Temperature variations at the nonextreme range modulate various processes of plant growth, development, and physiology, but how plants perceive and transduce these temperature signals is not well understood. Moderate cooling from 28°C to 22°C induces transcription of a number of genes in salicylic acid-dependent and -independent manners. Here, we report the study of the transcriptional control of the BON1-ASSOCIATED PROTEIN1 (BAP1) gene that is responsive to a moderate decrease of temperature as well as to many environmental stimuli. Using reporter genes under the control of series of regions of the BAP1 promoter, we identified a 35-bp fragment that is necessary and sufficient for the BAP1 transcript induction by a moderate cooling. This fragment also confers an induction of BAP1 by cold and reactive oxygen species-generating paraquat. Furthermore, the INDUCER OF CBF EXPRESSION1 (ICE1) protein that is involved in transcriptional control of cold responses is found to bind to a MYC element in this promoter and is required for the cooling induction of BAP1. The ice1 mutant has a low induction of BAP1 and enhanced resistance to a bacterial pathogen. Thus, responses to a moderate decrease in temperature may utilize components in the cold response as well as a potentiating signaling involving salicylic acid.

Plants, being sessile, have evolved to adapt to their environment to maximize their fitness and reproduction. One of the major environmental factors they monitor and respond to is temperature, which fluctuates daily and seasonally. Almost all processes of growth and development are modulated by temperature at the molecular, cellular, physiological, and ecological levels (Long and Woodward, 1988; Penfield, 2008). Transcriptional regulation is one of the major responses plants assume to achieve adaptation. Both cold acclimation and heat acclimation involve the upregulation of transcription of genes that are important for adaptation to extreme conditions (Hua, 2009). For cold responses, one transcriptional cascade has been identified by molecular and genetic studies on a number of cold-induced genes named COLD REGULATED (COR) or LOW TEMPERATURE INDUCED (Thomashow, 1999). This cascade includes the A/GCCGAC motif named C-REPEAT (CRT)/DEHYDRATION RESPONSIVE ELEMENT (DRE) that is found in the promoter region of many COR genes (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006). The CTR element is bound by AP2 domain-containing transcription factors CRT BINDING FACTOR (CBF)/DRE BINDING PROTEIN (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006). The CBF3 gene is transcriptionally regulated by a MYC-type transcription factor INDUCER OF CBF EXPRESSION1 (ICE1) through ICEr1 and ICEr2 sequences in its promoter (Chinnusamy et al., 2003). The significance of this transcriptional cascade is demonstrated by the profound effect on cold/freezing tolerance with altered expression of CBFs and ICE1 (Chinnusamy et al., 2003; Sung et al., 2003). For heat shock responses, transcriptional cascade has also been identified to control the expression of HEAT SHOCK PROTEIN (HSP; Vierling, 1991). Heat shock factors are transcription factors that bind to the heat shock element consisting of AGAAnTTCT found in promoters of many HSP genes (Kotak et al., 2007; von Koskull-Döring et al., 2007). Some of the heat shock factors have been demonstrated to be essential for thermotolerance (Sung et al., 2003; von Koskull-Döring et al., 2007).

Moderate temperature variations also greatly influence many aspects of growth and development such as growth rate (Cuadrado et al., 1989), flowering time (Blázquez et al., 2003), metabolism (Kaplan et al., 2004), hormonal responses (Larkindale and Huang, 2004), and circadian rhythms (Gardner et al., 2006). Additionally, they influence interaction between plants and other organisms, including plant disease resistance (Wang et al., 2009). Relatively less is known about the molecular mechanism underlying plants’
responses to these moderate temperature variations. Recently, it is shown that ARP6, a subunit of the SWR1 complex, represses expression of warm genes at low temperatures in Arabidopsis (*Arabidopsis thaliana*), likely through the temperature-sensitive occupancy of the alternative histone H2A.Z on promoter sequences (Kumar and Wigge, 2010). Our early studies with marker genes revealed shared and distinct mechanisms for responses to drastic and moderate decrease in temperature at the transcriptional level (Wang and Hua, 2009). Both a salicylic acid (SA)-dependent and an SA-independent pathway are found to function in cooling induction of genes. A small decrease of temperature from 28°C to 22°C induces COR15a expression in an SA-independent manner. The induction is mediated by the CBF genes and could contribute to the enhanced cold tolerance. It appears that some of the cooling responses may prepare plants to anticipate and prepare for extreme conditions.

We initiated an investigation on the SA-dependent transcriptional response to moderate temperature decrease in the *BONI-ASSOCIATED PROTEIN1 (BAP1)* gene. BAP1 is a membrane-associated C2 domain protein that negatively regulates defense responses (Hua et al., 2001; Yang et al., 2006; Yang et al., 2007). Its loss-of-function mutant has constitutive defense responses, and its overexpression in plants and yeast (*Saccharomyces cerevisiae*) suppresses programmed cell death induced by a number of reagents including pathogens, proapoptotic genes, and reactive oxygen species (ROS; Yang et al., 2007). BAP1 is itself induced by multiple stimuli including temperature variations, mechanical stress, and biotic stresses (op den Camp et al., 2003; Yang et al., 2006). The *BAP1* gene has a higher expression level at stable 22°C than at 28°C and is rapidly induced by a cooling from 28°C to 22°C. Interestingly, a number of genes involved in defense responses including *BONI, BAP1, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Wiermer et al., 2005)*, and *PHYTOLEXIN DEFICIENT4 (PAD4; Birger et al., 1999*) have differential expression at 28°C and 22°C (Yang and Hua, 2004). It is possible that this temperature modulation reflects a more critical role of these genes to regulate defense responses at 22°C than 28°C as the loss of *BONI* or *BAP1* function has more detrimental effects at 22°C than 28°C.

The cooling induction of *BAP1* provides an entry point to dissect the transcriptional response to moderate decrease in temperature in the SA-dependent manner. Here, we report the identification of a 35-bp fragment in the *BAP1* promoter as a cis-acting region to confer response to a cooling from 28°C to 22°C. This temperature-sensitive region also mediates responses to cold and ROS but not to wounding and pathogen infection. Furthermore, ICE1 is found to bind to this element and mediate the induction of *BAP1* by cooling, cold, and ROS. Thus, this study reveals a cooling induction utilizing the ICE1 protein, suggesting a common mechanism for responding to extreme and nonextreme temperatures.

**RESULTS**

**Induction of BAP1 Expression by a 28°C to 22°C Shift Requires the SA Pathway**

Earlier studies revealed both SA-dependent and -independent pathways for cooling induction from 28°C to 22°C (Wang and Hua, 2009). As *BAP1* expression is induced by SA (Yang et al., 2006), we determined whether the cooling induction of *BAP1* from 28°C to 22°C requires SA and the function of *PAD4*, a regulator of cell death and resistance. To this end, we used Arabidopsis plants either containing *nahG* coding for a bacterial enzyme degrading SA (Clarke et al., 2000) or with a loss-of-function (*pad4*) mutation (Jirage et al., 1999). The wild-type Columbia-0 (Col-0), *nahG*, and *pad4* plants were subjected to the 28°C to 22°C cooling treatment, and the *BAP1* induction was analyzed by RNA blots. In contrast to the wild type, neither *nahG* nor *pad4* had up-regulated *BAP1* expression 6 h after shift (Fig. 1A), indicating a requirement of SA and *PAD4* for this rapid induction.

*NPR1* mediates many aspects of SA responses and is a positive regulator of systemic acquired resistance (Durrant and Dong, 2004). RNA-blot analysis shows that the *npr1* mutant had the same induction of *BAP1* as the wild type, indicating that *NPR1* is not involved in this induction (Fig. 1A). A similar genetic requirement was observed in the up-regulation of the *BAP1* in the *bon1* mutant. *BAP1* is induced in the *bon1* mutant compared with the wild type and this induction is abolished in *bon1-1nahG* but not in *bon1-1npr1-1* (Fig. 1B). Thus, *BAP1* induction by cooling and the loss of *BON1* function are both mediated by a *NPR1*-independent SA function.

**Requirement of SA for BAP1 Induction by Multiple Stimuli**

The *BAP1* gene is induced by multiple stimuli including temperature variations, mechanical stresses, and biotic stresses (Yang et al., 2006). One common event following these stimuli might be the generation of ROS. Indeed, it has been shown that singlet oxygen species induces *BAP1* rapidly (op den Camp et al., 2003), and hydrogen peroxide also induces *BAP1* according the public array data (The Arabidopsis Information Resource array no. 185). To analyze the induction of *BAP1* by the other ROS superoxide, we treated plants with paraquat to induce the generation of superoxide in chloroplasts (Babbs et al., 1989). The *BAP1* transcript was greatly induced at 4 h after the spray with paraquat compared with the mock treatment (Fig. 1C). Thus, *BAP1* could be induced by various ROS.

We tested whether SA and *PAD4* are required for *BAP1* induction by other stimuli by comparing responses in *nahG* and *pad4* to those of the wild type. RNA-blot analysis showed that *BAP1* induction by cold, wounding, or paraquat was similar in *nahG* and *pad4* as in the wild type (Fig. 1, D–F). Thus, the 28°C to
Isolation of a Temperature-Responsive Element in the BAP1 Promoter

We determined that the induction of BAP1 by cooling is at the transcriptional level. A 2.1-kb fragment of the BAP1 promoter was fused to the reporter gene GUS and the pBAP1::GUS transgene (pBG) was transformed into wild-type Arabidopsis Col-0 plants. More than 50 transgenic plants were generated and 10 were tested for the induction of GUS by cooling treatment. Eight of the 10 lines showed an induction of GUS transcripts by a temperature shift from 28°C to 22°C for 6 h (Fig. 2A). Thus, BAP1 induction by cooling is at the transcriptional level and its promoter contains a temperature-responsive (TR) element(s).

To define this TR element in the BAP1 promoter, we generated a series of GUS promoter fusions with various lengths of the truncated BAP1 promoter (Fig. 2B). The five promoter fragments started from −902, −660, −553, −255, and −169 bp, respectively, and ended at 22°C induction of BAP1 appears to be unique among other stimuli in that it requires SA and PAD4.

Isolation of a Temperature-Responsive Element in the BAP1 Promoter

We determined that the induction of BAP1 by cooling is at the transcriptional level. A 2.1-kb fragment of
−1 bp relative to the translation start site and the transcriptional initiation site of BAP1 is 27-bp upstream of the translation start site. These fusions are named pbG4, pbG8, pbG5 pbG6, and pbG7, respectively.

All of these fusion constructs were transformed into wild-type Col-0 plants. More than 10 independent transgenic lines for each promoter deletion fusion were subject to cooling treatment, and the expression of both the endogenous BAP1 gene and the reporter GUS genes before and after the treatment were compared. We found that GUS staining was not sensitive and accurate enough to reflect the transcriptional regulation by the BAP1 promoter, so we used RNA-blot hybridization for all promoter analyses. The majority of each of the pbG4, pbG8, pbG5, and pbG6 lines (10 of 10, 10 of 10, five of 10, and eight of 10) showed GUS induction by the 28°C to 22°C shift (Fig. 2C). In contrast, none of the pbG7 lines exhibited induction by cooling (Fig. 2C) while the endogenous BAP1 exhibited induction. Thus, the TR elements reside in between −255 and −169 bp upstream to the translational start site of BAP1.

**Refining the TR Element to a 35-bp Fragment**

To determine whether this 87-bp fragment is sufficient to confer temperature response, we fused this fragment with a minimal 35S promoter to the GUS reporter gene and named it pbGES. Five independent transgenic lines were tested and four of them exhibited GUS induction after a 28°C to 22°C shift analyzed by RNA blots (Fig. 2D), indicating that this fragment contains the TR element. In general, a shorter promoter confers a relatively weaker induction than a longer promoter, suggesting that there are additional activating elements in the BAP1 promoter although this 87-bp fragment is the most prominent one responsive to cooling.

To refine the TR element, we divided this fragment into three overlapping subfragments named A, B, and C (Fig. 2E). Three repeats of A, four repeats of B, and four repeats of C were fused, respectively, to the GUS reporter gene with a minimal 35S promoter, and they were named pbGES1, pbGES2, and pbGES3 (Fig. 2E). None of the pbGES1 or pbGES2 transgenic lines had any induction of GUS by a 28°C to 22°C shift, while four of the 13 pbGES3 lines exhibited an induction of GUS similarly to the endogenous BAP1 gene (Fig. 2D). Therefore, the fragment ESA is responsive to a cooling of 28°C to 22°C downshift and contains the TR element. However, there was a lower proportion of transgenic lines of pbGES3 (four of 13) exhibiting induction compared with that of pbGES (four of five), indicating that ESB and ESC contain additional element(s) enhancing cooling induction by ESA.

**Requirement of the ESA for BAP1 Induction by Other Stimuli**

To determine whether BAP1 uses the same or different cis-element for induction by various stimuli, we assayed responses of pbG lines to other stimuli. A cold (4°C) treatment induced GUS expression in pbG4, pbG8, pbG5, and pbG6 lines but not pbG7 lines (Fig. 3A). Similarly, paraquat treatment induced GUS expression in pbG4, pbG8, pbG5, and pbG6 lines but not in the pbG7 lines (Fig. 3B), suggesting that cold and paraquat responses are mediated by the same ES fragment as cooling.

We found that the ESA fragment is sufficient for cold and paraquat induction. The GUS transcript was induced by cold and paraquat in the pbGES3 lines (Fig. 3, C and D) but not pbGES2 or pbGES3 lines (data not shown). Thus, the same ESA is responsible for cold and paraquat induction.

In contrast, wounding induction of BAP1 is not mediated by ESA. As this wounding response is rapid and robust, and we could directly utilize tissue staining to assay GUS activity. Transgenic lines of pbG6, pbG7, pbG8, and pbGES were damaged with needless syringe and GUS activities were assayed half an hour after wounding. All of the lines except for pbGES gave a strong GUS staining (Fig. 3E). Thus, wounding response is mediated by the 169-bp fragment 5′ to the ESA fragment.

SA induction of BAP1 does not appear to be mediated by the ESA element either. We treated three pbGES (1, 2, and 3) lines and two pbGES3 (1 and 2) lines with SA or water. Only the pbGES2 line exhibited a very weak GUS up-regulation and none of the others showed GUS induction upon SA treatment (Fig. 3F). Thus, the ESA fragment mediates BAP1 induction by some but not all stimuli.

**Potential cis-Elements in the ESA Sequences**

No obvious cold- or heat-inducible element is recognized in the ESA fragment. Therefore, we subjected it to plant cis-acting elements search through online softwares. Four motifs were found using PLACE (http://www.dna.affrc.go.jp/PLACE/index.html). The first one is A(A/T)TTCAAA starting from nucleotide position 2, an ethylene-responsive element initially found in the promoter of GLUTATHIONE S-TRANSFERASE1 (GST1; Itzhaki et al., 1994). The second is CAnTTG at nucleotide 6, a MYC binding site, conferring binding sites for bHLH transcription factors (Meshi and Iwabuchi, 1995). The third is (C/T)ACT at nucleotide 19, an element for mesophyll-specific expression in the promoter of phosphoenolpyruvate carboxylase gene (Gowik et al., 2004). The last one is AATACCTAAT, a Suc-responsive element conserved among Suc-regulated genes (Grierson et al., 1994). Two other elements were identified in ESA with PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). One is a CAAT box starting at position 5, a common cis-acting element in the promoter or enhancer regions. The other is similar to Gap-box CAAATGAA(A/G) A, part of a light-responsive element (Conley et al., 1994).
ICE1 Binds to the ESA Fragment

ICE1 is a MYC-type bHLH transcriptional activator that regulates the transcription of *CBF3* in the cold response by recognizing the consensus DNA sequence CAnnTG in its promoter. To test whether ICE1 binds to the potential MYC recognition site AaaTG in the ESA element, we carried out an electrophoresis mobility shift assay (EMSA). The ICE1-GST fusion protein (Miura et al., 2007) expressed and purified from *Escherichia coli* was incubated with a labeled DNA probe containing three copies of the ESA fragment (3ESA; Fig. 4A). The ESA probe was shifted to a large *M* in the presence of ICE1:GST (Fig. 4B). The shifted band was abolished by addition of increasing amount of cold unlabeled DNA of 3ESA (Fig. 4B), indicating that ICE1 is able to specifically bind to the ESA element. To examine if the binding requires to the MYC binding site CAAAAG, we mutated this motif into CTATGTG in ESA (Fig. 4A) and used this 3ESAasm as a competitor for the wild-type 3ESA probe in EMSA. In contrast to the wild-type 3ESA, competition was not observed with the unlabeled DNA of 3ESAasm (Fig. 4B), indicating that ICE1 binds specifically to the MYC recognition site in the ESA element.

ICE1 Is Associated with the Promoter Region of *BAP1* in Vivo

We carried out chromatin immunoprecipitation (ChIP) assay to determine whether ICE1 is associated with the promoter region of *BAP1* in vivo under cooling or cold treatment. To this end, we utilized transgenic lines of p35S::ICE1:GFP (Kanaoka et al., 2008) to facilitate the detection of ICE1. Cold and cooling induction was carried out by shifting 3-week-old plants grown at 22°C to 4°C or 2-week-old plants grown at 28°C to 22°C, respectively. Anti-GFP antibodies were used to immunoprecipitate (IP) ICE1:GFP and its associated chromatin from the treated tissues; and the presence of the *BAP1* promoter fragment in the IP was detected by PCR using a primer pair amplifying the promoter containing the ESA element. To this end, we utilized transgenic lines of p35S::ICE1:GFP (Kanaoka et al., 2008) to facilitate the detection of ICE1. Cold and cooling induction was carried out by shifting 3-week-old plants grown at 22°C to 4°C or 2-week-old plants grown at 28°C to 22°C, respectively. Anti-GFP antibodies were used to immunoprecipitate (IP) ICE1:GFP and its associated chromatin from the treated tissues; and the presence of the *BAP1* promoter fragment in the IP was detected by PCR using a primer pair amplifying the promoter containing the ESA element. To this end, we utilized transgenic lines of p35S::ICE1:GFP (Kanaoka et al., 2008) to facilitate the detection of ICE1. Cold and cooling induction was carried out by shifting 3-week-old plants grown at 22°C to 4°C or 2-week-old plants grown at 28°C to 22°C, respectively. Anti-GFP antibodies were used to immunoprecipitate (IP) ICE1:GFP and its associated chromatin from the treated tissues; and the presence of the *BAP1* promoter fragment in the IP was detected by PCR using a primer pair amplifying the promoter containing the ESA element.

Cooling and Cold Induction of *BAP1* Is Compromised in the *ice1* Mutant

We further tested the model that ICE1 is directly involved in the transcriptional regulation of *BAP1*...
We further tested whether ICE1 protein is required for BAP1 induction by other stimuli. The ice1-1 mutant and the wild type were sprayed with either 20 μm paraquat or 2 mM SA. RNA blots show that BAP1 induction by paraquat was totally abolished but its induction by SA was not altered in ice1-1 (Fig. 4, E and F). These results were consistent with the finding that the ESA element is required for BAP1 induction by ROS but not SA treatments (Fig. 3, D and F). Therefore, ICE1 might be involved in transcriptional regulation of BAP1 by temperature and ROS.

The ice1 Mutant Is More Resistant to Virulent Bacterial Pathogen Than the Wild Type

BAP1 is a negative regulator of cell death and SA-mediated disease resistance, and the bap1-1 mutant is more resistant to virulent bacterial pathogens (Yang et al., 2006). To determine if the low induction of BAP1 in the ice1-1 mutant renders the ice1-1 mutant more resistance to virulent pathogens, we assayed growth of Pseudomonas syringae pv tomato DC3000 in the ice1-1 mutant. As the ice1-1 mutant has more stomata that might facilitate bacterial invasion, we inoculated plants by dipping as well as vacuum infiltration to minimize the possible effects of easier entry of bacteria in ice1. In both methods, we saw an enhanced resistance to the bacterial pathogen in ice1. Compared with the wild type at 3 d after inoculation, there was a 7- and 8-fold decrease of bacterial growth in ice1-1 in dipping and infiltration, respectively, while there was, respectively, a 75- and 118-fold decrease in bap1-1 (Fig. 5). Thus, the ice1-1 mutant is more resistant to the bacterial pathogen than the wild type.

DISCUSSION

Earlier studies suggest multiple transcriptional pathways in plant responses to a moderate decrease in temperature. In this study, we analyzed the transcriptional induction of the BAPI gene by temperature as well as other stimuli in Arabidopsis. BAPI is a
We further showed genetically and biochemically that COR15a and a few other defense-related genes require SA (Wang and Hua, 2009). This study finds the SA-dependent induction of BAP1 utilizes ICE1, which raises the possibility that other SA-dependent cooling induction also requires ICE1. Although BAP1 can be induced by SA, this induction is not mediated by the same TR element for cooling. Therefore, SA and ICE likely operate independently on the promoter of BAP1, and they work together to promote its cooling induction. Intriguingly, cold induction of BAP1 utilizes the same TR element as cooling but does not require SA. Although we cannot exclude the possibility that a different motif in the same TR fragment mediates cold induction, it is likely that the same motif mediates both cold and cooling induction. In the latter case, induction is likely weaker by cooling than by cold and an additional factor mediating SA signals could work synergistically with ICE1 to fully activate cooling induction.

ICE1 is identified in this study as a transcription factor that mediates cooling induction of BAP1 possibly through directly binding to the MYC motif in the TR fragment. Originally isolated as a regulator of cold-induced transcription and freezing tolerance, ICE1 was later found to play additional roles in stomata differentiation (Kanaoka et al., 2008). Transcriptome analysis revealed that the ice1 mutation affects gene expression even when plants are not cold treated (Lee et al., 2005), suggesting a broader role of ICE1 in environmental response and development. Previous study of siz1 and ice1 also revealed an up-regulation of SA-related defense response genes such as PRI1 (Miura and Ohta, 2010). In this study, we found that the ice1-1 mutant has enhanced resistance to a virulent bacterial pathogen, similarly to the bap1 loss-of-function mutant although at a lesser degree. This phenotype is most likely due to postinvasion resistance as the ice1-1 mutant has more stomata than the wild type that presumably would make it more susceptible to pathogens. It raises a possibility that up-regulation of SA-related defense responses is due to down-regulation of the negative regulator of BAP1 or other regulators in ice1-1.

The cooling induction of BAP1 is likely carried out by ICE1 and its homologs such as SCRM2/ICE2 (Chinnusamy et al., 2003; Kanaoka et al., 2008). The ice1-1 mutation is dominant, possibly interfering with the function of itself and its homologs. We found that cooling induction of BAP1 was still present in the loss-of-function mutants ice1-2 and scrm2-1, suggesting that ICE1 has overlapping functions with SCRM2 or other homologs in regulating BAP1 induction. The lethality of the ice1-2 scrm2-1 double mutant prevented us to

**Figure 5.** The ice1-1 mutant is more resistant to a virulent *P. syringae* than the wild type. The virulent pathogen *P. syringae* pv. *tomato* DC3000 was inoculated either by vacuum infiltration (A) or dipping (B) at 10^6 colony-forming units/mL on the wild-type Col-0, ice1-1, and bap1-1 18-d-old plants. The amount of bacteria in plants 0 d postinoculation (DPI) and 3 d postinoculation were measured. Both ice1-1 and bap1-1 exhibited significant reduction of bacterial growth compared with the wild type in both inoculation methods. An asterisk indicates significant differences by the *t* test (*P* < 0.05).
vigorously test this hypothesis. Therefore, it has yet to be determined how much ICE1 and its homolog(s) each contribute to this induction.

The TR fragment also mediates BAPI induction by paraquat and this induction appears to be dependent on ICE1 as well. There are at least three known cis-elements for response to various ROS. One is the octa-nucleotide that is partly required for the induction of GST gene by hydrogen peroxide (Chen and Singh, 1999). The second is the as-1 element in the tobacco (Nicotiana tabacum) GST gene responsible for its induction by ROS (Garretón et al., 2002). The third one is a CORE element responsive to oxidative stress in the promoters of three antioxidant defense genes (Tsuchamoto et al., 2005). Although the ROS-responsive element needs to be further defined in the TR fragment, there is an interesting possibility that G box might be a new cis-element responsive to ROS. Intriguingly, ICE1 and its homolog(s) appear to be involved in ROS regulation of BAPI transcription. How the ROS and temperature signals interact and integrate in transcriptional regulation awaits further investigation.

In sum, we have revealed shared regulators for plants to respond to both moderate and drastic decrease in temperature, suggesting a common mechanism in temperature perception and signaling. In addition, the combinatory regulation by a decrease of temperature and SA on plant defense-related genes might enable plants to acquire enhanced resistance in cooling environment. Further study on the cooling induction of BAPI might facilitate the understanding of temperature sensing in plants and how temperature signal interacts with other environmental stimuli to regulate gene expression.

MATERIALS AND METHODS

Plant Material and Plant Treatment

Arabidopsis (Arabidopsis thaliana) plants were grown at 22°C or 28°C under 24 h (for growth) or 12 h (for pathogen test) of fluorescent light (100 µmol m⁻² s⁻¹) per day with 50% to 70% relative humidity. Arabidopsis seeds were either directly sowed on soil or selected on plates before being transferred to soil. Arabidopsis plants were exposed to different stimuli. Wounding treatment was done by squeezing leaves with a 1-mL needless syringe or cut with a pair of scissors. Temperature-shift assays were performed by moving plants from one growth environment to another while maintaining other parameters constant in growth chambers. For paraquat induction, a solution of 20 µM paraquat and 0.1% Tween 20 was sprayed onto Arabidopsis plants as previously described (op den Camp et al., 2005). For SA treatment, a solution of 2 mM SA and 0.1% Tween 20 was sprayed onto Arabidopsis plants (op den Camp et al., 2003). For RNA blot analysis at 4 h (paraquat) or 1 d (SA) after spray treatment used a solution with 0.1% Tween 20. Seedlings were collected for RNA gel blots were hybridized with rRNA bands to ensure equal loading. RNA gel blots were hybridized with rRNA bands to ensure equal loading. Temperature-shift assays were performed by moving plants from one growth environment to another while maintaining other parameters constant in growth chambers. For paraquat induction, a solution of 20 µM paraquat and 0.1% Tween 20 was sprayed onto Arabidopsis plants as previously described (op den Camp et al., 2005). For SA treatment, a solution of 2 mM SA and 0.1% Tween 20 was sprayed onto Arabidopsis plants (op den Camp et al., 2003). For RNA blot analysis at 4 h (paraquat) or 1 d (SA) after spray treatment used a solution with 0.1% Tween 20. Seedlings were collected for RNA gel blots were hybridized with rRNA bands to ensure equal loading. RNA gel blots were hybridized with rRNA bands to ensure equal loading. Temperature-shift assays were performed by moving plants from one growth environment to another while maintaining other parameters constant in growth chambers. For paraquat induction, a solution of 20 µM paraquat and 0.1% Tween 20 was sprayed onto Arabidopsis plants as previously described (op den Camp et al., 2005). For SA treatment, a solution of 2 mM SA and 0.1% Tween 20 was sprayed onto Arabidopsis plants (op den Camp et al., 2003). For RNA blot analysis at 4 h (paraquat) or 1 d (SA) after spray treatment used a solution with 0.1% Tween 20. Seedlings were collected for RNA gel blots were hybridized with rRNA bands to ensure equal loading. Arabidopsis (Arabidopsis thaliana) plants were grown at 22°C or 28°C under 24 h (for growth) or 12 h (for pathogen test) of fluorescent light (100 µmol m⁻² s⁻¹) per day with 50% to 70% relative humidity. Arabidopsis seeds were either directly sowed on soil or selected on plates before being transferred to soil.

Plasmid Construction

Standard molecular techniques were used (Sambrook et al., 1989). A BAPI promoter fragment of 2.1 kb was amplified from the bacterial artificial chromosome clone F22013 (from Arabidopsis Biological Resource Center) and fused to the GUS reporter gene in the binary vector pZPGUS2 (Diener et al., 2000) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with primers designed (op den Camp et al., 2003) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with primers designed (op den Camp et al., 2003) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with primers designed (op den Camp et al., 2003) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with primers designed (op den Camp et al., 2003) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with primers designed (op den Camp et al., 2003) to generate the pBG2 plasmid.

RNA-Blot Analysis

Total RNAs were extracted from 3-week-old plants using TRIReagents (Molecular Research) according to the manufacturer’s protocol. Twenty micrograms of RNA for each sample was resolved on 1.2% agarose gel containing 1.8% formaldehyde. Ethidium bromide was used to visualize the RNA bands to ensure equal loading. RNA gel blots were hybridized with gene-specific, 32P-labeled, single-stranded DNA probes.

GUS Activity Analysis

Plant tissues were lightly fixed half an hour later after wounding and incubated overnight at 37°C in staining solution as described previously (Hua et al., 2001). Tissues were then cleared through ethanol series.

EMSA

The ICE1-GST fusion protein was expressed and purified as previously described (Miura et al., 2007). EMSA was carried out similarly to previously described (Zhu et al., 2003). The DNA fragment containing the ESA element was labeled by the Klenow fragment (New England Biolabs) with [32P]-dCTP. Labeled DNA probes of 10 fmol were incubated with 0.2 µg of purified ICE1-GST fusion protein at 25°C for 20 min and the resulting DNA-protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel and visualized by autoradiograph. For the competition experiment, unlabeled competitors were incubated with purified ICE1-GST fusion proteins on ice for 15 min prior to the addition of labeled probe.

ChIP Assay

ChIP experiment was carried out following protocol previously described (Kwon et al., 2005; Sridhar et al., 2006). A BAPI-specific fragment containing the ESA element was amplified by using the following primers: 5’-ATGAA-CTAACACAGCAAAGAG-3’ and 5’-TACATTGGAAGGGTGACAAGG-3’; CBF3-specific fragment containing the ICE1 binding site was amplified by using the following primers: 5’-GCCAATAAATGCAGGTTAACATC-3’ and 5’-TGTTAATGGCAGCTAAACTATAC-3’. PCR reaction was carried out for 40 cycles of 50 s at 94°C, 50 s at 55°C, and 1 min at 72°C.

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