Induction of BAP1 by a Moderate Decrease in Temperature Is Mediated by ICE1 in Arabidopsis[1][C][OA]

Ying Zhu2,3, Huijun Yang2, Hyung-Gon Mang, and Jian Hua*
Department of Plant Biology, Cornell University, Ithaca, New York 14853

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 BAPI 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 up-regulation 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 transcriptional factors that bind to the heat shock element consisting of AGAAnnTCTCT 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 BON1, BAPI, ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Wiermer et al., 2005), and PHYTOLEXIN DEFICIENT4 (PAD4; Jirage 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 BON1 or BAP1 function has more detrimental effects at 22°C than 28°C.

The cooling induction of BAPI 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 BAPI 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, ICEI is found to bind to this element and mediate the induction of BAPI by cooling, cold, and ROS. Thus, this study reveals a cooling induction utilizing the ICEI protein, suggesting a common mechanism for responding to extreme and nonextreme temperatures.

RESULTS

Induction of BAPI 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 BAPI expression is induced by SA (Yang et al., 2006), we determined whether the cooling induction of BAPI 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 BAPI induction was analyzed by RNA blots. In contrast to the wild type, neither nahG nor pad4 had up-regulated BAPI 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 BAPI 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 BAPI in the bon1 mutant. BAPI is induced in the bon1-1 mutant compared with the wild type and this induction is abolished in bon1-1nahG but not in bon1-1npr1-1 (Fig. 1B). Thus, BAPI induction by cooling and the loss of BON1 function are both mediated by a NPR1-independent SA function.

Requirement of SA for BAPI Induction by Multiple Stimuli

The BAPI 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 BAPI rapidly (op den Camp et al., 2003), and hydrogen peroxide also induces BAPI according the public array data (The Arabidopsis Information Resource array no. 185). To analyze the induction of BAPI by the other ROS superoxide, we treated plants with paraquat to induce the generation of superoxide in chloroplasts (Babbs et al., 1989). The BAPI transcript was greatly induced at 4 h after the spray with paraquat compared with the mock treatment (Fig. 1C). Thus, BAPI could be induced by various ROS.

We tested whether SA and PAD4 are required for BAPI induction by other stimuli by comparing responses in nahG and pad4 to those of the wild type. RNA-blot analysis showed that BAPI 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
the BAPI 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, BAPI 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 BAPI promoter, we generated a series of GUS promoter fusions with various lengths of the truncated BAPI promoter (Fig. 2B). The five promoter fragments started from −902, −660, −553, −255, and −169 bp, respectively, and ended at

22°C induction of BAPI appears to be unique among other stimuli in that it requires SA and PAD4.

Isolation of a Temperature-Responsive Element in the BAPI Promoter

We determined that the induction of BAPI by cooling is at the transcriptional level. A 2.1-kb fragment of the BAPI 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, BAPI 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 BAPI promoter, we generated a series of GUS promoter fusions with various lengths of the truncated BAPI promoter (Fig. 2B). The five promoter fragments started from −902, −660, −553, −255, and −169 bp, respectively, and ended at

22°C induction of BAPI appears to be unique among other stimuli in that it requires SA and PAD4.

Isolation of a Temperature-Responsive Element in the BAPI Promoter

We determined that the induction of BAPI 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, 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 reporter 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 pBGESA, pBGESB, and pBGESC (Fig. 2E). None of the pBGESB or pBGESC transgenic lines had any induction of GUS by a 28°C to 22°C shift, while four of the 13 pBGESA 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 pBGESA (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 but not pBG7 lines (Fig. 3A). Similarly, paraquat treatment induced GUS expression in pBG4, pBG5, pBG6, and pBG7 lines but not in the pBG8 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 pBGESA lines (Fig. 3, C and D) but not pBGESB or pBGESC 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 pBGESA (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)TTCAA 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 CANNTG 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 AATACTAA, 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 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 (Kanoaka 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 fragment (3ESA; Fig. 4A). The ESA probe was shifted to a large band in the cold-treated 3ESA,competition was not observed with the unlabeled DNA of 3ESA (Fig. 4B), indicating that ICE1 binds specifically to the MYC recognition site in the ESA element.

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 band 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 CAAATG, we mutated this motif into CTAGG in ESA (Fig. 4A) and used this 3EAm 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 3EAm (Fig. 4B), indicating that ICE1 binds specifically to the MYC recognition site in 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
expression in cold and cooling responses by analyzing BAP1 induction in the ice1-1 mutant (Chinnusamy et al., 2003). For cold treatment, ice1-1 and the wild-type plants were grown at 22°C for 3 weeks before being shifted to 4°C for 6 h. For cooling induction, plants were grown at 28°C for 2 weeks before being shifted to 22°C for 9 h. RNA blots show that BAP1 inductions by cooling and cold treatments were both greatly reduced in ice1-1 (Fig. 4D), indicating that ICE1 is directly involved in BAP1 induction by cooling and cold.

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
negative regulator of programmed cell death and defense responses and it is induced by multiple environmental stimuli. Cooling from 28°C to 22°C and cold from 22°C to 4°C both induce BAP1, but the former not the latter induction is dependent on SA and PAD4. In addition, BAP1 is induced by other environmental stimuli including wounding, pathogen invasion, and ROS, which offers an opportunity to investigate temperature responses at the transcriptional level and potential interactions among different environmental stimuli.

With a series of promoter deletions, we identified a 35-bp TR element that is necessary and sufficient to confer induction by a moderate temperature cooling. We further showed genetically and biochemically that ICE1 binds to this fragment and regulates the induction of BAP1 by cold and cooling. A previous study revealed that CBFs, that bind the CRT/DRE elements, are directly involved in cooling induction of COR15a (Wang and Hua, 2009). In both cases, transcription factors formerly known to regulate transcription in cold responses are found to regulate transcriptional induction in response to a moderate decrease in temperature. Although two different transcription factors are utilized in cooling induction of COR15a and BAP1, respectively, they are in the same pathway in cold responses with ICE1 acting upstream of CBF3 by binding to its promoter directly. Thus, it appears that signaling cascade for cold responses is operating in cooling responses with more than one output into transcriptional regulation.

Transcriptional induction by a 28°C to 22°C cooling can be SA independent and SA dependent. Cooling induction of COR15a and a few other COR genes does not require SA while that of EDS1 and a few other defense-related genes requires 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 PRI (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
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 osr-1 element that is partly required for the induction of GST gene by hydrogen peroxide (Chen and Singh, 1999). The second is the as-l element in the tobacco (Nicotiana tabacum) GST gene responsible for its induction by ROS (Garretton 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 combinatorial 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 70% to 75% relative humidity. Arabidopsis seeds were either directly sown 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 1-mL needleless syringe or cut with a pair of scissors. Temperature-shift assays were performed by moving plants from one growth temperature 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., 2003). For SA treatment, a solution of 2 mM SA and 0.1% Tween 20 was sprayed on the Arabidopsis plants grown at 22°C. Mock treatment used a solution with 0.1% Tween 20. Seedlings were collected for RNA-blot analysis at 4 h (paraquat) or 1 d (SA) after spray.

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 F22O13 (from Arabidopsis Biological Resource Center) and fused to the GUS reporter gene in the binary vector pZIPGUS2 (Diener et al., 2000) to generate the pBG2 plasmid. A fragment of 1.662 bp in this promoter was amplified with a BamHI site introduced right before the start codon ATG. The 563-bp BamHI and SpeI fragment, the 255-bp BamHI and EcoRI fragment, and the 169-bp BamHI and SalI fragment were cloned, respectively, into the binary vector pZIPGUS2. Recombinant plasmids were designated as pBG4, pBG5, pBG6, and pBG7, respectively. The pBG8 plasmid contains a 762-bp fragment of the BAPI promoter (directly amplified from the bacterial artificial chromosome clone) in pZIPGUS2.

A 107-bp fragment (~106 to +1) of the cauliflower mosaic virus 35S promoter was amplified by PCR and inserted into the EcoRI site of the pBluescript vector to generate a p35S-mini construct. The 87-bp EcoRI and SalI fragment of the BAPI promoter was placed in front of the 35S mini promoter in p35S mini to generate the pE5 construct. Complementary strands of ESA, ESB, and ESC were annealed and ligated to the vector pBluescript. Three tandem copies of ESA and four tandem copies of ESB and ESC were obtained in plasmid and then placed in front of the 35S mini promoter in p35S-mini to generate pESA, pESB, and pESC, respectively. Cassette of the ES, ESA, ESB, and ESE-35S-mini promoters were each moved into the binary vector pZIPGUS2 to generate pGES, pGESA, pGESB, and pGESC constructs, respectively. These constructs were transformed into Agrobacterium GV3101 (Koncz and Schell, 1986) and transformed into wild-type Col-0 by the Agrobacterium-mediated floral-dip method (Clough and Bent, 1998).

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 gels containing 1% formaldehyde. Ethidium bromide was used to visualize the rRNA 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 after later 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 (Zhao et al., 2003). The DNA fragment containing the ESA element was labeled by the Klenow fragment (New England Biolabs) with 32P-dCTP. EMSA was carried out following protocol previously described (Hua et al., 2001). EMSA was carried out similarly to previously described (Zhao 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′-ATGACAATTACACAGCAAAAGC-3′ and 5′-TCACAGGAAAGCGTGACAAGG-3′. BAPI-specific fragment containing the ICE1 binding site was amplified by using the following primers: 5′-GCACATAGGATTCATGATATC-3′ and 5′-TGTAATGCCCACGTTAACTTAC-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.

ACKNOWLEDGMENTS

We thank Dr. J. Zhu, Dr. Torri, Dr. K. Miura, and Dr. P. Hasegawa for strains and constructs.

Received November 15, 2010; accepted November 19, 2010; published November 22, 2010.

LITERATURE CITED


