SIZ1, a small ubiquitin-related modifier ligase, controls cold signaling through regulation of salicylic acid accumulation

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Summary

Low temperature induces several genes to acquire plant cold tolerance. Here, we demonstrate that accumulation of SA (salicylic acid) is involved in the regulation of the *DREB1A /CBF3* regulon and plant tolerance to cold stresses. The SA-accumulating mutant *siz1* exhibits sensitivity to chilling and freezing conditions and decreased expression of *DREB1A /CBF3* and its regulon genes. Reduction of SA levels in *siz1* by *nahG* restored cold sensitivity and down-regulation of these genes.

Database analyses and RT-PCR analysis revealed that the *ice1* mutation also increased expression of SA-responsive genes. As well as *siz1*, another SA accumulating mutant *acd6* exhibited freezing sensitivity and the sensitivity was suppressed in *acd6 nahG* plants. Taken together, these data indicate that SA is involved in regulation of cold signaling.

Keywords: cold response, cold signaling, salicylic acid, SUMO

Abbreviations:

ABA, abscisic acid; ACD, accelerated cell death; COR, cold responsive; CPR, constitutive expresser of *PR*; DREB1/CBF, dehydration responsive element (DRE)/C-repeat (CRT) binding proteins; GA, gibberellic acid; HPY, high ploidy; ICE1, for inducer of DREB1/CBF expression 1; MMS, methyl methanesulfonate sensitive; PR, pathogenesis-related; SA, salicylic acid; SIZ, SAP and Miz; SUMO, small ubiquitin-related modifier

Introduction

Low temperature is one of the significant limitations to agricultural yields and crop productivity, especially when frost occurs during reproductive development. Additionally, cold limits distribution of plant species (Guy, 1990). To adapt to cold stress, plant cells synthesize and accumulate cryoprotectant solutes and cryoprotective proteins that stabilize cellular membranes and enhance antioxidative mechanisms (Mahajan and Tuteja, 2005).

Recent studies have revealed that several components play important roles in cold signaling and cold tolerance (Zhu et al., 2007). Numerous transcription factors that facilitate cold signaling and tolerance have been identified (Zhu et al., 2007). The best-characterized transcription factors are ICE1 (inducer of DREB1/CBF expression 1; Chinnusamy et al., 2003) and DREB1/CBF (dehydration responsive element (DRE)/C-repeat (CRT) binding proteins; Stockinger et al., 1997; Liu et al., 1998). ICE1, a MYC-type transcription factor, binds to canonical *MYC cis*-elements in the promoter of *DREB1A/CBF3* (Chinnusamy et al., 2003). The DREB1A/CBF3 proteins transactivate cold-inducible and ABA (abscisic acid)-independent expression of *COR* (cold responsive) genes, which contain *DRE/CRT cis*-elements in their promoters (Yamaguchi-Shinozaki and Shinozaki, 1994).

To activate *DREB1A/CBF3*-dependent cold signaling, ICE1 is posttranslationally modified by SUMO (small ubiquitin-related modifier; Miura et al., 2007a; Miura and Hasegawa, 2008). SUMO conjugation to substrate proteins (sumoylation) is a reversible posttranslational modification (Miura et al., 2007b). Sumoylation of ICE1 positively regulates expression of *DREB1A/CBF3* and the genes of its regulon and increases cold tolerance (Miura et al., 2007a). In general, SUMO modification modulates transcription factor activity to coordinate the gene expression that is necessary for development and for hormonal and environmental responses (Miura et al., 2007b). Sumoylation undergoes in a series of biochemical steps; SUMO-specific E1-activation, E2-conjugation, and E3-ligation enzymes (Miura et al., 2007b). Two SUMO E3 ligases, SIZ1 (SAP and Miz1) and HPY2 (high ploidy2)/AtMMS21 (methyl methanesulfonate sensitive21), have been identified in *Arabidopsis* (Miura et al., 2005; Ishida et al., 2009). The *Arabidopsis* SIZ1 has been functionally linked to many plant responses, including phosphate starvation, growth, cold signaling, basal thermotolerance, SA (salicylic acid)-mediated signaling for plant defense, flowering, and ABA (Miura et al., 2005; Yoo et al., 2006; Catala et al., 2007; Lee et al., 2007; Miura et al., 2007a; Saracco et al., 2007; Jin et al., 2008; Miura and Hasegawa, 2009; Miura et al., 2009).

During chilling conditions, wild-type *Arabidopsis* shoots slowly accumulate free SA and glucosyl SA (Scott et al., 2004). *Arabidopsis* plants transformed with the bacterial SA hydroxylase gene *nahG* have reduced amounts of SA (Yamamoto et al., 1965) and grow much larger than wild-type plants at 5°C (Scott et al., 2004). The SA-deficient *eds5* (enhanced disease susceptibility 5) mutant likewise exhibits more growth than wild type under cold condition (Scott et al., 2004). In contrast to *nahG* and *eds5* plants, the *cpr1* (constitutive expresser of *PR* (pathogenesis-related) gene1) mutation enhances SA accumulation and causes a strong reduction in growth under low temperatures (Scott et al., 2004). These results suggest that SA is one of phytohormones contributing to growth inhibition under low temperatures. Both winter and spring wheat has decreased whole plant survival after freezing stress when SA is hydroponically applied (Horváth et al., 2007). However, if SA is sprayed onto the leaves, freezing tolerance is promoted in winter wheat (Tasgín et al., 2003), suggesting that the effect of SA might be tissue-specific.

To understand the relationship between the internal SA level and cold response/signaling, we investigated the freezing and chilling tolerances of *siz1-2, nahG,* and *siz1-2 nahG* plants. *siz1-2* accumulates SA, while *siz1-2 nahG* has reduced internal SA levels (Yoo et al., 2006; Lee et al., 2007). The SA-accumulating mutant *siz1-2* exhibited enhanced cold sensitivity, as described

previously (Miura et al., 2007a), but cold sensitivity of *siz1-2* was rescued by reducing SA. Expression levels of cold-responsive genes such as *DREB1A/CBF3, COR47,* and *KIN1* were down-regulated in *siz1-2*, but were similar to wild type in *siz1-2 nahG* plants. Genevestigator database analysis revealed that several SA-inducible genes are also up-regulated in the *ice1* mutant as well as in the *siz1-2* (Lee et al., 2007) under unstressed condition, suggesting that SIZ1-ICE1-dependent cold signaling also regulates SA accumulation. As well as *siz1* mutant, another SA-accumulating mutant *acd6* (accelerated cell death 6) exhibited freezing sensitivity, which was suppressed in *acd6 nahG* plants. These results SA plays a role in regulation of cold signaling and are located down-stream of ICE1.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana siz1-2 (Miura et al., 2005), *nahG* (van Wees and Glazebrook, 2003), *ice1* (Chinnusamy et al., 2003), *siz1-2 nahG* (Lee et al., 2007), *acd6* and *acd6 nahG* (Rate et al., 1999) mutants in the Columbia background (Col-0) were used in this research. Plants grown for 3 weeks in soil at 23°C were used for non-acclimated plants. For cold acclimation, 3-week-old plants

were incubated at 4°C for 7 days. Whole plant freezing tests were performed as described (Miura et al., 2007a). For chilling assays, 3-week-old plants were incubated at 4°C for 4 weeks.

Measurement of electrolyte leakage

Electrolyte leakage test was performed as described (Miura et al., 2007a). Briefly, a leaf harvested from fifth and sixth rosette leaves of 3-week old non- or cold-acclimated plants was used. The leaf was put into a tube containing 200 μL deionized water, and an ice chip was added to the tubes to initiate nucleation. The tubes was incubated in a refrigerated circular bath (TRL-N11LP, Thomas, Tokyo, Japan) programmed by continuous reduction of -1°C per 30 min. Each tube was removed from the bath and incubated on ice to thaw, and 15 mL of deionized water was added and shaken overnight. The conductivity of the solution was determined with conductivity meter CD-4302 (Lutron).

RNA isolation and real-time quantitative PCR analysis

For quantitative RT-PCR analysis, 3 μg of RNA from 10-day-old seedlings with or without cold treatment were used as template for first-strand cDNA synthesis using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Real-time PCR was performed with SYBR

Premix Ex Taq (Takara) and gene specific primers, *ACT2, DREB1A/CBF3, COR47,* and *KIN1* (primer sequence information was described in Miura et al., 2007a), *PR1* (Tsuda et al., 2008), and *At1g17170* (Yoon et al., 2008). PCR products were detected by Thermal Cycler Dice Real Time System (Takara). Relative differences in expression were calculated as described (Miura et al., 2007a).

***In vivo* analysis of sumoylation profiles**

Seeds were sown onto half Murashige and Skoog medium containing 1% sucrose. Ten-day-old seedlings were incubated at 4ºC for 3 h. Total protein was extracted in lysis buffer [50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.2 mM sodium orthovanadate, 100 mM NaF, 10% glycerol, 0.2% Triton X-100, 5 mM DTT, 5 mM *N*-ethylmaleimide, and 1 x protein inhibitor cocktail (Roche)]. Samples were spun and the concentration of proteins in the supernatant was determined (Miura et al., 2007). Two hundred micrograms of protein was loaded onto an SDS-PAGE gel, and the protein gel blot was probed with the AtSUMO1 antibody and was detected using ECL plus (GE healthcare). To raise AtSUMO1 antibody, the recombinant His-SUMO1 protein (a vector was kindly provided by Dr. HP Stuible, Colby et al., 2006) was purified by HIS-Select Nickel Affinity Gel (Sigma) according to the manufacturer's instructions. Affinity purified protein was separated by SDS-

PAGE and the corresponding band was cut. The gel was used to raise antiserum (BioRegenerations).

Results

Reduction of SA by *nahG* enhances cold tolerance in *siz1 nahG*

We prepared plants with or without cold acclimation and treated plants at several freezing temperatures as described (Miura et al., 2007a). Plants with the *nahG* expression exhibited more tolerant to freezing than wild-type plants both before and after cold acclimation (Figure 1A-C). *siz1-2* plants were freezing-sensitive. Interestingly, *siz1-2 nahG* plants recovered the enhanced freezing sensitivity of *siz1-2* plants (Figure 1A-C). Electrolyte leakage from non- and cold-acclimated *siz1-2* was higher than wild-type plant (Figure 1D) as described (Miura et al., 2007a). *siz1-2* plants were impaired in cold acclimation capacity compared with wild-type plants (Figure 1D); electrolyte leakage from cold-acclimated wild-type plants was less than half of that from non-acclimated wild-type plants, but the leakage from cold-acclimated *siz1-2* was more than half of that from non-acclimated *siz1-2*.

Three-week-old wild-type, *siz1-2, nahG,* and *siz1-2 nahG* plants were incubated at 4°C for 4 weeks to check chilling sensitivity. *nahG* plants grew better after the chilling treatment than wild-type plants (Figure 1E) as described (Scott et al., 2004), even though no significant difference between wild-type and *nahG* plants under normal condition (not shown). On the other hand, leaves of *siz1-2* exhibited necrosis, which is a typical symptom of chilling sensitivity, but necrosis was not observed in the other genotypes (Figure 1E). The *nahG* expression suppressed *siz1-2* hypersensitivity to chilling (Figure 1E). These results suggest that SIZ1 controls cold tolerance through regulation of SA accumulation.

Down-regulation of cold inducible genes in *siz1* is suppressed by *nahG*

Induction of cold-responsive genes through cold signaling is important for cold tolerance (Zhu et al., 2007). Low temperatures transiently induce *DREB1/CBF* expression and increase transcript levels of *DREB1/CBF*-regulon genes. Of the three *DREB1/CBF* genes, *DREB1A/CBF3* is mainly controlled by sumoylated ICE1 and is substantially down-regulated in *siz1* (Chinnusamy et al., 2003; Miura et al., 2007a). So we tested expression level of *DREB1A/CBF3* and its regulon genes, *COR47,* and *KIN1* in wild-type, *siz1-2, nahG,* and *nahG siz1-2* seedlings. Transcript accumulation of these genes was down-regulated in *siz1-2* and was recovered by the introduction of

nahG into *siz1-2* (Figure 2). Plants with the *nahG* expression exhibited both increased cold tolerance (Figure 1) and increased transcript levels of these genes under low temperature conditions (Figure 2) compared with wild-type plants.

Expression of SA regulated genes is controlled by SIZ1-ICE1-dependent pathway

Because SIZ1 mediates sumoylation of ICE1 to control cold signaling (Miura et al., 2007a), we hypothesized that ICE1-dependent cold signaling is mediated by SA. Microarray analyses reveal that several SA-inducible and pathogen defense genes were up-regulated in *siz1-2* under normal conditions (Lee et al., 2007). For example, genes encoding pathogenesis-related PR1 protein, chitinase, osmotin-like protein, glutathione-*S*-transferase, and peroxidases were highly expressed in *siz1-2* plants (Lee et al., 2007). To compare expression levels of these genes under cold and *Pseudomonas syringae* treatment, in the *ice1* and *cpr5* mutants, we extracted the expression patterns of these genes from Genevestigator database (Fig. 3; Zimmermann et al., 2004). The *cpr5* mutant accumulates high levels of SA without any treatment (Bowling et al., 1997). The expression patterns of the genes highly expressed in *siz1-2* mutant were almost overlapped with those in the *cpr5* mutant, demonstrating that these genes are typical SA- and disease-responsive genes (Figure 3A). Interestingly, most of these genes were up-regulated in *ice1* as well as in *cpr5* and *siz1-2*

mutants under unstressed condition (Figure 3A). To confirm up-regulation of SA-regulated genes in *ice1*, expression levels of *PR1* and *At1g17170* were measured by real-time quantitative RT-PCR analysis. The expression of these genes was up-regulated in *ice1* as were in *siz1-2* (Figure 3B). Cold treatment also increased expression levels of these genes (Figure 3A), consistent with cold-induced accumulation of SA (Scott et al., 2004). These results implicate that there is a relationship between ICE1-dependent cold signaling and SA accumulation and/or responses and SA should be downstream of ICE1 in cold signaling.

Because SIZ1 is a SUMO E3 ligase and sumoylated proteins were substantially decreased *in planta* (Miura et al., 2005), SUMO1 conjugation pattern in wild-type, *siz1-2*, *nahG,* and *siz1-2 nahG* plants was investigated. As described (Miura et al., 2007), SUMO1 conjugation was increased by cold treatment (Figure 4). Similar pattern was observed in *nahG* plants (Figure 4). Because SUMO E3 ligase activity is disrupted by the *siz1-2* mutation, SUMO1 conjugation was substantially decreased in *siz1-2 nahG* as well as *siz1-2* (Figure 4). These results suggest that accumulation of SUMO1 conjugation is not a major factor to enhance SA accumulation.

SA is one of factors to regulate freezing tolerance

To confirm that SA plays a role in regulation of cold signaling, freezing sensitivity of another SA accumulating mutant *acd6* was investigated. As well as *siz1-2* (Figure 1), the *acd6* mutant exhibited freezing sensitivity before and after cold acclimation (Figure 5A, B). This sensitivity was also suppressed by introduction of *nahG*. We also investigated whether expression of cold regulated genes are affected by the *acd6* mutation. Expression of *DREB1A/CBF3, COR47,* and *KIN1* was down-regulated in *acd6* and was recovered by the introduction of *nahG* into *acd6* (Figure 5C). These results are similar results as *siz1* mutant showed (Figure 1, 2). These results demonstrate that accumulation of SA reduces cold tolerance and expression level of cold-inducible genes.

Discussion

Here we demonstrate that internal SA accumulation caused by the *siz1* and *acd6* mutation is involved in regulation of cold signaling and tolerance. Reduction of internal SA in *siz1-2 nahG* or *acd6 nahG* enhanced cold tolerance (Figure 1, 5A, 5B) and expression of cold-responsive genes (Figure 2, 5C). Because SA is induced by cold temperature (Scott et al., 2004), *nahG* plants showed more tolerant to chilling and freezing stresses, compared to wild-type plants (Figure 1).

The *ice1* mutation also enhances SA-responsive genes (Figure 3), suggesting that internal SA is involved in regulation of ICE1-dependent cold signaling.

DREB1/CBFs are important transcription factors for cold signaling. Overexpression of *DREB1/CBF* genes in *Arabidopsis* can confer enhanced freezing tolerance, but causes severe growth retardation under normal growth conditions (Gilmour et al., 2004). This result implicates that *DREB1/CBF* regulates two different physiological processes, induction of cold tolerance and growth retardation. The growth retardation of *DREB1B/CBF1*overexpressing plants is caused, in part, by accumulation of DELLA proteins (Archard et al., 2008), whose degradation by the proteasome is promoted by GA (gibberellic acid; Dill et al., 2004). Thus, the double-DELLA mutation *gai-t6 rga-24* suppresses the growth inhibition phenotype of *DREB1B/CBF1*-overexpressing plants, as does the application of GA (Archard et al., 2008). The DREB1/CBF-dependent signaling pathway modulates accumulation of DELLA proteins to promote plant growth inhibition (Archard et al., 2008).

On the other hand, DREB1/CBFs also induce expression of the DREB1/CBF regulon, such as cold-responsive genes, and the accumulation of soluble sugars for cold tolerance (Gilmour et al.,

2004). The DREB1/CBF regulon is independent of DELLA accumulation (Archard et al, 2008), indicating that this DREB1/CBF-dependent signaling pathway for cold tolerance constitutes a different pathway form the DELLA-mediated growth retardation pathway. And SA may be regulated by this signaling pathway for cold tolerance, independent of DELLA-mediated growth retardation.

Our present results demonstrate an aspect that ICE1-dependent cold signaling represses accumulation of SA. Accumulation of SA drastically reduced cold tolerance and the expression of *DREB1A/CBF3* and its regulon genes (Figure 1, 2). This reduction was suppressed by removal of SA (Figure 1, 2). Because the *ice1* mutation up-regulates expression of SA-inducible genes, ICE1-dependent pathway may suppress SA accumulation. SA accumulated mutants, such as *siz1-2, acd6, cpr1,* and *cpr5,* exhibits dwarf-like phenotype (Bowling et al., 1994; Bowling et al., 1997; Rates et al., 1999; Saracco et al., 2007). And the *ice1* mutant also shows dwarf-like phenotype (Chinnusamy et al., 2003). It is likely that there is a linkage between SA accumulation and cold signaling and that SA is located at downstream of ICE1 in cold signaling.

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Reference

Archard P, Gong F, Cheminant S, Alioua M, Hedden P, Genschik P. The cold-inducible CBF1 factoer-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. Plant Cell 2008;20:211-229.

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell 1994;6:1845-1857.

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 1997;9:1573-1584.

Catala R, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH. The Arabidopsis E3 ligase SIZ1 regulates plant growth and drought responses. Plant Cell 2007;19:2952-2966.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu J-K. ICE1: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis.* Genes Dev 2003;17:1043-1054.

Dill A, Thomas SG, Hu J, Steber CM, Sun TP. The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell 2004;16:1392-1405.

Gilmour SJ, Fowler SG, Thomashow MF. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol 2004;54:767-781.

Guy CL. Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol 1990;41:187-223.

Horváth E, Pál M, Szalai G, Páldi E, Janda T. Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. Biol Plant 2007;51:480-487.

Ishida T, Fujiwara S, Miura K, Stacey N, Yoshimura M, Schneider K, Adachi S, Minamisawa K, Umeda M, Sugimoto K. SUMO E3 ligase HIGH PLOIDY2 regulates endocycle onset and meristem maintenance in *Arabidopsis*. Plant Cell 2009;PMID:19666737.

Jin JB, Jin YH, Lee J, Miura K, Yoo CY, Kim WY, van Oosten M, Hyun Y, Somers DE, Lee I, Yun D-J, Bressan RA, Hasegawa PM. The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. Plant J 2008;53:530-540.

Lee J, Nam J, Park HC, Na G, Miura K, Jin JB, Yoo CY, Baek D, Kim DH, Jeong JC, Kim D, Lee SY, Salt DE, Mengiste T, Gong Q, Ma S, Bohnert HJ, Kwak SS, Bressan RA, Hasegawa PM, Yun D-J. Salicylic acid-mediated innate immunity in Arabidopsis is regulated by SIZ1 SUMO E3 ligase. Plant J 2007;49:79-90.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 1998;10:1391-1406.

Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 2005;444:139-158.

Miura K, Hasegawa PM. Regulation of cold signaling by sumoylation of ICE1. Plant Signal Behav 2008;3:52-53.

Miura K, Hasegawa PM. Sumoylation and abscisic acid signaling. Plant Signal Behav 2009:4: in press.

Miura K, Rus A, Sharkhuu A, Yokoi S, Karthikeyan AS, Raghothama KG, Baek D, Koo YD, Jin JB, Bressan RA, Yun D-J, Hasegawa PM. The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proc Natl Acad Sci USA 2005;102:7760-7765.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun D-J, Hasegawa PM. SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* expression and freezing tolerance in *Arabidopsis*. Plant Cell 2007a;19:1403-1414.

Miura K, Jin JB, Hasegawa PM. Sumoylation, a post-translational regulatory process in plants. Curr Opin Plant Biol 2007b;10:495-502.

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM. Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proc Natl Acad Sci USA 2009;106:5418-5423.

Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT. The gain-of-function Arabidopsis mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell 1999;11:1695-1708.

Saracco SA, Miller MJ, Kurepa J, Vierstra RD. Genetic analysis of SUMOylation in Arabidopsis: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. Plant Physiol 2007;145:119-134.

Scott IM, Clarke SM, Wood JE, Mur LAJ. Salicylate accumulation inhibits growth at chilling temperature in Arabidopsis. Plant Physiol 2004;135:1040-1049.

Stockinger EJ, Gilmour SJ, Thomashow MF. *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 1997;94:1035-1040.

Tasgín E, Atící Ö, Nalbantoglu B. Effects of salicylic acid and cold on freezing tolerance in winter wheat leaves. Plant Growth Regul 2003;41:231-236.

Tsuda K, Sato M, Glazebrook J, Cohen JD, Katagiri F. Interplay between MAMP-triggered and SA-mediated defense responses. Plant J 2008;53:763-775.

van Wees SCM, Glazebrook J. Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J 2003;33:733-742.

Yamaguchi-Shinozaki K, Shinozaki K. A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 1994;6:251-264.

Yamamoto S, Katagiri M, Maeno H, Hayashi O. Salicylate hydroxylase, a monooxygenases requiring flavin adenine dinucleotide. J Biol Chem 1965;240:3408-3413.

Yoo CY, Miura K, Jin JB, Lee J, Park HC, Salt DE, Yun D-J, Bressan RA, Hasegawa PM. SIZ1 small ubiquitin-like modifier E3 ligase facilitates basal thermotolerance in *Arabidopsis* independent of salicylic acid. Plant Physiol 2006;142:1548-1558.

Yoon JM, Oliver DJ, Shanks JV. Phytotransformation of 2,4-dinitrotoluene in *Arabidopsis thaliana*: toxicity, fate, and gene expression studies *in vitro*. Biotech Prog 2008;22:1524-1531.

Zhu J, Dong C-H, Zhu J-K. Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Curr Opin Plant Biol 2007;10:290-295.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 2004;136:2621-2632.

Legends of figures

Figure 1. *nahG* recovers cold sensitivity of *siz1*

Non-acclimated (A) or cold-acclimated (B) wild-type (WT), *siz1-2*, *nahG*, and *siz1-2 nahG* plants were exposed for 2 h to the temperature indicated. Photographs are taken 7 day after freezing treatment. (C) Survival ratio was determined for non-acclimated or cold-acclimated plants after freezing treatment. Data are means ± SD (n = 3). (D) Electrolyte leakage from non- or cold-acclimated wild-type, *siz1-2*, *nahG*, and *nahG siz1-2* plants after exposure to -4°C. Data are means ± SD (n = 4). (E) Three-week-old wild-type, *siz1-2*, *nahG*, and *siz1-2 nahG* plants were incubated at 4°C for 4 weeks to investigate chilling sensitivity. Illustration is of representative plants after chilling treatment. All tested plants (n = 12 for each genotype) exhibited similar phenotype.

Figure 2. *nahG* restores down-regulation of cold-inducible genes in *siz1*

Ten-day-old seedlings grown at 24°C were incubated at 4°C for the indicated time. Relative mRNA levels of *DREB1A/CBF3*, *COR47*, and *KIN1* were determined by quantitative RT-PCR analyses. Data are means ± SD (n = 3).

Figure 3. SA-inducible genes were up-regulated in *ice1*

(A) Relative mRNA levels of pathogen-related genes, which are up-regulated in the *siz1-2* mutant (Lee et al., 2007), were obtained from Genevestigator database. Expression levels in the *ice1, cpr5* mutants, *DREB1B/CBF2*-overexpression line, and after *Pseudomonas syringae* or cold treatment were listed. (B) RNA from four-week-old plants grown at 22°C was used as a template for cDNA synthesis. Relative mRNA levels of *PR1* and *At1g17170* were determined by quantitative RT-PCR analysis. Data are means ± SD (n = 3).

Figure 4. Cold inducible SUMO conjugation is decreased in *siz1-2 nahG* plants as well as in *siz1-2.* Shown are in planta sumoylation profiles of 10-day-old wild-type, *siz1-2, nahG,* and *siz1-2 nahG* seedlings that were grown on medium at 23ºC and then incubated at 0ºC for 3 h. Immunoblot analysis was performed with anti-SUMO1 antibody.

Figure 5. SA accumulating mutant, *acd6,* exhibits cold sensitive and the sensitivity is recovered by introduction of *nahG*.

(A) Non-acclimated or cold-acclimated wild-type (WT), *acd6, nahG,* and *acd6 nahG* plants were exposed for 4 h to the temperature indicated. Photographs are taken 7 day after freezing treatment.

(B) Survival ratio was determined for non-acclimated or cold-acclimated plants after freezing

treatment. Data are means ± SD (n = 3). (C) Ten-day-old seedlings grown at 24°C were incubated at 4°C for the indicated time. Relative mRNA levels of *DREB1A/CBF3*, *COR47*, and *KIN1* were determined by quantitative RT-PCR analyses. Data are means ± SD (n = 3).

Figure1

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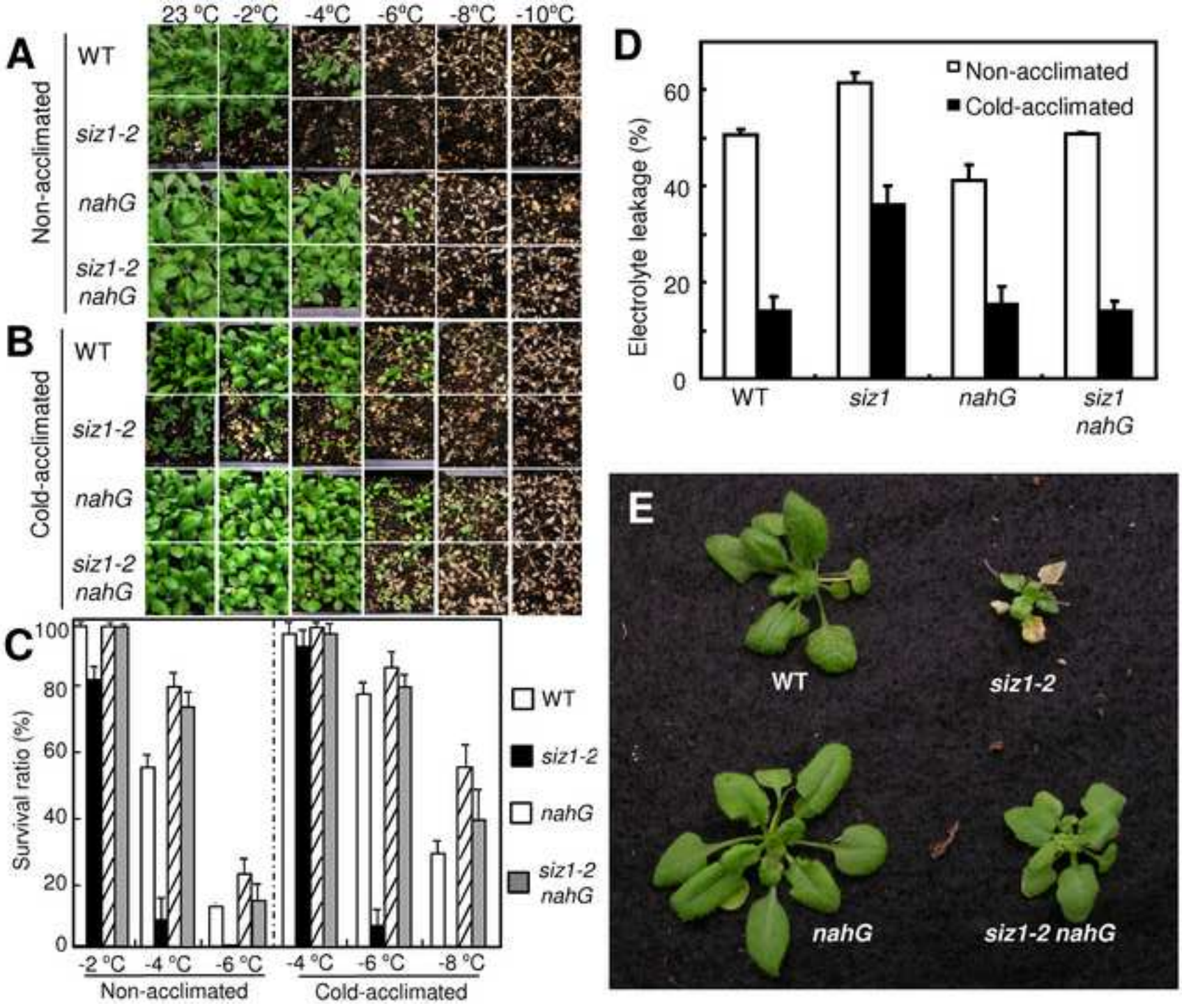

Figure2

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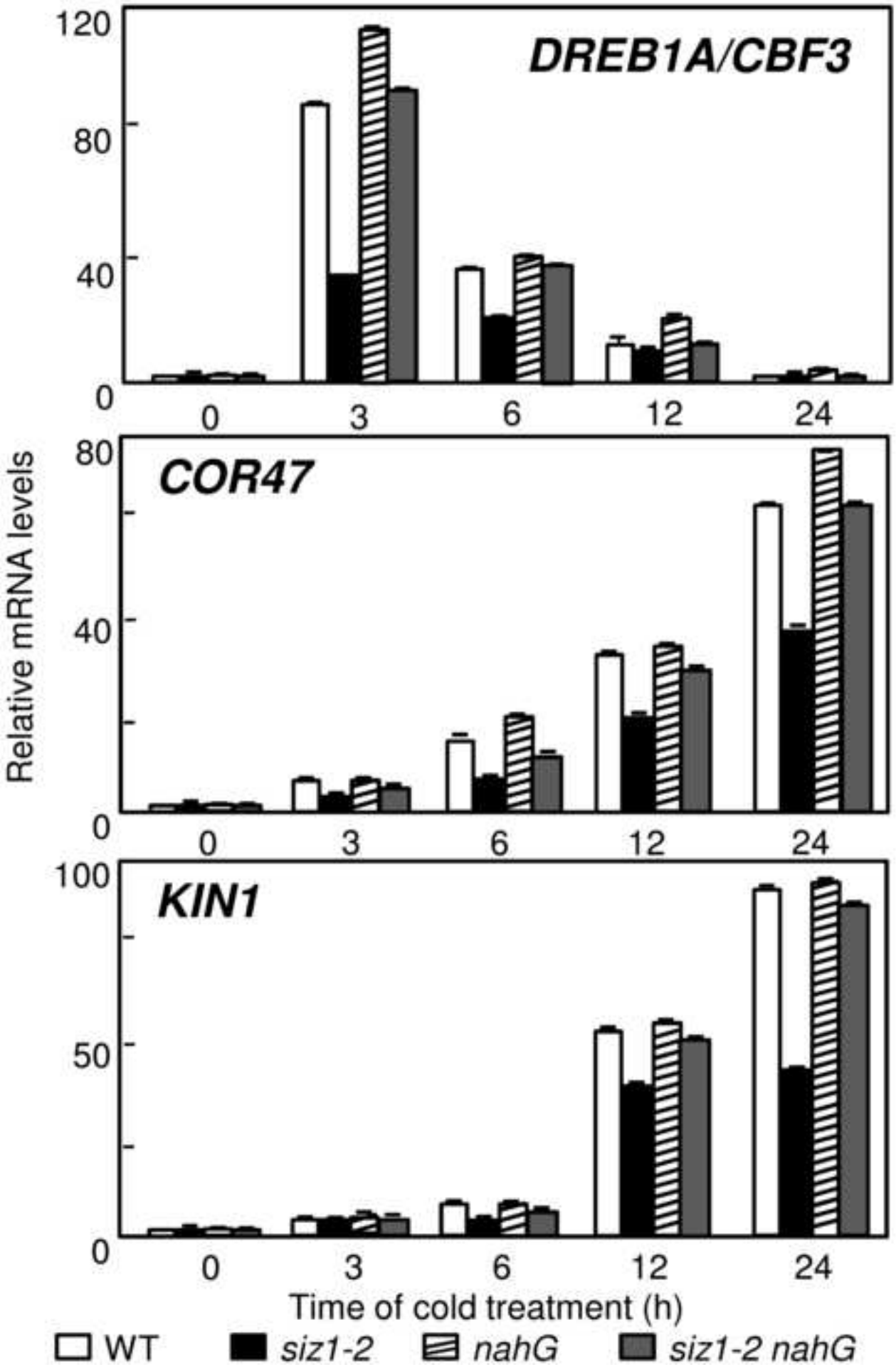

Figure3

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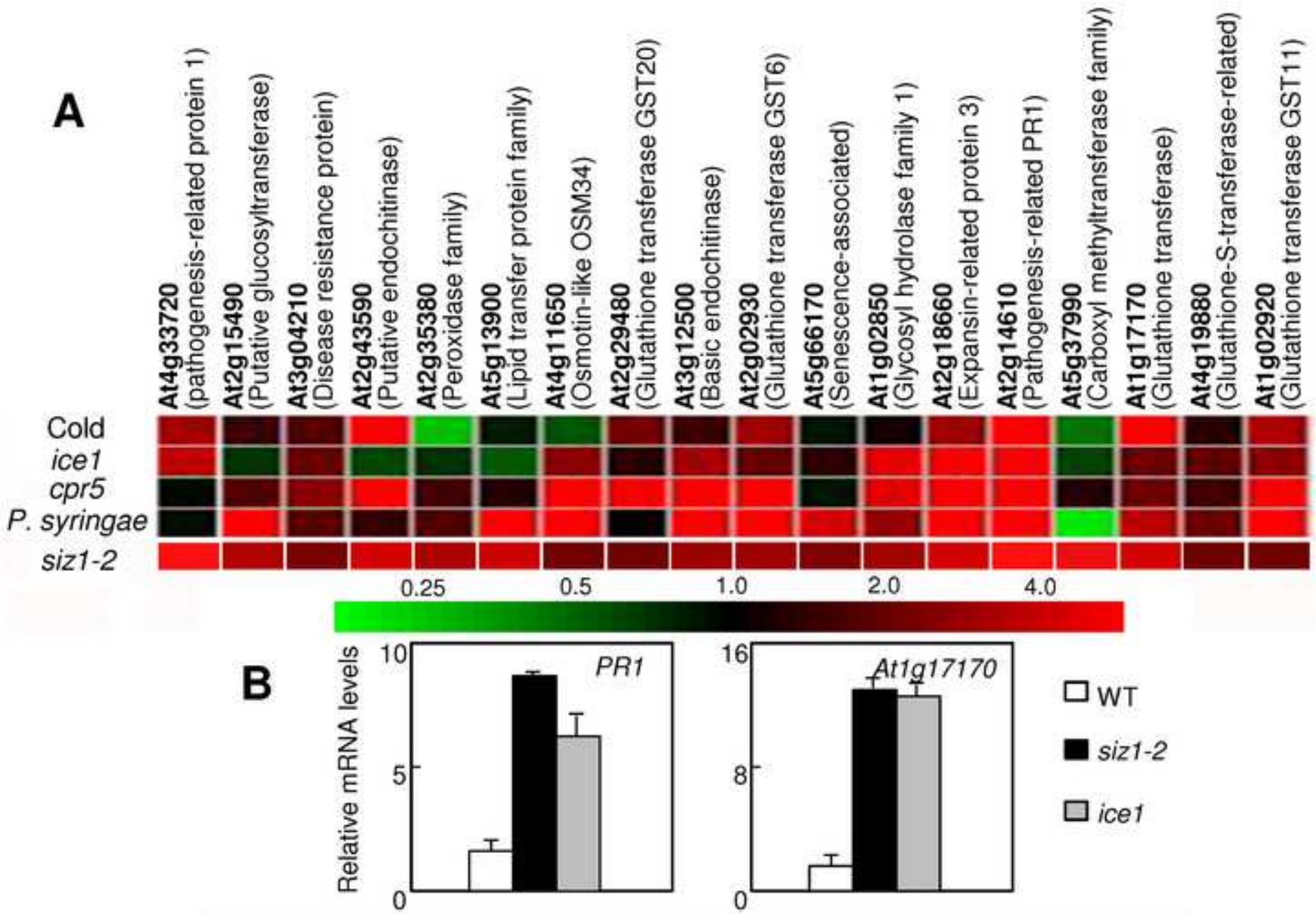

Figure4
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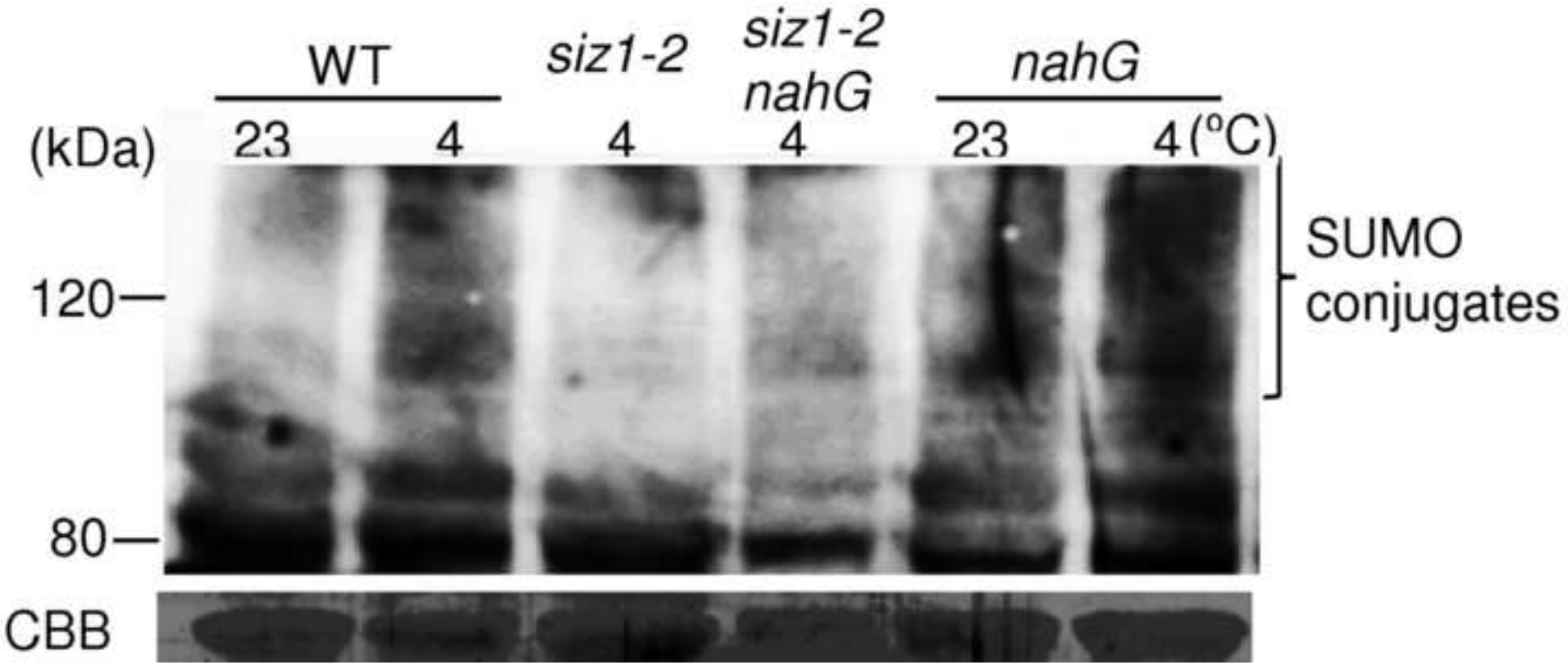

Figure5

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