtain more insight into cellular conditions under abiotic stresses, metabolomic investigations have been performed in Arabidopsis and other plant species (Schauer and Fernie, 2006). Under stress conditions, plants appear to re-organize their metabolic network in order to adapt to such conditions (Kaplan *et al.*, 2004). Using metabolic changes as a 'map' or 'marker', factors regulating metabolic movements were investigated in combination with other 'omic' analyses, such as transcriptome experiments (Saito *et al.*, 2008). For example, CBF/DREB1 transcription factors were shown to play important roles in cold acclimation and cold tolerance in Arabidopsis (Cook *et al.*, 2004). Sulfur deprivation activates glucosinolate (GSL) synthesis in Arabidopsis. Detailed analysis of time-series data for the metabolite and transcript levels by self-organizing mapping suggested that the MYB28 and MYB29 transcription factors and dozens of downstream enzymes are involved in the production of GSL in the sulfur-starvation stress response (Hirai *et al.*, 2005, 2007). The function of MYB28 in GSL synthesis was confirmed by the fact that the gene knockout mutant and transgenic plants over-expressing MYB28 had lower and higher levels, respectively, of both GSL and transcripts for GSL biosynthetic enzymes (Hirai *et al.*, 2007).

INTERACTIONS BETWEEN ABIOTIC AND BIOTIC STRESSES

An emerging topic in the stress responses of plants is interaction between abiotic stress (or ABA) and biotic stress responses. Biotic stress responses, or resistance responses to pathogens or viruses, are mainly regulated by the phytohormones SA, JA and ET. According to our current understanding of defense response systems, SA is more involved in the defense response against biotrophic or hemibiotrophic pathogens, while JA and ET take part in the defense system against necrotrophic pathogens (Pieterse *et al.*, 2009), although these classifications are controversial. As mentioned above, many studies have suggested complicated interactions between ABA and these plant hormones. Recent studies showed that ABA is implicated in the disease-resistance response more directly than expected, and ABA action in the defense system is under spatial and temporal regulation (Asselbergh *et al.*, 2008; Ton *et al.*, 2009). One recent remarkable finding is that ABA plays important roles in the defense system by closing stomata upon pathogen attack (Melotto *et al.*, 2006). In contrast, ABA increases the susceptibility to pathogens mainly by inhibiting SA action. Intriguingly, the pathogenic bacterium *Pseudomonas syringae* induced several genes related to ABA biosynthesis and signaling, presumably to suppress the host defense system (de Torres-Zabala *et al.*, 2007). Yasuda *et al.* (2008) attempted to dissect the interaction between ABA and SA, and showed that these hormones interact antagonistically at both the biosynthetic and signaling levels. The temporal action of plant hormones in defense systems was further emphasized by the study of the *constitutive disease susceptibility 2-1D* (*cds2-1D*) mutant,

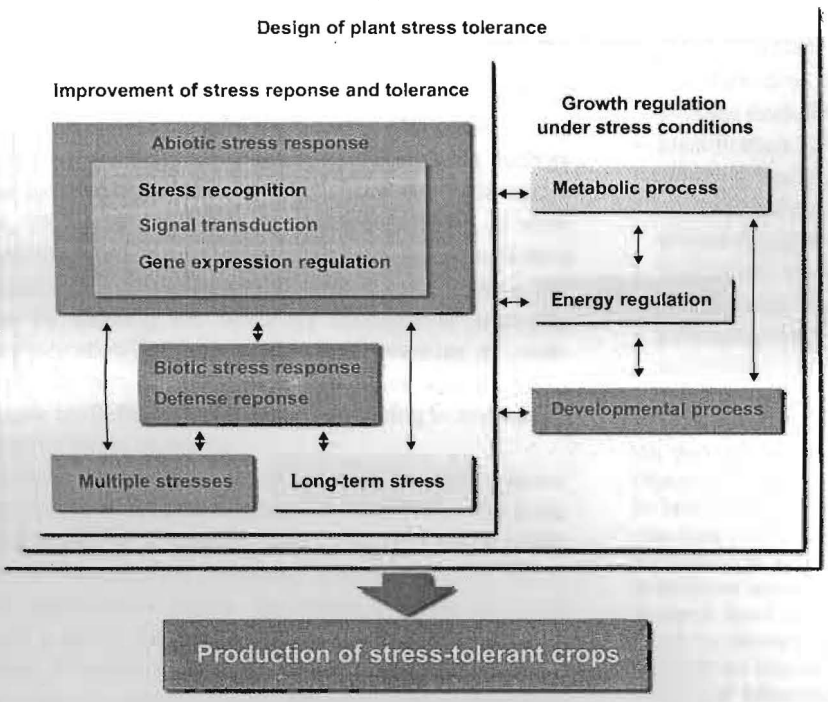

Figure 4. Design of stress-tolerant crops.
To produce crops tolerant to various abiotic and biotic stresses and provide high yields, comprehensive knowledge on many processes involved not only in stress responses but also in energy regulation, metabolic processes and developmental processes, are required. Integration all of these will offer us appropriate sites and ways for manipulation of plant systems.

which increased the expression of *NCED5* and enhanced disease susceptibility (Fan *et al.*, 2009). Asselbergh *et al.* (2008) proposed that ABA is the integrative signal mediator that regulates global cellular responses. This proposal appears to be consistent with accumulated evidence that ABA is implicated in biotic stress responses in many host–pathogen combinations, and is attractive because ABA is thought to be the connecting molecule between stress responses and various developmental processes.

STRESS-TOLERANCE MECHANISMS IN DIVERSE PLANT SPECIES

Knowledge obtained from the study of plant species living under extreme environmental conditions has provided important information on stress tolerance mechanisms. One of the most studied groups are halophytes, such as mangroves, which grow under high-salinity conditions. Generally, the mechanisms by which halophytes tolerate high salinity differ among species, but common features can be found in their tight regulation of internal Na^+, K^+ and Cl^- content (Flowers and Colmer, 2008). However, halophytes exhibit tolerance to other abiotic stresses as well. Among them, *Thellungiella halophila*, or salt cress, has been used as a model plant because it is closely related to Arabidopsis. Detailed analysis of salt cress revealed that this halophyte has additional features, including the accumulation of more proline under stress conditions, high selectivity for ions, and sensitive stomatal regulation (Inan *et al.*, 2004). Given the high similarity to Arabidopsis, transcriptome analyses of salt cress have been performed by adapting the genome-related information on Arabidopsis, revealing both common and distinct expression profiles of stress-related transcripts (Taji *et al.*, 2004; Gong *et al.*, 2005). Detailed analysis of the differences between glycophytes and halophytes will provide information on the molecular basis of salinity tolerance and other stress tolerance attributes of halophytes. Du *et al.* (2008) attempted to identify salt cress genes that confer salinity stress tolerance to Arabidopsis and obtained several candidates. Such an approach is useful to understand the molecular basis of salt tolerance in halophytes.

Quantitative trait locus (QTL) analysis among tolerant and intolerant species of crops is now receiving much attention. A major benefit of QTL-based approaches is that they may enable production of stress-tolerant crops by combining or 'pyramiding' QTLs for various stress tolerances. Several QTL studies relating to various abiotic stress tolerances have been reported (Takeda and Matsuoka, 2008). For example, Ren *et al.* (2005) identified the *SKC1* locus encoding a high-affinity K^+ transporter (HKT)-type sodium transporter by analyzing a QTL for salinity tolerance using salt-tolerant and salt-susceptible rice varieties. Such genes or loci can be used to improve the salinity tolerance of rice.

STRATEGIES IN CROP BREEDING BASED ON GENOME INFORMATION

One purpose of studying abiotic stress responses in plants is to improve the abiotic stress tolerance of crops by means of genetic manipulation. The results of basic research using Arabidopsis have been applied to improving stress tolerance

in other plant species, including crop plants. These studies have been summarized very well in several recent reviews (Flowers, 2004; Bartels and Sunkar, 2005; Umezawa *et al.*, 2006b). In brief, ectopic expression of components involved in abiotic stress responses has led to improved stress tolerance. However, constitutive ectopic expression of these components often causes reduced plant growth, presumably due to an adverse effect of accumulated factors on cellular functions or energy consumption. Such unfavorable effects can be suppressed by using appropriate promoters, such as tissue-specific or abiotic stress-inducible ones (Umezawa *et al.*, 2006b). Alternatively, careful manipulation of transcription factors can also be used. Some transcription factors or signal transducers may be converted to constitutive active forms by deleting the inhibitory domains or changing phosphorylation-accepting amino acid residues to phosphorylation-mimicking amino acid residues (e.g. serine to asparatic acid). Such strategies are promising techniques to improving stress tolerance.

However, improvement of crops using the above strategies will require further research. The biggest concern is the differences in the conditions between laboratories and crop fields. In many reports describing stress tolerance of genetically manipulated plants, the levels of plant tolerance against a stress were examined only over short periods. In contrast, in the field, plants are subjected to various stresses simultaneously and the constraints extend throughout their lifetimes in some cases. Combined abiotic stresses have been reported to cause unexpected physiological changes in plant cells (Larkindale *et al.*, 2005; Mittler, 2006). In addition, as discussed earlier, abiotic stress and ABA responses interact with the defense response against pathogens in a highly complicated manner. The molecular basis for this interaction has not yet been elucidated. Therefore, transgenic crops harboring a gene or genes designed to improve the tolerance to a specific stress might encounter unexpected problems. To overcome these difficulties, we need to fully understand whole stress-response system of plants (Figure 4).

PERSPECTIVES

As described above, our understanding of abiotic stress responses has taken a big leap forward in the last decade, namely the post-genome era. However, we still have several critical problems to overcome in molecular breeding of stress-tolerant plants. Dinneny *et al.* (2008) clearly demonstrated that different differentiated cells in roots responds differently to various abiotic stresses, suggesting that different cell types respond differently to abiotic stresses. Our understanding of the whole-plant stress response mechanism is very limited. To dissect this system, we need to investigate stress responses in differentiated cells, tissues and organs, and to connect the data relevantly. Systems biological and mathematic biological approaches will be required to integrate the data and to draw a complete overall picture of the abiotic stress response in plants.

Challenges to be met for integrated knowledge of plant abiotic stress responses and tolerance include:

- Identification of sensors and signaling pathways for abiotic stresses.
- Understanding the molecular basis of interplay among stresses (including biotic stresses).
- Identification of key factors in the connection between abiotic stress responses and developmental processes.
- Addressing how local abiotic stress signals are processed and transduced to other parts of the plant body.
- Examining long-term plant responses under multiple abiotic stress conditions in nature.
- Establishment of experimental conditions that mimic field conditions.

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The results consistent with Umezawa's work (Umezawa *et al.*, 2009) were reported recently by Vald *et al.* and Fujii *et al.* confirming the model.

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