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Regulatory network of gene expression in the drought and cold stress responses

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Molecular and genomic studies have shown that several genes with various functions are induced by drought and cold stresses, and that various transcription factors are involved in the regulation of stress-inducible genes. The products of stress-inducible genes function not only in stress tolerance but also in stress response. Genetic studies have identified many factors that modify the regulation of stress responses. Recent progress has been made in analyzing the complex cascades of gene expression in drought and cold stress responses, especially in identifying specificity and crosstalk in stress signaling.

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Abbreviations

ABA	abscisic acid
aba	*ABA-deficient*
ABF	ABRE-binding factor
abi	*ABA-insensitive*
ABRE	ABA-responsive element
AREB	ABRE-binding protein
bHLH	basic helix–loop–helix
bZIP	basic-domain leucine zipper
CBF	*C-REPEAT-BINDING FACTOR*
CRT	C-repeat
DRE	dehydration-responsive element
DREB	DRE-binding protein
ERD1	*EARLY RESPONSIVE TO DEHYDRATION1*
ERF	ethylene-responsive element binding factor
fry1	*fiery1*
hos	*high expression of osmotically responsive genes*
ICE1	*INDUCER OF CBF EXPRESSION1*
los	*low expression of osmotically responsive genes*
LTI	*LOW-TEMPERATURE-INDUCED*
LUC	luciferase
NCED	9-*cis*-epoxycarotenoid dioxygenase
RD	*RESPONSIVE TO DEHYDRATION*
SnRK	SNF1-related protein kinase

Introduction

Environmental stresses, such as drought, high salinity and low temperature, have adverse effects on plant growth and seed production. Plants respond and adapt to these stresses through various biochemical and physiological processes, thereby acquiring stress tolerance. Many genes respond to drought, salt and/or cold stress at the transcriptional level, and the products of these genes function in the stress response and tolerance [1–3]. Transcriptome analyses using microarray technology [4–6] have identified several genes that are induced by abiotic stresses, and these genes have been classified into two major groups [1–3]. One group encodes products that directly protect plant cells against stresses, whereas the products of the other group regulate gene expression and signal transduction in abiotic stress responses. Molecular and genomic analyses have shown that several different transcriptional regulatory systems are involved in stress-responsive gene induction. Several different sets of *cis*- and *trans*-acting factors are known to be involved in stress-responsive transcription. Some of them are controlled by abscisic acid (ABA) but others are not, indicating the involvement of both ABA-dependent and -independent regulatory systems for stress-responsive gene expression [1–3,7•]. Many genes are induced by both drought and cold stress, suggesting the existence of crosstalk between the drought and cold-stress signaling pathways. The use of mutants isolated from transgenic *Arabidopsis* that contain promoter::luciferase (LUC) constructs provides a powerful method for the analysis of stress signaling pathways [7•,8]. In this short review, we highlight recent progress in understanding the regulation of gene expression in response to drought and/or cold stress, and in revealing complex gene networks for specificity and crosstalk in abiotic-stress-responsive gene expression.

Transcriptome analysis of stress-inducible gene expression using microarray technology

Microarray technology using cDNAs or oligonucleotides has become a powerful and useful tool for analyzing the gene expression profiles of plants that are exposed to abiotic stresses, such as drought, cold and high salinity, or to ABA treatment [4,5,9,10••,11•,12,13•–15•]. Potential *cis*-acting DNA elements have been analyzed by comparing their expression profiles with those of the promoter

sequences of stress-inducible genes [9,10••,11•,13•]. Microarray technology is also useful in identifying target genes for stress-related transcription factors, opening a way to analyze gene networks in abiotic stress responses. There are two major forms of microarray technology: the cDNA-spotted glass microarray and the oligonucleotides-based microarray, which is used by GeneChip [4–6]. Both kinds of microarray have been used to analyze gene-expression profiles under conditions of drought, cold and high salinity stress. First, cDNA microarrays were used to identify drought-, cold- and high-salinity-inducible genes [9,10••,11•,12]. Recently, 299 drought-inducible genes, 54 cold-inducible genes, 213 high-salinity-stress-inducible genes and 245 ABA-inducible genes were identified using a cDNA microarray containing around 7000 independent *Arabidopsis* full-length cDNA groups [10••,11•]. These cDNAs were collected from stress-treated plants as well as normally grown plants. Thousands of stress-inducible genes were identified using a GeneChip array containing oligonucleotides for about 8000 independent *Arabidopsis* genes. The identification of these stress-inducible genes was based on the annotation of these genes on partial genomics sequences [15•]. Many differences in the lists of stress-inducible genes were found between cDNA microarray and GeneChip analyses. This is mainly due to the different sets of genes that are arrayed in the two systems (only 1919 genes are represented in both systems) and different conditions of plant growth and stress treatments (K Maruyama *et al.*, unpublished data).

More than half of the drought-inducible genes are also induced by high salinity and/or ABA treatments, indicating the existence of significant crosstalk among the drought, high-salinity and ABA responses [10••,11•]. By contrast, only 10% of the drought-inducible genes were also induced by cold stress. Many transcription-factor genes were found among the stress-inducible genes, suggesting that various transcriptional regulatory mechanisms function in the drought-, cold- or high-salinity-stress signal transduction pathways [10••,11•,13•–15•]. These stress-inducible transcription factors include members of the DRE-binding protein (DREB) family, the ethylene-responsive element binding factor (ERF) family, the zinc-finger family, the WRKY family, the MYB family, the basic helix–loop–helix (bHLH) family, the basic-domain leucine zipper (bZIP) family, the NAC family, and the homeodomain transcription factor family. These transcription factors could regulate various stress-inducible genes cooperatively or separately, and may constitute gene networks. Functional analysis of these stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to drought, cold and high-salinity stresses.

Transcriptome analysis has also revealed many genes that respond to rehydration after drought stress, indicating their involvement in the process of recovery from abiotic stress [16••]. The products of these genes are thought to function not only in recovery from stress but also in cell growth and elongation. The expression and function of the rehydration-inducible proline dehydrogenase gene (*ProDH*) has been precisely analyzed. This gene is involved in the degradation of the proline that accumulates during dehydration [17]. Promoter analysis of the *ProDH* gene revealed an important *cis*-acting element, ACTCAT, that is involved in rehydration-inducible gene expression [18•]. Many rehydration-inducible gene promoters contain the ACTCAT motif. There are probably many other genes that are induced during the deacclimation process after cold stress. Similar molecular and genomic analyses will help to reveal total plant responses not only during abiotic stress but also during recovery from stress.

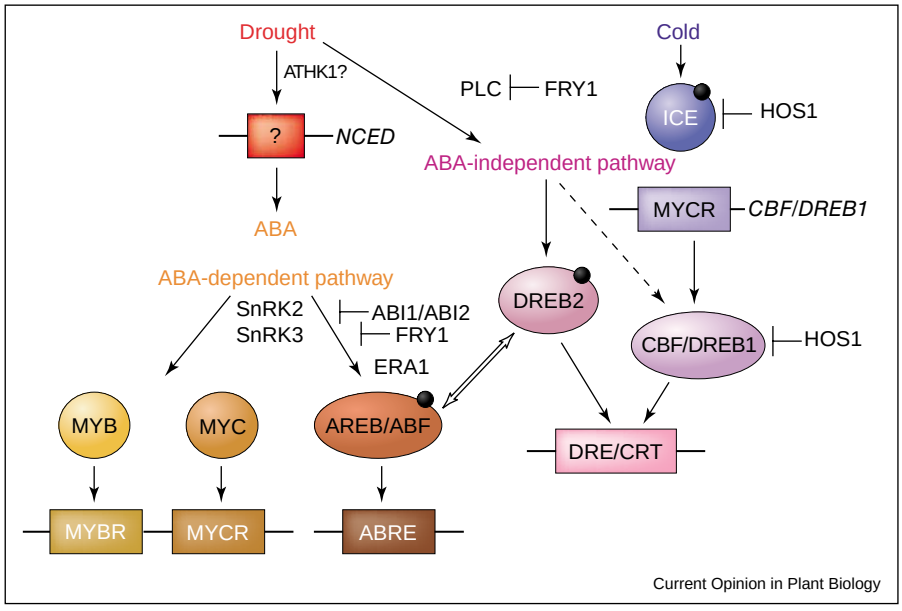
Systems that regulate gene expression in response to drought and cold stress: identification of *cis*-acting elements and their DNA-binding proteins in ABA-independent pathways

The *cis*-acting elements of some genes that have a typical stress-inducible expression profile and the transcription factors that affect the expression of these genes have been analyzed precisely [1–3]. Promoter analyses of drought- and/or cold-inducible genes have provided at least four independent regulatory systems for gene expression [2].

The promoter of a drought-, high-salinity- and cold-inducible gene, *RESPONSIVE TO DEHYDRATION29A* (*RD29A*)/*COLD-REGULATED78* (*COR78*)/*LOW-TEMPERATURE-INDUCED78* (*LTI78*), has been found to contain two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT), that are involved in stress-inducible gene expression [19]. ABRE and DRE/CRT are *cis*-acting elements that function in ABA-dependent and ABA-independent gene expression in response to abiotic stress, respectively (Figure 1). Transcription factors belonging to the ERF/APETALA2 (AP2) family that bind to DRE/CRT have also been isolated and termed *C-REPEAT-BINDING FACTOR* (*CBF*)/*DREB1* and *DREB2* [20,21]. The conserved DNA-binding motif of *DREB1A*/*CBF3* and *DREB2* is A/GCCGAC [22]. The *CBF*/*DREB1* genes are quickly and transiently induced by cold stress, and their products activate the expression of target stress-inducible genes. The *DREB2* genes are induced by dehydration, leading to the expression of various genes that are involved in drought-stress tolerance [21]. The inducible expression of *CBF*/*DREB1* also activates target genes that are involved in acquired stress tolerance.

Overexpression of *CBF*/*DREB1* in transgenics increased tolerance of freezing, drought and high salt concentrations

Figure 1



Regulatory network of gene expression in response to drought and cold stresses: specificity and crosstalk of gene networks. *Cis*-acting elements that are involved in stress-responsive transcription are shown in boxes. Transcription factors that control stress-inducible gene expression are shown in circles or ovals. Small shaded circles indicate the modification of transcription factors in response to stress signals for their activation, such as phosphorylation. The upper part of the figure shows transcription cascades that are involved in rapid and emergency responses to drought and cold stress, such as those involving ICE, DREB2 or NCED. Lower parts of the figure show transcription cascades that are involved in slow and adaptive processes in stress responses, such as those involving AREB/ABF, MYB, MYC and CBF/DREB1. A two-component histidine kinase 1 (ATHK1) is thought to function as an osmosensor, and phospholipase C (PLC) is thought to function upstream of the DREB2 system [2]. HOS1 functions as a negative regulator of ICE and CBF/DREB1 [49]. FRY1 functions as a negative regulator of drought, cold and ABA responses [46]. SnRK2- and SnRK3-like protein kinases are involved in ABA signaling [40–42]. ABI1/ABI2 functions as a negative regulator for ABA signaling. A dashed arrow indicates that CBF4/DREB1D are involved in gene expression in response to drought stress [25•]. The open double-headed arrow suggests crosstalk between DREB2 and AREB/ABF that is based on DRE/CRT's acting as a coupling element for ABRE [55••]. ERA1, ENHANCED RESPONSE TO ABA1; MYBR, MYB recognition site; MYCR, MYC recognition site.

[21,23,24], suggesting that the CBF/DREB1 proteins function without modification in the development of cold-stress tolerance [21]. Many *CBF/DREB1* target genes have been identified using both cDNA and GeneChip microarrays ([9,14•]; K Maruyama *et al.*, unpublished data). Most of these target genes contain DRE- or DRE-related CCGAC core motif sequences in their promoter regions. By contrast, overexpression of *DREB2* in transgenics does not improve stress tolerance, suggesting that DREB2 proteins require posttranslational activation [21]. However, one of the *CBF/DREB1* genes, *CBF4/DREB1D*, is induced by osmotic stress, suggesting the existence of crosstalk between the CBF/DREB1 and the DREB2 pathways [22,25•]. The DREB2 protein is expressed under normal growth conditions and is activated in the early stage of the osmotic stress response through posttranslational modification. The dehydration-inducible CBF/DREB1 may function in next stage of the osmotic stress response. Rice homologs for *CBF/DREB1* and *DREB2*, four *OsDREB1*s and one *OsDREB2* have been identified in the rice genome sequence and function in stress-inducible gene expression [26•]. Overexpression of *OsDREB1A* in *Arabidopsis* revealed that this gene has a similar function to that of its *Arabidopsis* homolog in stress-responsive gene expression and stress tolerance. This indicates that similar transcription factors function in dicotyledons and monocotyledons.

Recently, the *INDUCER OF CBF EXPRESSION1* (*ICE1*) gene was identified through the map-based cloning of the *Arabidopsis ice1* mutation [27], which affected the expression of the CBF3/DREB1A promoter::LUC transgene [28••]. *ICE* encodes a MYC-type bHLH transcription factor that regulates the expression of *CBF3/DREB1A* but not of other *CBF/DREB1* genes. Overexpression of *ICE1* in transgenics resulted in improved freezing tolerance, supporting an important role for *ICE1* in the cold-stress response (Figure 1). Molecular analysis of the *DREB1C/CBF2* promoter has identified a *cis*-acting element that is involved in cold-inducible gene expression and its DNA-binding protein. The DNA-binding protein has been cloned and shown to be a MYC-type bHLH transcription factor that is different from ICE1 (Y Imura *et al.*, unpublished data). These results suggest the redundant involvement of MYC-type bHLH transcription factors in the upregulation of *CBF/DREB1* genes. A cold

signal is necessary for the activation of the ICE proteins but the mechanism of this signal remains to be solved.

Several drought-inducible genes do not respond to either cold or ABA treatment, suggesting the existence of an ABA-independent pathway in the dehydration stress response. These genes include *EARLY RESPONSIVE TO DEHYDRATION1* (*ERD1*), which encodes a Clp protease regulatory subunit, ClpD [29]. The *ERD1* gene is not only induced by dehydration but also upregulated during natural senescence and dark-induced senescence [30•]. Promoter analysis of the *ERD1* gene in transgenic plant indicates that the *ERD1* promoter contains *cis*-acting element(s) that are involved not only in ABA-independent stress-responsive gene expression but also in senescence-activated gene expression. Analysis of the *ERD1* promoter also identified two novel *cis*-acting elements that are involved in induction by dehydration stress and in dark-induced senescence [30•]. Cloning of the DNA-binding proteins for these *cis*-elements will reveal this novel regulatory system (Figure 1).

Cis-acting elements and transcription factors involved in ABA-dependent gene expression

ABA is synthesized *de novo* mainly in response to drought and high-salinity stress but not in response to cold stress. Many stress-inducible genes are regulated by the endogenous ABA that accumulates during drought and high-salinity stress [1,2]. Recently, genes that are involved in ABA biosynthesis have been identified through genetic and genomics analyses [7•,31]. Several of these genes are induced by drought and high salinity but not by cold stress. This indicates important roles for ABA in drought-stress responses. The genes encoding 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme of ABA biosynthesis, are strongly induced by drought stress [32–34]. However, regulatory factors that control the drought-inducible expression of the *NCED* gene have not been elucidated (Figure 1).

ABRE is a major *cis*-acting element in ABA-responsive gene expression (Figure 1). Two ABRE motifs are important in the ABA-responsive expression of the *Arabidopsis* gene *RD29B* [35]. The bZIP transcription factors ABRE-binding protein (AREB)/ABRE-binding factor (ABF) can bind to ABRE and activate ABA-dependent gene expression [35,36]. The AREB/ABF proteins have reduced activity in ABA-deficient *aba2* mutants and in ABA-insensitive *abi1* mutants, and enhanced activity in the ABA-hypersensitive *era1* mutant. Hence, activation of the AREB/ABF proteins has been shown to require an ABA-mediated signal [35], which is probably ABA-dependent phosphorylation. Overexpression of *ABF3* or *AREB2/ABF4* caused ABA hypersensitivity, reduced transpiration rate and enhanced drought tolerance of the transgenics [37•].

The induction of the drought-inducible gene *RD22* is mediated by ABA, and this gene requires protein biosynthesis for its ABA-dependent expression [2,38]. A MYC transcription factor, *RD22BP1 (AtMYC2)*, and a MYB transcription factor, *AtMYB2*, have been shown to bind *cis*-elements in the *RD22* promoter and cooperatively activate *RD22* ([38]; Figure 1). These MYC and MYB proteins are synthesized after the accumulation of endogenous ABA, indicating that their role is in a late stage of the stress responses. Microarray analysis of *MYC/MYB*-overexpressing transgenics revealed target genes for MYC/MYB, such as the alcohol dehydrogenase gene and ABA- or jasmonic-acid (JA)-inducible genes [39••]. Overexpression of both *AtMYC2* and *AtMYB2* not only caused an ABA-hypersensitive phenotype but also improved the osmotic-stress tolerance of the transgenic plants [39••].

Recently, the type-2 SNF1-related protein kinase (SnRK2) that is activated by ABA (OPEN STOMATA1 [OST1]/SnRK2E) has been shown to function upstream of ABA-responsive expression of *RD22* and *RD29B* and of stomatal closure in an ABA signal transduction pathway [40,41]. The *ost1/srk2e* mutant has a wilty phenotype. The SnRK3-like protein kinase, PKS3 (a SALT OVERLY SENSITIVE2 [SOS2]-like protein kinase), functions in general ABA signaling, and interacts with a SOS3-like calcium-binding protein (SCaBP5) and ABI2, a 2C-type protein phosphatase [42]. These protein kinases may be involved in the activation of transcription factors in ABA-responsive gene expression.

In addition to the transcription factors that bind to *cis*-acting elements of stress-inducible genes, many genes for transcription factors of various types are induced by drought and/or cold stress. As described above, microarray analyses have also identified many stress-inducible transcription-factor genes. These transcription factors probably function in stress-inducible gene expression, although their target genes have not yet been identified. Among them, *Arabidopsis* ATHB6, which contains the homeodomain, functions downstream of ABI1 as a negative regulator in the ABA signal transduction pathway [43•]. The soybean transcription factor SCOF-1, a zinc-finger protein, can activate stress-inducible genes and increase freezing tolerance in transgenic plants. SCOF-1 interacts with the GBF-type bZIP protein SGBF-1, which binds to the G-box and ABRE [44].

Genetic analysis of drought and cold-stress signaling pathways

A unique mutant screening system using transgenic *Arabidopsis* plants with a firefly *LUC* reporter gene under the control of the *RD29A* promoter has been developed to screen mutants that have defects in their abiotic-stress signal transduction pathways [45]. Using this system, many *Arabidopsis* mutants have been isolated that have altered induction of stress-responsive genes by drought,

high-salinity, cold and ABA. These mutants exhibited altered expression of the *RD29A::LUC* gene at a constitutive (*cos*), high (*hos*), or low (*los*) level in response to various abiotic-stress or ABA treatments [45]. The mutated genes are thought to function not only upstream of *RD29A* induction in signal transduction pathways but also in posttranscriptional regulation of the activation of the DREB1/CBF, DREB2 and/or AREB/ABF transcription factors.

The occurrence of mutations with differential responses to drought, cold, high-salinity and/or ABA treatments predicts crosstalk among the drought, salinity, cold and ABA signal transduction pathways [31]. The loci of the mutations, such as *fiery1* (*fry1*) [46], *fry2* [47,48], *hos1* [49], *los1* [50], *los2* [51], *los5/aba3* [52], *los6/aba1* [53] and *sad1* [54], have been cloned. The roles of their gene products have been described precisely in recent reviews by Zhu and his colleagues [7•,8]. Among them, three gene products are thought to function directly in transcription. The *hos1* mutation enhances the expression of stress-inducible genes and functions as a negative regulator of a cold-specific signal transduction pathway [49]. HOS1 encodes a novel protein that contains a RING-finger motif, which may function in the degradation of CBF/DREB1 and ICE proteins (Figure 1). It is located in the cytoplasm but moves to the nucleus upon cold treatment, and is thought to control the expression of *CBF/DREB1*. LOS2 is involved in cold-responsive gene expression and encodes a bi-functional enolase [51]. This enzyme is thought to function as a transcription factor that controls STZ/ZAT10, a zinc-finger transcriptional repressor, and to repress *RD29A* expression. FRY2/CPL1 also functions as a transcriptional repressor and contains a region that is partially homologous to the catalytic domain of RNA polymerase II carboxy-terminal domain phosphatases, which are found in humans and yeast [47,48]. FRY2 also contains two double-stranded RNA-binding domains, which may function in mRNA processing. The mutant screening system using transgenic *Arabidopsis* plants containing stress-inducible promoter::LUC provides a powerful tool not only for the analysis of signal transduction pathways upstream of gene expression but also for studies of the posttranscriptional regulation of gene expression. Recently, ICE1 has been identified using the *CBF3/DREB1A* promoter::LUC system as described above [28••].

Complexity of stress-responsive gene networks: specificity and crosstalk

Four transcriptional regulatory systems have been reported, two of them are ABA-independent whereas two are ABA-dependent. Crosstalk between these regulatory systems has, however, been suggested by genetic and molecular analyses. Genomic analyses of stress-inducible genes using microarrays have recently revealed crosstalk in stress-responsive gene expression [10••,11•,15•]. Most drought-inducible genes are also induced by high-salinity stress, and many drought-inducible genes are also induced by ABA. However, only 10% of drought-inducible genes are also cold-inducible. In fact, ABA is involved in osmotic-stress-responsive gene expression but does not play an important role in the low-temperature stress response.

Crosstalk occurs at the interaction between different *cis*-acting elements. Many drought- and cold-inducible genes contain both DRE/CRT and ABRE motifs in their promoters. These *cis*-acting elements are thought to function independently. However, precise analysis of these *cis*-acting elements in *RD29A* gene expression revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought stress [55••]. This indicates that interactions between different transcription machineries function to provide crosstalk between different stress signaling pathways. Recently, an osmotic-stress inducible *CBF4/DREB1D* gene has been identified [25•]. Genes of the *CBF/DREB1* family are mainly induced by cold stress, but the drought-inducible gene *CBF4* functions to provide crosstalk between DREB2 and CBF/DREB1 regulatory systems. The drought-inducible expression of *CBF4* is controlled by ABA-dependent pathways, suggesting that CBF4 may function in the slow response to drought that relies on the accumulation of ABA. A maize DRE-binding protein, DBF1, has been shown to function as a transcriptional activator of the *rab17* promoter by ABA [56]. This also suggests the existence in some plants of an ABA-dependent pathway for the regulation of stress-inducible genes that involves DRE/CRT. Cold-induced gene expression through DRE/CRT is greatly enhanced by a light signal mediated by phytochrome B, which indicates crosstalk between abiotic-stress and light-signaling pathways [57•].

The *fry1* mutation results in elevated levels of inositol 1,4,5-triphosphate (Ins[1,4,5]P_3) in both control and ABA-treated plants. It also causes the super-induction of ABA- and stress-inducible genes in response to ABA, salt and dehydration, and gives rise to plants that are less tolerant to freezing, drought and salt stress [46]. The *FRY1* gene encodes an inositol polyphosphate 1-phosphatase that is involved in Ins(1,4,5)P_3 signaling. *FRY1* functions as a negative regulator of ABA and stress signaling, and provides genetic evidence that phosphoinositols have an important role in ABA and stress-related signal transduction in plants (Figure 1). Biochemical analysis has shown that phospholipase C functions upstream of *RD29A* expression in osmotic stress signaling [58], which may be negatively regulated by FRY1.

Conclusions and perspectives

Molecular and genomic analyses have revealed many genes that are induced by abiotic stress and their functions in stress-responsive gene expression and stress

tolerance. The many stress-inducible genes include those that encode signaling molecules, such as enzymes involved in phospholipid metabolism and various protein kinases including mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases (CDPKs), receptor-like kinases and histidine kinases [2,7•,8]. Transgenic plants and T-DNA or transposon-tagged mutants have been analyzed to reveal the function of stress-responsive loci in *Arabidopsis*. The reverse genetics approach will be even more important in extending our understanding of regulatory factors in stress signaling. Genetic analysis of regulatory factors using the stress-inducible promoter::LUC system is promising, and can be used to reveal not only upstream stress signaling pathways but also posttranscriptional regulatory systems that modify abiotic stress responses. In the future, a combination of molecular, genomic and genetic analyses will be used to elucidate the complex systems that regulate the responses of gene expression to abiotic stresses.

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- •• of outstanding interest

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