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SIZ1-Mediated Sumoylation of ICE1 Controls *CBF3/DREB1A* Expression and Freezing Tolerance in *Arabidopsis* [W][OA]

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SIZ1 is a SUMO E3 ligase that facilitates conjugation of SUMO to protein substrates. *siz1-2* and *siz1-3* T-DNA insertion alleles that caused freezing and chilling sensitivities were complemented genetically by expressing *SIZ1*, indicating that the SIZ1 is a controller of low temperature adaptation in plants. Cold-induced expression of *CBF/DREB1*, particularly of *CBF3/DREB1A*, and of the regulon genes was repressed by *siz1. siz1* did not affect expression of *ICE1*, which encodes a MYC transcription factor that is a controller of *CBF3/DREB1A*. A K393R substitution in ICE1 [ICE1(K393R)] blocked SIZ1-mediated sumoylation in vitro and in protoplasts identifying the K393 residue as the principal site of SUMO conjugation. SIZ1-dependent sumoylation of ICE1 in protoplasts was moderately induced by cold. Sumoylation of recombinant ICE1 reduced polyubiquitination of the protein in vitro. *ICE1(K393R)* expression in wild-type plants repressed cold-induced *CBF3/DREB1A* expression and increased freezing sensitivity. Furthermore, expression of *ICE1(K393R)* induced transcript accumulation of *MYB15*, which encodes a MYB transcription factor that is a negative regulator of *CBF/DREB1*. SIZ1-dependent sumoylation of ICE1 may activate and/or stabilize the protein, facilitating expression of *CBF3/DREB1A* and repression of *MYB15*, leading to low temperature tolerance.

INTRODUCTION

Frosts cause substantial agricultural yield losses, particularly if a freezing event occurs during reproductive development. Low temperatures also substantially limit the geographic locations where crops can be grown (Guy, 1990). Temperate plants have evolved a capacity to survive freezing exposure through adaptive processes, many of which are initiated in response to low temperature exposure, a phenomenon referred to as cold acclimation (Guy et al., 1985; Thomashow, 1999). Research over the last decades has exploited the genetic variation for cold acclimation that exists in temperate flora and has provided comprehensive characterization of freezing stress injury and comparative physiological and biochemical dissection of low temperature hardiness processes (Guy et al., 1985; Thomashow, 1999). Recently, the molecular genetic tractability of *Arabidopsis thaliana* has led to a more insightful understanding of critical cold acclimation and tolerance processes (Thomashow, 1999). Cellular processes that contribute to cold acclimation include protection and stabilization of cellular membranes, enhancement of antioxidative mechanisms, and synthesis and accumulation of cryoprotectant solutes and unique cryoprotective proteins (Mahajan and Tuteja, 2005).

Low temperatures initiate signaling pathways that control the expression of genes encoding determinants that are necessary for chilling tolerance (Gong et al., 2002; Hsieh et al., 2002), cold acclimation, and freezing tolerance (Guy et al., 1985; Lang and Palva, 1992; Knight et al., 1999; Thomashow, 1999; Chinnusamy et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006). Cold signaling is transduced through intermediate processes, such as inositol 1,4,5-triphosphate–mediated Ca^{2+} signatures, reactive oxygen species, abscisic acid (ABA), and mitogen-activated protein kinase signaling cascades, and RNA metabolism (Kovtun et al., 2000; Teige et al., 2004; Chinnusamy et al., 2006). Numerous transcription factors that facilitate cold signaling and control expression of genes in cold regulons have been identified (Shinozaki et al., 2003; Cook et al., 2004; Gilmour et al., 2004; Kaplan et al., 2004; Chinnusamy et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006).

The most characterized transcription factors that regulate cold signaling include the C-Repeat (*CRT*)/dehydration responsive element (*DRE*) binding proteins CBF/DREB1 (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu et al., 1998; Thomashow 1999). Three CBF/DREB1 proteins, CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A, transactivate cold-dependent and ABA-independent expression of *COR/RD/LTI* (for cold-responsive/responsive to dehydration/low-temperature-induced)

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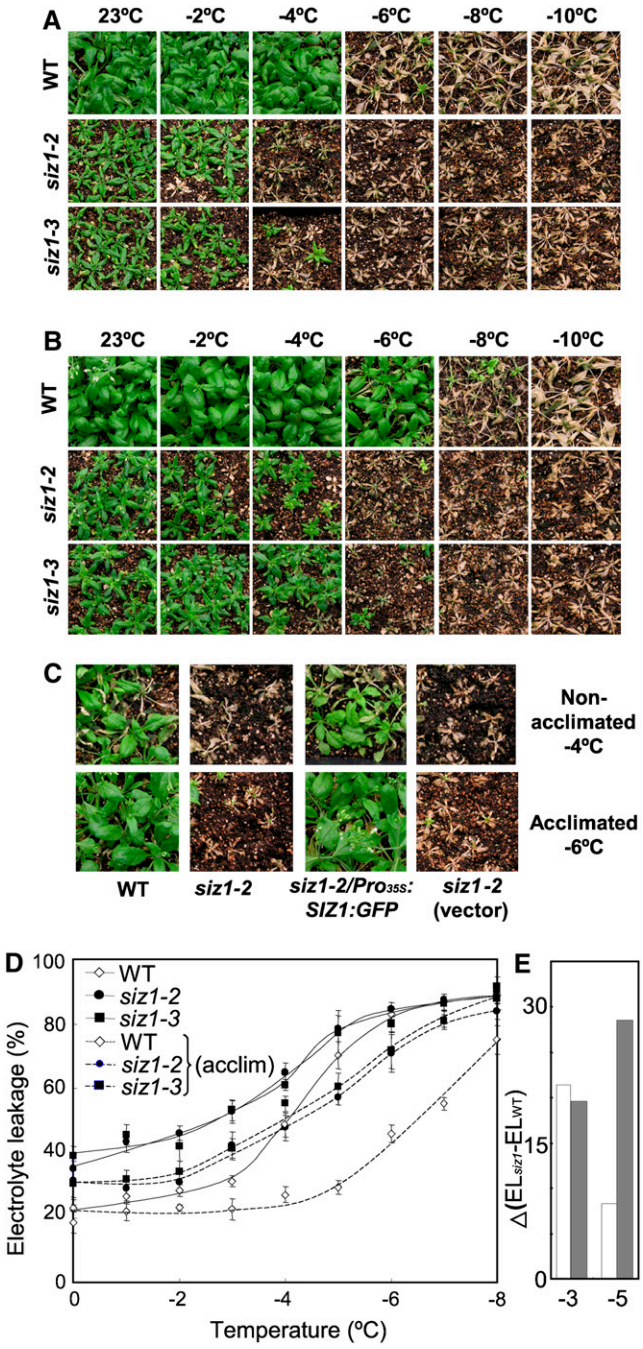

Figure 1. *SIZ1* Mediates Freezing Tolerance.

(A) and **(B)** Nonacclimated **(A)** or cold-acclimated **(B)** wild-type (Col-0), *siz1-2*, and *siz1-3* plants were exposed for 1 h to the temperature indicated, and photographs are of plants 7 d after freezing treatment.

(C) Freezing sensitivity of *siz1-2* plants is suppressed by *Pro35S:SIZ1:GFP* expression. Photographs are of nonacclimated or acclimated plants 7 d after 1 h of incubation at −4 or −6°C, respectively.

(D) Electrolyte leakage from nonacclimated or acclimated (acclim) wild-type, *siz1-2*, and *siz1-3* plants after exposure to the temperature indicated (programmed to cool at 2°C h^{-1}). Data are means ± SE ($n = 4$ leaves, each from a different plant).

genes through the interaction between the AP2/ERF DNA binding domain of the transcription factor and core *CRT/DRE cis*-elements (A/GCCGAC) that are present in promoters of the target genes (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Thomashow, 1999). Low temperatures transiently induce expression of all *CBF/DREB1* transcription factors within minutes (Vogel et al., 2005). Overexpression of each *CBF/DREB1* constitutively induces the *CBF/DREB1* regulon and enhances plant freezing tolerance, indicating that these transcription factors are sufficient for cold acclimation (Gilmour et al., 2000, 2004). However, a loss-of-function *cbf2* mutation causes freezing tolerance and cold hyperinduction of *CBF1/DREB1B*, *CBF3/DREB1A*, and *CBF/DREB1* regulon expression, suggesting that *CBF2/DREB1C* negatively regulates *CBF1/DREB1B* and *CBF3/DREB1A* (Novillo et al., 2004). *CBF3/DREB1A* may negatively regulate *CBF2/DREB1C* (Chinnusamy et al., 2003, 2006).

Direct regulators of *CBF/DREB1* expression are HOS1, ICE1, and MYB15 (Agarwal et al., 2006; Chinnusamy et al., 2006; Dong et al., 2006). The HOS1 (for high expression of osmotically responsive genes) RING-type ubiquitin E3 ligase negatively regulates cold-induced *CBF/DREB1* expression (Ishitani et al., 1998). ICE1 (for inducer of CBF/DREB1 expression 1) is a MYC-like basic helix-loop-helix transcription factor that activates *CBF/DREB1* expression in response to low temperatures (Chinnusamy et al., 2003). ICE1 binds to canonical *MYC cis*-elements (CANNTG) in the *CBF3/DREB1A* promoter to induce expression, which then leads to induction of *CBF/DREB1* regulon expression (Chinnusamy et al., 2003; Lee et al., 2005). ICE1 protein apparently is a focal controller of *CBF3/DREB1A*, *CBF/DREB1* regulon gene expression, and cold tolerance responses (Chinnusamy et al., 2003). Recently, it was determined that HOS1 negatively regulates ICE1 function in low temperature adaptation (Dong et al., 2006). HOS1 migrates to the nucleus in response to cold treatment and polyubiquitinates ICE1, targeting this transcription factor for proteasome degradation (Lee et al., 2001; Dong et al., 2006). MYB15 binds to *CBF/DREB1* promoter regions and represses expression of *CBF/DREB1* and the *CBF/DREB1* regulon and negatively regulates freezing tolerance (Agarwal et al., 2006). ICE1 also physically interacts with MYB15 and attenuates *MYB15* expression (Agarwal et al., 2006). Together, these results indicate that the ubiquitin E3 ligase HOS1, MYC transcription factor ICE1, and MYB transcription factor MYB15 function in a regulatory cascade to modulate expression of *CBF3/DREB1A*, and perhaps other *CBF/DREB1s*, to control plant responses to low temperatures.

SUMO (for small ubiquitin-related modifier) conjugation to protein substrates (sumoylation) is a reversible posttranslational modification that is regulated by environmental stimuli in animals and yeasts (Johnson, 2004). Sumoylation/desumoylation of substrates affects critical and diverse processes, such as innate immunity, chromosome segregation and cell division, DNA repair, nucleocytoplasm trafficking, subnuclear targeting,

(E) Electrolyte leakage difference between nonacclimated (white bars) or acclimated (gray bars) *siz1* and wild-type plants (EL_{siz1}-EL_{wt}) after exposure to the temperature indicated as in **(D)**.

transcriptional regulation, and ubiquitin-mediated protein degradation by proteasomes (Gill, 2005; Hay, 2005). PIAS (for protein inhibitor of activated STAT)/Siz (for SAP and Miz) proteins are SUMO E3 ligases that mediate the final step of SUMO conjugation (Kahyo et al., 2001; Johnson and Gupta, 2001; Kotaja et al., 2002; Takahashi et al., 2003; Hay, 2005). Transcription factors are direct targets of SUMO conjugation that is mediated by PIAS/Siz proteins (Gill, 2005). SUMO conjugation affects transcription factor function through activation, repression, or protein stabilization processes. Yeast SUMO E3 ligases, Siz1 and 2, facilitate cell division at low temperatures (Johnson and Gupta, 2001). SUMO conjugation/deconjugation in plants has been implicated in responses to heat shock, oxidative stress, hypoxia, phosphate limitation, ABA, flowering, and pathogen defense (Kurepa et al., 2003; Lois et al., 2003; Murtas et al., 2003; Miura et al., 2005; Yoo et al., 2006; Lee et al., 2007). Recently, the *Arabidopsis* SUMO E3 ligase SIZ1 has been shown to participate in responses to phosphate starvation, salicylic acid–mediated signaling in plant defense, and basal thermotolerance (Miura et al., 2005; Yoo et al., 2006; Lee et al., 2007).

This study establishes that SIZ1 (Miura et al., 2005) is a regulator of cold acclimation by controlling ICE1 activity, *CBF/DREB1* expression, particularly *CBF3/DREB1A*, and target gene function. A K393R mutation blocks sumoylation of ICE1, represses expression of *CBF3/DREB1A* and its regulon genes, and reduces freezing tolerance. We present evidence that sumoylation of ICE1 represses polyubiquitination of the protein that leads to enhanced stability of ICE1 at low temperatures. Sumoylation of ICE1 also represses expression of the negative regulator *MYB15*. Together, these results indicate that SIZ1-mediated SUMO conjugation/deconjugation of ICE1 is a key process that initiates many changes in gene expression that are required for low temperature tolerance.

RESULTS

SIZ1 Regulates Freezing and Chilling Tolerances

The *siz1-2* and *siz1-3* T-DNA insertion alleles, which impair SIZ1 SUMO E3 ligase function (Miura et al., 2005), caused freezing sensitivity based on survival (Figures 1A to 1C) and electrolyte leakage assays (Figure 1D). Electrolyte leakage from unacclimated *siz1* plants was approximately twice that of the wild type at 0 to −3°C (Figure 1D) even though leakage was similar at 23°C (data not shown; Lee et al., 2007). *siz1* plants were impaired in cold acclimation capacity compared with wild-type plants (Figure 1D), particularly at temperatures below −3°C (Figure 1E). The

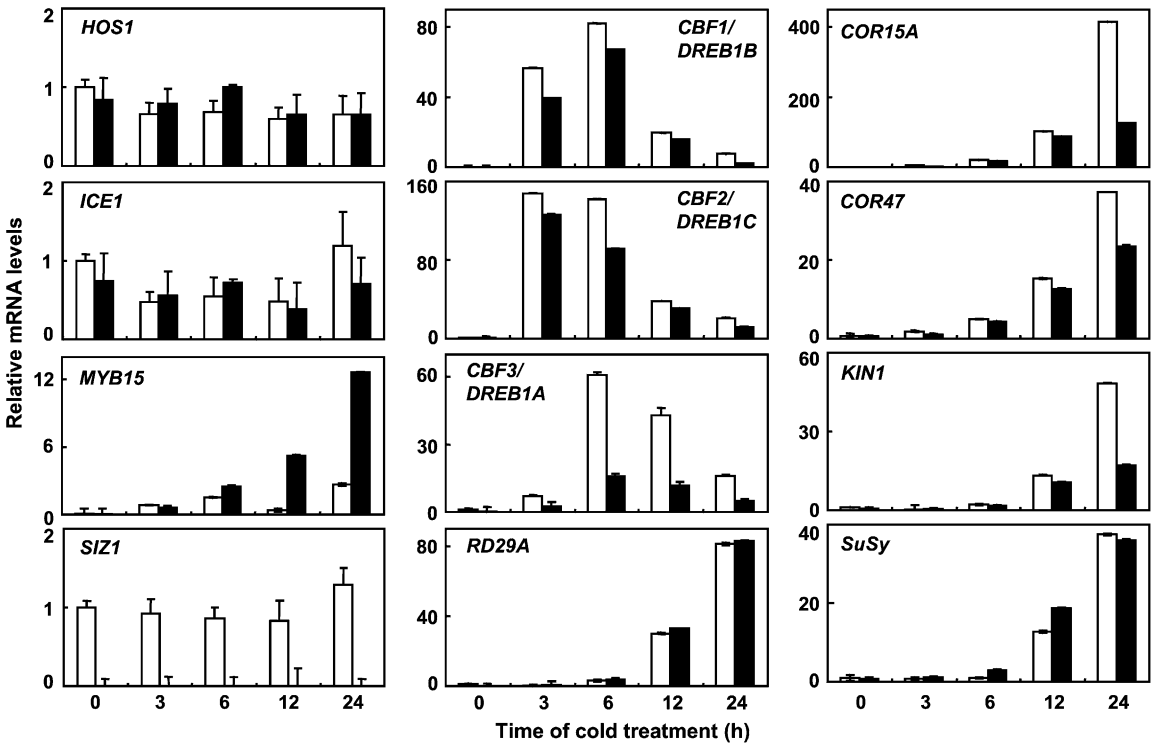

Figure 2. *SIZ1* Is a Positive Regulator of *CBF/DREB1* Expression, Particularly of *CBF3/DREB1A*.

Relative mRNA levels in wild-type (white bars) and *siz1-2* (black bars) seedlings were determined by quantitative RT-PCR analyses. Ten-day-old seedlings that were grown at 23°C were incubated at 0°C for the indicated time. Transcript abundances of *SIZ1*; *CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A*; *CBF/DREB1* regulon genes *COR15A*, *COR47*, *KIN1*, and *RD29A*; *SuSy* (cold induced but independent of *CBF/DREB1*); and *CBF/DREB1* regulators *ICE1*, *HOS1*, and *MYB15* are illustrated. Data are means ± SD ($n = 4$).

siz1 plants also were chilling sensitive, which was evident by comparison with the wild type after prolonged exposure to 4°C (see Supplemental Figure 1 online). Leaf chlorosis and necrosis of *siz1* plants were visible after 3 weeks of chilling, but chlorophyll content of the wild type did not decline even after 6 weeks of chilling (see Supplemental Figures 1B and 1C online). Expression of the wild-type allele $Pro_{CaMV35S}$*:SIZ1:GFP* suppressed the freezing sensitivity of *siz1-2* plants, further confirming that *SIZ1* is required for low temperature tolerance (Figure 1C).

SIZ1 Controls *CBF/DREB1* and *CBF/DREB1* Regulon Gene Expression

In wild-type plants, low temperatures of 10°C or less induce *CBF/DREB1* expression within 15 min, and transcript accumulation increases with lower temperatures (Jaglo-Ottosen et al., 1998; Chinnusamy et al., 2003). Cold (0°C)–induced *CBF/DREB1* transcript accumulation in *siz1-2* seedlings was less than in the wild type over a 24-h time frame (Figure 2, middle panels). The *siz1* mutation affected expression of *CBF3/DREB1A* more than that of *CBF1/DREB1B* or *CBF2/DREB1C* (Figure 2, middle panels).

Transcript accumulation of the *CBF/DREB1* regulon genes *COR15A*, *COR47*, and *KIN1* began 6 to 12 h after exposure of plants to cold (Gilmour et al., 1998; Figure 2, right panels). Low temperature–induced *COR15A*, *COR47*, and *KIN1* transcript accumulation was less in *siz1-2* than in wild-type seedlings (Figure 2, right panels). However, expression of *RD29A*, another *CBF/DREB1* regulon gene, was similar in plants of both genotypes (Figure 2, middle panels). This gene may be regulated by other processes, as the *hos9* mutation increases *RD29A* expression independent of CBF/DREB1 (Zhu et al., 2004). Sucrose synthase (*SuSy*), which is cold regulated through a *CBF/DREB1*-independent pathway (Gilmour et al., 2000), exhibited similar expression patterns in wild-type and *siz1-2* seedlings (Figure 2, right panels). These data indicate that *SIZ1* controls cold signaling through the regulation of *CBF/DREB1* expression, although not all genes of the regulon are affected. Cold-induced *CBF3/DREB1A*, *CBF1/DREB1C*, *COR47*, and *COR15A* expression also was repressed in *siz1-3* plants (data not shown).

A positive regulator, ICE1, and negative regulators, HOS1 and MYB15, of *CBF/DREB1* expression and freezing responses have been identified (Lee et al., 2001; Chinnusamy et al., 2003; Agarwal et al., 2006). *HOS1* and *ICE1* transcript accumulation was not substantially affected by cold and was similar in wild-type and *siz1-2* seedlings (Figure 2, left panels). *HOS1* mRNA abundance transiently decreases immediately after cold exposure but is similar to precold levels after 3 h (Lee et al., 2001), which was the first treatment time point in this experiment (Figure 2, left panels). *ICE1* transcript abundance was similar in wild-type and *siz1* plants even with cold treatment (Lee et al., 2005; Figure 2, left panels). On the other hand, *MYB15* mRNA abundance was moderately upregulated by cold in wild-type seedlings (Agarwal et al., 2006) but was induced substantially in *siz1* seedlings (Figure 2, left panels). The timing of cold-responsive *MYB15* expression in *siz1* seedlings resembled that of *COR* and *KIN1* transcript accumulation (i.e., occurred later during the low temperature incubation period relative to *CBF/DREB1* expression). These results indicate that SIZ1 negatively regulates *MYB15* expression but does not alter appreciably the transcript level of *HOS1* or *ICE1*.

SIZ1 Facilitates Cold-Induced Accumulation of SUMO Conjugates

Anti-SUMO1 conjugation products were accumulated in wild-type seedlings within 1 h after exposure to 0°C but to a much lesser extent in *siz1-2* seedlings, at comparable times during cold treatment (Figure 3). Anti-SUMO1 detects both SUMO1 and SUMO2 (Murtas et al., 2003), which are the most closely related by sequence comparison among the eight family members (Kurepa et al., 2003) and may be functionally redundant (Kurepa et al., 2003). These data indicate that cold induces SIZ1-mediated SUMO1/2 conjugation in planta that is due presumably to the E3 ligase activity of SIZ1 (Miura et al., 2005). Heat shock and other environmental stresses also cause an increase in SUMO1/2 conjugation products (Kurepa et al., 2003; Miura et al., 2005; Yoo et al., 2006), implicating that sumoylation/desumoylation of protein targets may be a posttranslational regulatory process in abiotic stress signaling of plants.

SIZ1-Mediated Sumoylation of ICE1 Facilitates *CBF3/DREB1A* Expression

The *siz1* mutation did not affect *ICE1* expression (Figure 2), which infers that SIZ1 is not a transcriptional regulator of ICE1 but could regulate activity by posttranslational modification. SUMOplot (http://www.abgent.com/tool/sumoplot) predicted that ICE1 contains one SUMO conjugation motif (ΨKXE; Minty et al., 2000) with K393 as the acceptor residue. SIZ1 was necessary for sumoylation of ICE1 in vitro (Figure 4A) but did not facilitate

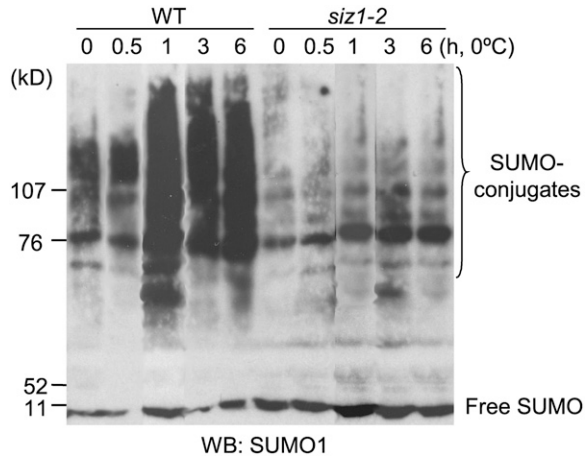

Figure 3. Cold Stress Induces SUMO1/2 Conjugate Accumulation That Is Facilitated by SIZ1.

Shown are in planta sumoylation profiles of 10-d-old wild-type and *siz1-2* seedlings that were grown on medium at 23°C (0 time) and then incubated at 0°C for the time indicated. Total protein was separated by SDS-PAGE, transferred, and detected with anti-SUMO1 that interacts with both SUMO1 and SUMO2 (Murtas et al., 2003).

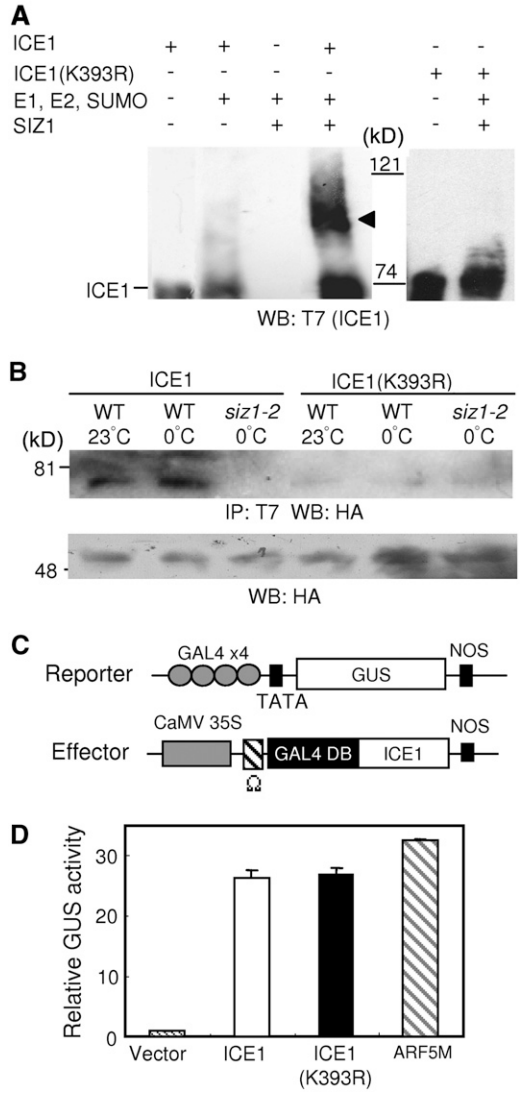

Figure 4. SIZ1 Facilitates Sumoylation of ICE1.

(A) SIZ1 mediates in vitro sumoylation of ICE1. Affinity-purified recombinant GST-T7-ICE1 or GST-T7-ICE1(K393R) was used as a substrate in an in vitro sumoylation assay; reaction mixture contained yeast GST-ScAos1 (E1), GST-ScUba2 (E1), ScUbc9 (E2), and His-ScSmt3 (SUMO) and *Arabidopsis* GST-SIZ1 (E3) recombinant proteins (Takahashi et al., 2003; Miura et al., 2005), without (−) or with (+) protein. ICE1(K393R) has an amino acid substitution of the residue that is predicted to be a SUMO conjugation site in ICE1. Unconjugated and SUMO-conjugated ICE1 proteins were detected with anti-T7 (T7-ICE1). Arrowhead indicates sumoylated ICE1 and WB: T7 designates a protein gel blot detected with anti-T7.

(B) SIZ1-mediates SUMO1 conjugation to ICE1 in protoplasts. Wild-type or *siz1-2* protoplasts were cotransformed to express T7-SUMO1 and HA-ICE1 or HA-ICE1(K393R) at 23°C for 36 h. Protoplasts were then incubated at 0°C or maintained at 23°C for 1 h and then harvested immediately thereafter. Soluble extracts were immunoprecipitated with anti-T7 Tag Agarose (Novagen) to obtain T7-SUMO1 conjugates (IP: T7). Anti-HA was used to detect T7-SUMO1-HA-ICE1 conjugates on the immublot (WB: HA; top panel). To confirm equivalent expression of HA-ICE1 in protoplasts, crude extract from protoplasts was separated by SDS-PAGE, and ICE1 was detected by protein gel blot analysis with anti-HA (WB: HA; bottom panel).

(C) Schematic representation of the effector and reporter plasmids used in the GAL4 transient expression assay (Chinnusamy et al., 2003; Tiwari et al., 2003). The *ICE1* or *ICE1(K393)* open reading frame was inserted into the effector plasmid. GAL4 DB is the DNA binding domain of the yeast transcription factor GAL4; *GUS* is the reporter gene; NOS is the terminator signal of the nopaline synthase gene; and Ω is the translational enhancer of *Tobacco mosaic virus*.

(D) Relative GUS activities after transfection with an effector vector (vector control), *ICE1*, *ICE1(K393R)*, or *ARF5M* (positive control; Tiwari et al., 2003). *Renilla* luciferase activity was used for normalization; relative GUS expression $[(GUS_{sample}/LUC_{sample})/(GUS_{vector}/LUC_{vector})]$ was calculated. Data are means ± SE ($n = 3$).

SUMO conjugation to the ICE1(K393R) variant protein (Figure 4A). K-to-R substitutions in the sumoylation motifs of substrate proteins effectively block isopeptide linkage of SUMO to the target protein (Gostissa et al., 1999; Sachdev et al., 2001; Lin et al., 2003; Long et al., 2004a; Gill, 2005). These results indicate that SIZ1 facilitates sumoylation of ICE1 and identifies K393 as the SUMO conjugation residue in the protein.

T7:SUMO1 and *HA:ICE1* or *HA:ICE1(K393R)* cDNAs were cotransformed into protoplasts isolated from wild-type or *siz1-2* plants (Jin et al., 2001) to assess if SIZ1 facilitates sumoylation in planta. SUMO1 conjugation to ICE1 was moderately induced by cold treatment in the wild type but was not evident in cold-treated *siz1-2* protoplasts (Figure 4B). Also, the K-to-R mutation blocked in vivo sumoylation of ICE1 (Figure 4B). Together, these results indicate that SIZ1 facilitates sumoylation of ICE1 at the K393.

Transient expression of *ICE1* but not of *ICE1(K393R)* in wild-type protoplasts induced *CBF3*/*DREB1A* transcript abundance in response to cold (see Supplemental Figure 2 online). Conversely, *ICE1(K393R)* but not *ICE1* expression moderately increased *MYB15* mRNA abundance (see Supplemental Figure 2 online). These results and those illustrated in Figure 2 are consistent with the premise that cold-induced, SIZ1-mediated sumoylation of K393 in ICE1 induces *CBF3*/*DREB1A* and represses *MYB15* expression.

The GAL4 DNA binding system was used to evaluate the effect of K393R on ICE1 transcriptional activator function (Chinnusamy et al., 2003; Tiwari et al., 2003). The effector plasmid was constructed by fusing the cDNA encoding *ICE1*, *ICE1(K393R)*, or *ARF5M* (positive control; Tiwari et al., 2003) to the DNA binding domain of the yeast GAL4 transcriptional activator (Figure 4C). Expression of this cassette is driven by the 35S promoter of *Cauliflower mosaic virus* (CaMV). The effector plasmid [*ICE1*, *ICE1(K393R)*, or *ARF5M*], the Pro_{GAL4}:β-*glucuronidase* (*GUS*) reporter plasmid (Tiwari et al., 2003), and the plasmid encoding *Renilla* luciferase driven by the 35S promoter for normalization (Chinnusamy et al., 2003) were cotransformed into *Arabidopsis* protoplasts (Jin et al., 2001). Transient expression of *ICE1* increased GUS reporter activity ~20-fold (Figure 4D) as reported (Chinnusamy et al., 2003). Expression of *ICE(K393R)* activated *GAL4*-responsive transcription to an equivalent level of *ICE1*

(Figure 4D). Sumoylation/desumoylation seems not to regulate ICE1 transactivation activity by the criteria of this assay.

SIZ1-Dependent SUMO Conjugation to ICE1 Reduces Polyubiquitination in Vitro

Cold treatment induces HOS1 to accumulate in the nucleus, where the E3 ligase facilitates ubiquitination of ICE1, which targets the protein for proteasome degradation (Dong et al., 2006). ICE1 destabilization leads to reduced *CBF3*/*DREB1A* expression and freezing sensitivity. Recombinant T7-ICE1 was incubated together with other components of the in vitro sumoylation assay mixture, including SIZ1 as the SUMO E3 ligase (Miura et al., 2005), and then purified using anti-T7. Unsumoylated or SUMO-conjugated ICE1 was then added to an in vitro ubiquitination assay mixture that included HOS1 as the ubiquitin E3 ligase (Dong et al., 2006; Figure 5A). Sumoylated ICE1 was less polyubiquitinated than unsumoylated ICE1 (Figure 5A). However, K393 was not the primary ubiquitination site in ICE1 (Figure 5B). These results indicate that sumoylation of K393 reduces polyubiquitination of ICE1 that could increase ICE1 stability, thereby increasing *CBF3*/*DREB1A* expression and low temperature tolerance.

Sumoylation of ICE1 Mediates Freezing Tolerance

Overexpression of *ICE1* (*Pro*$_{CsV}$*:ICE1*) but not *ICE(K393R)* [*Pro*$_{CsV}$*:ICE1(K393R*)] increased freezing tolerance of transformed wild-type plants (Figures 6A and 6B). Increased abundance of transgene transcripts was detected in plants of the different overexpression lines by RT-PCR (see Supplemental Figure 3 online). Freezing tolerance of *ICE1*-overexpressing plants obtained in this research (Figure 6A) was similar to that of *ICE1*-overexpressing plants reported in a prior publication (Chinnusamy et al., 2003; Figure 6A, ICE1oe). *ICE1(K393R)*-expressing plants were more sensitive to freezing stress than wild-type or vector control plants but less sensitive than *siz1-2* plants (Figures 6A and 6B). Cold-induced transcript abundance of *CBF3*/*DREB1A* and the regulon genes *COR15A* and *COR47* was greater in *ICE1*-overexpressing plants and less in *ICE1(K393R)* plants relative to the vector control wild type (Figure 6C). *CBF1*/*DREB1B* and *CBF2*/*DREB1C* expression was upregulated in *ICE1*-overexpressing plants, as reported (Chinnusamy et al., 2003), but was moderately downregulated in *ICE1(K393R)* transgenic plants relative to plants transformed with the vector cassette. *MYB15* was moderately upregulated by *ICE1(K393R)* overexpression (Figure 6C). These results indicate that sumoylation of ICE1 facilitates *CBF3*/*DREB1A* expression and freezing tolerance and that substitution of K393 to R represses *CBF3*/*DREB1A* and increases *MYB15* expression.

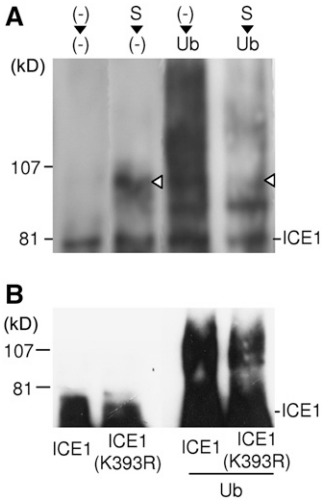

Figure 5. Sumoylation of ICE1 Inhibits Polyubiquitination of the Transcription Factor.

(A) Affinity-purified recombinant GST-T7-ICE1 was incubated in an in vitro sumoylation (S) mixture (Miura et al., 2005) or not incubated (–). Proteins were immunopurified with anti-T7 Tag agarose (Novagen), washed three times, and eluted. Eluted proteins were subjected to an in vitro ubiquitination assay (Ub) (Dong et al., 2006) or not (–). Proteins were separated by SDS-PAGE and identified by immunoblot analysis using anti-T7. Arrowheads indicate sumoylated ICE1.

(B) Recombinant GST-T7-ICE1 and GST-T7-ICE1(K393R) was not or was subjected to an in vitro ubiquitination assay (Ub). Proteins were separated by SDS-PAGE and detected by immunoblot analysis with anti-T7.

DISCUSSION

Genetic and biochemical evidence indicates that the SUMO E3 ligase SIZ1 controls low temperature–dependent *CBF*/*DREB1* regulon expression through the induction of *CBF*/*DREB1*, most substantially *CBF3*/*DREB1A*, and facilitates chilling and freezing responses, including cold acclimation. The MYC-like transcription factor ICE1, which is necessary for low temperature induction of *CBF*/*DREB1* (Chinnusamy et al., 2003), is a direct target of SIZ1-mediated SUMO1 conjugation. SIZ-mediated SUMO1 conjugation to K393 affects ICE1 activity to control *CBF3*/*DREB1A* expression (Figures 2 and 6C; see Supplemental Figure 2 online). MYB15 is a negative regulator of *CBF*/*DREB1* expression presumably through an interaction with elements in the promoter regions, although there is no direct evidence of transrepressor function (Agarwal et al., 2006). Expression of *MYB15* is upregulated in *siz1* plants or by ICE1(K393R) expression (Figures 2 and 6C; see Supplemental Figure 2 online), indicating that unsumoylated ICE1 induces *MYB15* expression. Thus, SIZ1-mediated SUMO1 conjugation to ICE1 facilitates induction of *CBF3*/*DREB1A* expression, repression of *MYB15*, and cold tolerance.

SIZ1 Transduces Cold Signals and Mediates Low Temperature Tolerance

SIZ1-mediated SUMO1 conjugation to ICE1 is induced by cold treatment (Figure 4B) and induces *CBF3*/*DREB1A* expression (Figure 6C; see Supplemental Figure 2 online). These results indicate that SIZ1-mediated sumoylation of ICE1 is an early event in low temperature responses of plants and induces signal cascades that regulate expression of genes that are necessary for cold tolerance. However, it is still not established how cold regulates SIZ1 E3 ligase function. *SIZ1* expression is constitutive

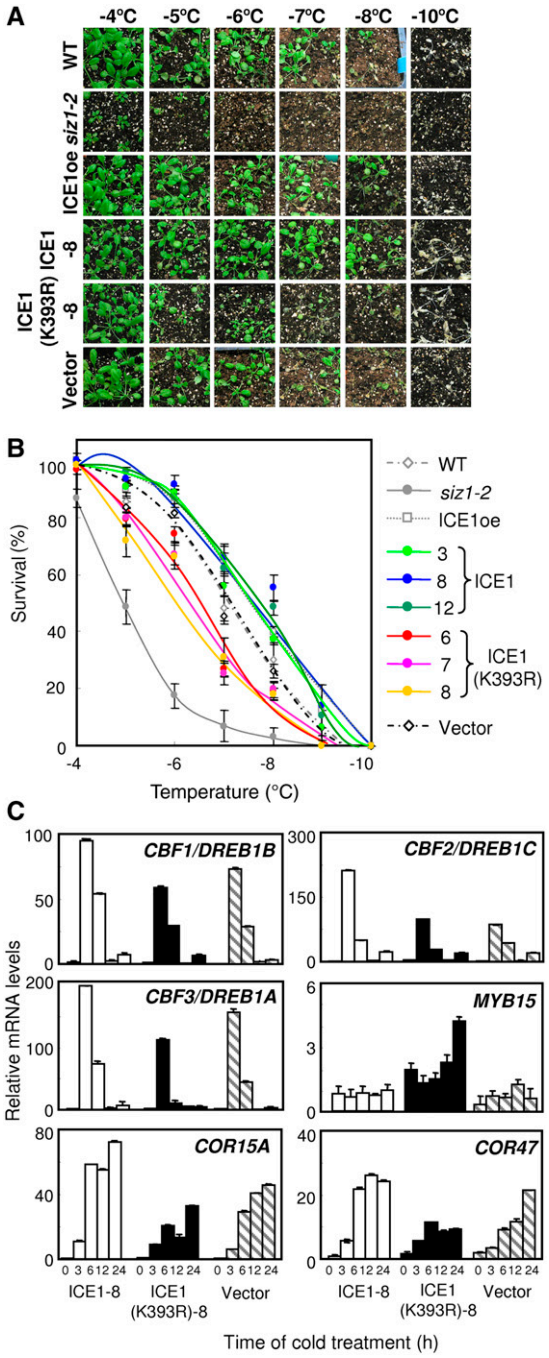

Figure 6. ICE1 Sumoylation Facilitates Freezing Tolerance and Enhances *CBF3/DREB1A* and *CBF/DREB1* Regulon Expression.

(A) Photographs are of representative plants 7 d after 1-h exposure to the indicated temperature. P_{CsV}*:ICE1* (line 8) or P_{CsV}*:ICE1(K393R)* (line 8) was transformed into Col-0 plants. pCsV1300 transgenic lines were generated for use as a vector control. The ICE1 overexpressing line (ICE1oe) was used as a positive control (Chinnusamy et al., 2003).

(B) Survival was determined for 20 plants after freezing treatment at the indicated temperature. Data are means ± SE calculated from data of five independent experiments and three independent *ICE1* (3, 8, and 12) and *ICE1(K393R)* (6, 7, and 8) expressing lines.

(C) Relative mRNA levels in plants of *ICE1* (white bars) or *ICE1(K393R)* (black bars) overexpressing lines or a vector control line (hatched bars) were determined by quantitative PCR. Seedlings were either untreated (0 h) or treated with low temperature (0°C) for the indicated time. Data are means ± SD ($n = 4$).

and unaffected by cold, indicating that transcriptional regulation does not directly control SIZ1 function in low temperature tolerance. Transcriptome profiling analyses reveal that genes encoding SUMO, SUMO E1, SUMO E2, and SUMO proteases are not induced by low temperature stress (Lee et al., 2005), inferring that cold-induced sumoylation of ICE1 is not due to transcriptional regulation of the SUMO conjugation/deconjugation cycle. Identification of the regulators that control sumoylation in any organism remains elusive. Thus, it remains unclear how this posttranslational control process transduces specific regulatory inputs (Hay, 2005).

It is plausible that low temperatures directly or indirectly induce posttranslational modification of SIZ1 that alters the regulatory function of the protein. The human PIASy is sumoylated at K35, which is necessary for PIASy E3 ligase activity that activates the transcription factor Tcf-4 (Ihara et al., 2005). Since *Arabidopsis* SIZ1 contains predicted sumoylation motifs, it is possible that SUMO conjugation regulates the function of the protein. TGF-β activates the p38 MAP kinase cascade that stabilizes the PIASxβ protein and enhances *PIASx*β gene expression (Ohshima and Shimotohno, 2003). PIASxβ facilitates sumoylation of Smad4, which is necessary for transactivation function (Ohshima and Shimotohno, 2003).

Posttranscriptional control of SIZ1 function could be a result of subnuclear targeting. SIZ1 is located in nuclear speckles at 23°C (Miura et al., 2005), which is analogous to PIAS proteins that localize to mammalian promyelocytic leukemia (PML) nuclear bodies (Sachdev et al., 2001; Seeler and Dejean, 2003). The precise function of PML nuclear bodies is not clear, but these are implicated in both gene activation and repression because transcription factors and transcriptional coregulators, including chromatin-modifying proteins, colocalize to PML bodies (Ching et al., 2005). Cytochemical analyses and genetic evidence indicate that PML bodies interact with specific genes and are associated with transcriptionally active chromatin (Wang et al., 2004; Ching et al., 2005). Recently, subnuclear compartmentalization processes have been implicated in gene activation (Sharrocks, 2006). It is possible that low temperature affects subnuclear localization of SIZ1 and/or of ICE1 that alters *CBF/DREB1* expression.

SIZ1-Mediated Sumoylation of ICE1 May Facilitate and Stabilize Transcription Factor Activity

ICE1 preferentially induces *CBF3/DREB1A* expression within minutes after exposure of plants to low temperatures (Baker et al., 1994; Chinnusamy et al., 2003; Gilmour et al., 2004). The paradigm is that cold stimulates ICE1 transcription factor activity, by some yet unknown processes, which facilitates *CBF3/*

DREB1A expression (Chinnusamy et al., 2003; Dong et al., 2006; Figure 7). SUMO conjugation can enhance or repress transcription factor activity (Gill, 2005; Hay, 2005). For instance, sumoylation of NFAT-1 (for nuclear factor of activated T cells), p53 tumor suppressor, Smad4, and Tcf-4 is necessary for transcription factor activity (Ohshima and Shimotohno, 2003; Gill, 2005; Hay, 2005; Ihara et al., 2005). Likewise, sumoylation of transcription factors represses activity through processes that involve transcriptional coregulatory complexes (Johnson, 2004; Gill, 2005; Hay, 2005).

Our results indicate that the ICE1(K393R) substitution does not alter transactivation activity of the MYC factor in the GAL4 assay (Figure 4D), even though expression of *CBF3*/*DREB1A* is downregulated in ICE1(K393R)-overexpressing plants (Figure 6C). Interestingly, the *ice1* mutation (R236H) does not affect ICE1 transactivation activity but represses *CBF3*/*DREB1A* expression (Chinnusamy et al., 2003). Mammalian PIAS3 interacts with the coactivator p300/CBP and the transcription factor Smad3 (Long et al., 2004b). Recruitment of the p300/CBP coactivator to chromatin stimulates PIAS3-mediated transcriptional activation of Smad3 in response to TGF-β (Long et al., 2004b). Therefore, it is possible that SIZ1 may be involved in recruitment of coactivator complex subunits to the *CBF3*/*DREB1A* chromatin to facilitate induction of expression by ICE1.

Later in the cold episode, ICE1 activity decreases, at least in part, are attributable to proteolysis facilitated by HOS1, a RING finger ubiquitin E3 ligase, which is a negative regulator of *CBF*/*DREB1* expression (Dong et al., 2006). Degradation of ICE1 presumably contributes to lower *CBF3*/*DREB1A* expression that occurs after the initial induction by low temperatures. SUMO and ubiquitin can interact competitively or cooperatively on the same substrate to regulate protein function and biological processes (Hay, 2005; Ulrich, 2005). Sumoylation of IκBα, Smad4, or NEMO reduces ubiquitin-mediated degradation of these proteins (Ulrich, 2005). Sumoylation of ICE1 at K393 attenuates polyubiquitination of the transcription factor in vitro (Figure 5A). Perhaps SUMO conjugation to ICE1 prevents access of the ubiquitination complex, which may include HOS1, to a target residue(s) in the protein (Pichler et al., 2005) and thereby reduces proteasome degradation (Dong et al., 2006). Thus, SUMO or ubiquitin conjugation to ICE1 results in either transcription factor activity or destabilization (i.e., inactivation) causing opposing effects on *CBF3*/*DREB1A* expression.

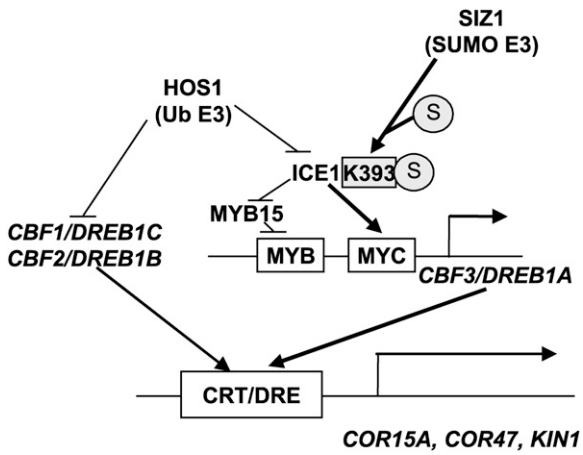

Figure 7. A Model of SIZ1 Regulation of Cold Signaling and Tolerance.

SIZ1-dependent sumoylation of ICE1 at K393 regulates expression of *CBF3*/*DREB1A* and cold tolerance. SUMO conjugation to ICE1 induces *CBF3*/*DREB1A* expression, and then CBF3/DREB1A induces cold-responsive genes, such as *COR15A*, *COR47*, and *KIN1* (Figures 2 and 6C), and cold tolerance (Figures 1 and 6). Sumoylation may enhance protein stability to block polyubiquitination of ICE1 (Figure 5). Furthermore, SUMO conjugation to ICE1 represses *MYB15* expression (Figure 6C). Sumoylation of ICE1 is a key regulatory process in cold signaling and tolerance.

SUMO Modification of ICE1 Represses *MYB15* Expression

Sumoylation of ICE1 also negatively affects *MYB15* transcript abundance, which presumably facilitates *CBF3*/*DREB1A* expression (Figure 6C; see Supplemental Figure 2 online). *MYB15* represses *CBF*/*DREB1*, particularly *CBF3*/*DREB1A*, expression (Agarwal et al., 2006). It is yet to be determined whether SUMO-conjugated ICE1 directly or indirectly represses *MYB15*. Sumoylation of the same transcription factor can result in induction or repression of different target genes (Gill, 2005; Hay, 2005). SUMO modification of p53 enhances transcription of p21, an inhibitor of cyclin-dependent kinase (Gostissa et al., 1999). Interestingly, sumoylation of p53 represses expression of the human ribosomal gene cluster gene that inhibits DNA replication (Schmidt and Müller, 2002). The overall effect of p53 sumoylation, however, is to mediate p53-dependent G1 cell cycle arrest (Müller et al., 2000; Megidish et al., 2002). Also, PIAS-mediated sumoylation of Smad4 causes activation or repression of specific genes (Ohshima and Shimotohno, 2003; Long et al., 2004a). Cold-induced *MYB15* expression occurs much more rapidly and intensively in *siz1* seedlings than in the wild type. Sumoylated ICE1 may negatively regulate *MYB15*, but *ICE1(K393R)* expression enhances *MYB15* transcript abundance (Figure 6C; see Supplemental Figure 2 online).

CBF/*DREB1* gene function is tightly regulated both transcriptionally and posttranscriptionally (Thomashow, 1999; Lee et al., 2001; Chinnusamy et al., 2006), presumably because uncontrolled *CBF*/*DREB1* activation may be disadvantageous in particular environments. In fact, constitutive *CBF*/*DREB1* overexpression results in dwarfism (Gilmour et al., 2004), and hyperexpression of *CBF*/*DREB1* caused by a *hos1* mutation results in freezing sensitivity (Ishitani et al., 1998). The results presented herein confirm that ICE1 is a key regulator of *CBF*/*DREB1* expression and cold tolerance and that sumoylation is a critical process in determining ICE1 activity (Figure 7). SIZ1-mediated sumoylation may facilitate *CBF*/*DREB1* expression, ICE1 stability, and *MYB15* repression. SUMO1/2 conjugation to K393 of ICE1 is the apparent biochemical basis for these positive and negative control processes. Presumably, the relative affect of cold exposure on these positive and negative control processes can provide a fine-tuned balance of phenotypic responses that enable fitness to an environment that experiences different low temperature episodes.

METHODS

Plant Freezing Assay

Wild-type (ecotype Col-0), *siz1-2*, and *siz1-3* plants were grown for 3 weeks (from sowing) in soil at 23°C under a long-day photoperiod (16 h light/8 h dark). Plants grown under these conditions were considered nonacclimated. For cold acclimation, 3-week-old plants were incubated at 4°C for 7 d under a long-day photoperiod. Plant freezing tests were performed as described (Zhu et al., 2004). Briefly, plants were exposed to low temperatures in a controlled temperature chamber (model Tenney-JR; Tenney Engineering). For Figure 1, plants were exposed to 4°C for 0.5 h, then 0°C for 1 h, and then the programmed cycle of temperature reduction (−2°C over 30 min and then maintenance of that temperature for 1 h). The cycle was repeated until the desired temperature was reached. At 0°C, ice chips were spread over the plants to facilitate uniform nucleation. For Figure 6, cold-acclimated (4°C) plants were exposed to 0°C for 0.5 h, and ice chips were sprinkled over these plants before the chamber was programmed to cool at -1°C h^{-1}. After a freezing treatment, plants were incubated at 4°C for 1 d and then returned to 23°C, and survival was determined 7 d later.

Electrolyte Leakage from Leaves

Electrolyte leakage from fully developed rosette leaves of 3-week-old plants was assessed as described (Zhu et al., 2004), with modifications. Each leaf (5th or 6th rosette leaf) was placed into a tube containing 200 μL deionized water, an ice chip was added to initiate nucleation, and the tube was incubated in a refrigerated circular bath (NESLAB RTE-120). The temperature of the bath was programmed for a temperature decrease from 0 to −8°C by a continuous reduction of −1°C over 30 min until the desired temperature was reached. Each tube was removed from the refrigerated bath and then placed immediately into ice to facilitate gradual thawing. Deionized water (14 mL) was added to the sample that was then shaken overnight, after which the conductivity of the solution was determined with the Accumet model 20 pH/conductivity meter (Fisher Scientific). The tube was then incubated at 90°C for 1.5 h and cooled to room temperature, and conductivity of the solution was determined (i.e., 100% conductivity). Conductivities of four leaves, each from a different plant, were evaluated at the different temperature minima.

Quantitative RT-PCR

Total RNA was isolated using TRIZOL reagents (Invitrogen) according to the manufacturer's protocol. Three micrograms of RNA were used as template for first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen) and an oligo(dT_{21}) primer. Primer pairs for real-time PCR (see Supplemental Table 1 online) were designed as described (Miura et al., 2005). Real-time PCR amplification, validation experiments, and calculation of relative differences in expression level were performed as per instructions (User Bulletin 2 for ABI PRISM 7700 sequence detection system; Applied Biosystems) (Miura et al., 2005). Briefly, real-time PCR amplification was performed with 20 μL of reaction solution, containing 5 μL of 50-fold–diluted cDNA, 0.3 μM of each primer, and 1× QuantiTect SYBR Green PCR Master Mix (Qiagen). Analysis was performed using the Applied Biosystems PRISM 7700 sequence detection system. Relative transcript abundance was calculated using the comparative C_T method (User Bulletin 2 for ABI PRISM 7700 sequence detection system). For a standard control, expression of *Actin2* was used. After calculation of ΔC_T ($C_{T,\text{gene of interest}} - C_{T,actin2}$), $\Delta\Delta C_T$ [$\Delta C_T - \Delta C_{T,WT(0\ h)}$] was calculated as instructed. The relative expression level was calculated as $2^{-\Delta\Delta CT}$. A $2^{-\Delta\Delta CT}$ value for the wild type without cold treatment (0 h) was normalized to 1[$2^{-\Delta\Delta CT(\Delta CT,WT(0\ h)-\ \Delta CT,WT(0\ h))} = 2^0 = 1$].

In Vivo Analysis of Sumoylation Profiles

Seeds were sown onto 1× Murashige and Skoog salts medium containing 3% sucrose (Miura et al., 2005). Ten-day-old seedlings were incubated at 0°C for the times indicated prior to harvesting. Total protein was extracted as described (Murtas et al., 2003, 2005). Total protein (200 μg) was separated by SDS-PAGE and then blotted. The gel blot was probed with anti-SUMO1 (kindly provided by G. Coupland; Murtas et al., 2003) and was detected using ECL plus (Amersham).

Purification of Recombinant Proteins and in Vitro SUMO Conjugation Assay

To introduce the T7 tag (MASMTGGQQMG) into pGEX-5X-1 (Amersham), pET-21a (Novagen) and pGEX-5X-1 were digested with *Nde*I and *Eco*RI, respectively, and then incubated with T4 DNA polymerase for blunt-end ligation. The blunt-ended vectors then were digested with *Xho*I. The DNA fragment containing the T7 sequence was separated on an agarose gel, collected using the QIAquick gel extraction kit (Qiagen) and ligated into the digested pGEX-5X-1. The resulting construct was named pGEX-5X-T7. The *ICE1* open reading frame was amplified from cDNA (synthesized from RNA of 3-h cold-treated [0°C] seedlings) using *pfu* DNA polymerase (Stratagene), and the primers ICE1-T7F and ICE1-HAR (see Supplemental Table 2 online). The PCR product and pGEX-5X-T7 were digested with *Eco*RI and *Xho*I and then ligated. The construct was designated as pGST-T7-ICE1. To make an ICE1(K393R) protein, $AA_{1178}G$ was replaced with AGG by site-directed mutagenesis using the primers ICE1K393RF and ICE1K393RR (see Supplemental Table 2 online) (Miura et al., 2005). The PCR product was cloned into pGEX-5X-T7. The resulting plasmid construct was named pGST-T7-ICE1(K393R). ICE1 recombinant protein constructs were transformed into *Escherichia coli* BL21(DE3). *E. coli* cells containing pGST-T7-ICE1 or pGST-T7-ICE1(K393R) were incubated at 37°C until cells reached mid-log phase of growth. Then, 0.1 mM isopropylthio-β-galactoside was added, and cells were incubated at 18°C for 16 h. Crude extracts were purified using Glutathione Sepharose 4 Fast Flow (Amersham). Yeast proteins and *Arabidopsis thaliana* SIZ1 protein were purified as described (Takahashi et al., 2003; Miura et al., 2005).

The in vitro sumoylation assay was performed as described (Takahashi et al., 2003; Miura et al., 2005). Briefly, 3 μg of GST-T7-ICE1 or GST-T7-ICE1(K393R) was added to a reaction mixture containing 1.8 μg GST-ScUba2, 0.85 μg GST-ScAos1, 0.17 μg ScUbc9, 3 μg His-ScSmt3, 2 μg GST-AtSIZ1, 50 mM Tris-HCl, pH 7.4, 10 mM ATP, 2 mM DTT, and 5 mM $MgCl_2$. The reaction mixture (30 μL) was incubated at 37°C for 1.5 h. Proteins were separated by SDS-PAGE, and immunoblot analysis performed with anti-T7 antibody (Bethyl Laboratories) was performed.

In Vitro Ubiquitination of ICE1

Arabidopsis MBP-HOS1 (cDNA clone was kindly provided by Jian-Kang Zhu; Dong et al., 2006) was purified using amylose resin (New England Biolabs) as instructed. Purified GST-T7-ICE1 was sumoylated in vitro as described above. ICE1 was purified using T7-Tag antibody agarose (Novagen). The ICE1 was washed three times and eluted according to the manufacturer's instructions. ICE1 protein was added to the ubiquitination buffer containing 0.2 μg of human E1 and 0.4 μg of E2 UbcH5b (Boston Biochemicals), 1 μg of E3 (MBP-HOS1), 10 μg of bovine ubiquitin (Sigma-Aldrich), 1 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT. The mixture was incubated at 30°C for 4 h, and samples were separated by electrophoresis on 8% SDS gels.

Protoplast Purification and Immunoprecipitation Analysis

The *ICE1* coding region was amplified with *pfu* DNA polymerase and the primers ICE1-HAF and ICE1-HAR (see Supplemental Table 2 online). The

*Bam*HI- and *Xho*I-digested PCR product was inserted into the plasmid p326-HAN (Jin et al., 2001) to produce a chimeric *HA-ICE1* or *HA-ICE1(K393R)* fusion under the control of the 35S promoter. These plasmids were purified using the Qiagen plasmid maxi kit according to the manufacturer's instructions. *Arabidopsis* protoplasts were prepared from 2-week-old wild-type or *siz1-2* seedlings with Cellulase Onozuka R-10 and Macerozyme R-10 (Yakult Pharmaceutical) as described (Jin et al., 2001). The fusion constructs were introduced into *Arabidopsis* protoplasts by polyethylene glycol–mediated transformation (Jin et al., 2001). After transformation with *T7:SUMO1* and *HA:ICE1* or *HA:ICE1(K393R)*, protoplasts were incubated for 36 h at 23°C. Cells were then maintained at 23°C or incubated at 0°C for 1 h and then immediately harvested for protein isolation.

Immunoprecipitation was performed essentially as described (Jin et al., 2001). T7-Tag antibody agarose (Novagen) was added to the lysate. After incubation at 4°C for 3 h, the agarose was washed three times. After elution from T7 antibody agarose, immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting using anti-HA.

Transgenic Plants

The *ICE1* coding region was amplified with *pfu* DNA polymerase and the primers ICE1-expF and ICE1-EGR (see Supplemental Table 2 online). pGEX-T7-ICE1 or pGEX-T7-ICE1(K393R) cDNA clone was used as a template for PCR. The binary vector pCsV1300 (promoter from cassava vein mosaic virus) was digested with *Bam*HI. The *Bam*HI-digested PCR product was inserted to construct pCsV1300-ICE1 or pCsV1300-ICE1(K393R). Each binary vector was mobilized into *Agrobacterium tumefacians* GV3101 and transformed into wild-type plants by the floral dip method (Miura et al., 2005). Hygromycin-resistant plants were selected, and diagnostic PCR was performed with the primers ICE1K393RF and NOS-R (see Supplemental Table 2 online).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *SIZ1*, AAU00414; *ICE1*, AAP14668; and *HOS1*, AAB87130.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *siz1* Plants Are Chilling Sensitive.

Supplemental Figure 2. Sumoylation of ICE1 Induces *CBF3*/*DREB1A* and Represses *MYB15* Expression.

Supplemental Figure 3. ICE1 or ICE1(K393R) Expression in Transgenic Wild-type Plants.

Supplemental Table 1. Primer Sequences Used to Detect *SIZ1* or Genes Involved in Cold Signaling by the Quantitative PCR Method.

Supplemental Table 2. Primer Sequences Used for Construction of Vectors.

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