SIZ1 Small Ubiquitin-Like Modifier E3 Ligase Facilitates Basal Thermotolerance in Arabidopsis Independent of Salicylic Acid\textsuperscript{1[W][OA]}

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Small ubiquitin-like modifier (SUMO) conjugation/deconjugation to heat shock transcription factors regulates DNA binding of the peptides and activation of heat shock protein gene expression that modulates thermal adaptation in metazoans. SIZ1 is a SUMO E3 ligase that facilitates SUMO conjugation to substrate target proteins (sumoylation) in Arabidopsis (Arabidopsis thaliana). siz1 T-DNA insertional mutations (siz1-2 and siz1-3; Miura et al., 2005) cause basal, but not acquired, thermosensitivity that occurs in conjunction with hyperaccumulation of salicylic acid (SA). NahG encodes a salicylate hydroxylase, and expression in siz1-2 seedlings reduces endogenous SA accumulation to that of wild-type levels and further increases thermosensitivity. High temperature induces SUMO1/2 conjugation to peptides in wild type but to a substantially lesser degree in siz1 mutants. However, heat shock-induced expression of genes, including heat shock proteins, ascorbate peroxidase 1 and 2, is similar in siz1 and wild-type seedlings. Together, these results indicate that SIZ1 and, by inference, sumoylation facilitate basal thermotolerance through processes that are SA independent.

High temperature stress adversely affects organisms by causing membrane integrity loss, reactive oxygen species (ROS) production, protein inactivation and denaturation, and metabolic and cellular disequilibria, which ultimately lead to cell death (Berry and Björkman, 1980; Quinn, 1988; Lindquist, 1992; Dat et al., 1998b; Los and Murata, 2000; Iba, 2002). Plants have an innate capacity to survive high temperature stress (basal thermotolerance) and can sense and acclimate to high temperatures with metabolic and cellular adjustments that impart a capacity to tolerate heat extremes that were previously lethal (acquired thermotolerance; Vierling, 1991; Alfonso et al., 2001; Clarke et al., 2004; Larkindale et al., 2005). Acquired thermal tolerance responses are coordinated by signaling pathways that regulate heat tolerance determinants to limit stress damage and facilitate reestablishment of cellular homeostasis for survival and growth (Clarke et al., 2004; Larkindale et al., 2005). Thermal adaptation responses include membrane compositional changes necessary for maintenance of functional integrity, activation of oxidative defensive systems through ethylene and salicylic acid (SA), and production of heat shock proteins (HSPs) necessary for cellular protection (Quinn, 1988; Boston et al., 1996; Schöffl et al., 1998; Larkindale and Knight, 2002; Baniwal et al., 2004; Clarke et al., 2004; Larkindale et al., 2005).

Heat shock transcription factor (HSF) activation that facilitates transient production of HSPs is a well-characterized process in acquired thermotolerance (Pirkkala et al., 2001; Larkindale et al., 2005). Vertebrate HSF1, which is the ortholog of yeast (Saccharomyces cerevisiae), Drosophila melanogaster, and Caenorhabditis elegans HSF, exists at ambient temperature as an inactive monomer that is complexed with HSP90 in the cytosol (Zou et al., 1998; Liu and Thiele, 1999; Guo et al., 2001; Hu and Mivechi, 2003). Heat shock causes disruption of the complex, leading to the formation of activated HSF1 homotrimers that migrate to the nucleus (Zandi et al., 1997) and facilitate HSP transcription through interaction of HSF1 trimers with heat shock elements (5′-AGAAnnTCTT-3′) in the promoters (Pelham, 1982; Westwood and Wu, 1993; Zuo

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SUMO conjugation of substrates occurs independently and does not appreciably affect HSP expression; however, hsfs1 hsfs3 double mutation substantially reduces heat shock-induced HSP expression (Lohmann et al., 2004). Hsf1 hsfs3 marginally affects thermotolerance, even though high temperature induction of HSP101 expression is substantially less (Lohmann et al., 2004). A tomato (Solanum lycopersicum esculentum) hsfs1 mutation is reported to cause thermosensitivity (Mishra et al., 2002).

HSPs are molecular chaperones that reduce protein denaturation, target denatured proteins for proteasome degradation, facilitate protein folding necessary for proper maturation or renaturation, and regulate the activity of HSFs to control HSP gene expression during thermotolerance acquisition (Johnson and Craig, 1997; Lee and Goldberg, 1998; Lee and Vierling, 2000; Frydman, 2001; Kim et al., 2002). Plant HSPs presumably mediate high temperature stress tolerance, but this is inferred largely because orthologs in other organisms have a thermal adaptive function (Vierling, 1991; Ellis, 2000; Hartl and Hayer-Hartl, 2002). Only the HSP100 family of plant HSPs, which are members of the ClpB chaperone family of ATPases that facilitate disaggregation of denatured proteins, are established functional determinants of acquired thermotolerance (Hong and Vierling, 2000; Questisch et al., 2000; Lee et al., 2005). Hsa32 (heat shock-associated) protein is necessary for maintenance of acquired thermotolerance in Arabidopsis (Charn et al., 2006). HOT2, HOT3, and HOT4 are genetic loci that facilitate acquired thermotolerance in Arabidopsis but map to positions in the genome that do not encode HSPs (Hong et al., 2003).

Basal thermotolerance is comparatively less understood than acquired thermotolerance (Hong and Vierling, 2000; Larkindale et al., 2005). HSP101 is an essential determinant for basal thermotolerance of seed germination (Hong and Vierling, 2000), and ethylene, SA, and ROS signaling functions in basal thermotolerance at different plant developmental stages (Clarke et al., 2004; Larkindale et al., 2005). The numerous cellular and metabolic processes involved in basal thermotolerance implicate that a signaling network composed of numerous regulators is necessary to exercise concerted control over effector determinants in a developmental context (Larkindale et al., 2005).

Sumoylation is a posttranslational modification process that conjugates the small ubiquitin-like modifier (SUMO) peptide to the K residue in the Ψ-K-X-D/E (Ψ, large hydrophobic residue; X, any residue) target motif of protein substrates (Bernier-Villamor et al., 2002; Melchior et al., 2003; Schmidt and Müller, 2003; Johnson, 2004). SUMO conjugation of substrates occurs in a series of biochemical steps that are mediated by E1-activating, E2-conjugating, and E3-ligation enzymes. SUMO has been linked in fungi and metazoans to processes such as innate immunity, cell cycle progression, thermal adaptation, DNA repair, nucleocytoplasmic trafficking, subnuclear targeting, ubiquitination antagonism, and transcriptional regulation (Mao et al., 2000; Saitoh and Hinche, 2000; Freiman and Tjian, 2003; Bohren et al., 2004; Dohmen, 2004; Johnson, 2004; Gill, 2005; Hay, 2005; Shuai and Liu, 2005; Zhao and Blobel, 2005; Hietakangas et al., 2006). Conjugation of SUMO to human (h) HSF1, hHSF2, and hHSF4b and Xenopus HSF2 regulates DNA binding and HSP expression (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2003, 2006; Hilgarth et al., 2003, 2004). In plants, high temperature induces SUMO1/2 conjugation to peptides, inferring that sumoylation may be involved in responses to high temperatures (Kurepa et al., 2003; Miura et al., 2005).

Arabidopsis SIZ1 is an ortholog of mammalian PIAS (protein inhibitor of activated signal transducer and activator of transcription) and yeast Siz family SUMO E3 ligases that facilitate sumoylation of transcription factors (Gill, 2005; Hay, 2005; Miura et al., 2005). Loss-of-function analyses described herein establish that the independent dysfunctional T-DNA insertion alleles siz1-2 or siz1-3 (Miura et al., 2005) cause thermosensitivity. Experimental results indicate that SIZ1 is a positive regulator of processes that are necessary for basal thermotolerance through functions that are independent of SA. However, SIZ1, dependent or independent of sumoylation function, does not regulate acquired thermotolerance as it does in fungi and metazoans (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2003; Hilgarth et al., 2003, 2004). Apparently, SUMO conjugation/deconjugation facilitates high temperature tolerance in plants through processes that have yet to be identified in other organisms.

RESULTS
The SUMO E3 Ligase SIZ1 Facilitates High Temperature Tolerance

Ten-day-old siz1-2 and siz1-3 seedlings exhibited substantial thermosensitivity, as determined by using a heat shock survival assay (Fig. 1, A and B). siz1 seedlings rapidly developed severe heat shock symptoms in response to high temperature that were not evident in wild type. Substantial reduction in leaf surface area (shrinkage) was visible immediately after treatment, followed by severe chlorosis 24 h later. siz1 seedlings that developed these extreme symptoms did not survive (Fig. 1, A and B). The maximal heat shock temperature (4-h treatment) that wild-type and siz1 seedlings could survive was 43°C and 40°C, respectively (data not shown).

Thermosensitivity of siz1-2 and siz1-3 seedlings was evident also in a hypocotyl elongation assay (Fig. 1C). High soil temperatures early in seedling development can restrict hypocotyl elongation, which may prevent...
or delay shoot emergence from the soil (Lin et al., 1984; Hong and Vierling, 2000). Hypocotyl elongation of siz1 seedlings was sensitive to even a brief exposure to 39°C (Fig. 1C). siz1 seedlings also exhibited a reduction in hypocotyl elongation at 37°C and 38°C, but sensitivity relative to wild type was less than at 39°C. Similar results in heat shock-sensitive phenotypes caused by independent siz1 alleles (Fig. 1) are indicative that SIZ1 functions in thermotolerance.

During latter stages of seed maturation, HSP101 accumulates and facilitates basal thermotolerance during germination (Hong and Vierling, 2001). siz1 seeds were sensitive to heat shock administered after imbibition and stratification, inhibiting both germination rate and seedling development (Fig. 2; Columbia [Col-0] and siz1-2, P < 0.01; Col-0 and siz1-3, P < 0.05). siz1-2 caused greater seed germination sensitivity than siz1-3 (Fig. 2; siz1-2 and siz1-3, P < 0.05). Heat treatment did not significantly alter seedling viability after germination (data not shown) but impaired development (Fig. 2A) and increased leaf chlorosis of siz1-2 and siz1-3 seedlings relative to wild type (data not shown).

SIZ1 Mediates Basal Thermotolerance through a SA-Independent Process(es)

Plants, like most other organisms, exhibit both basal (innate) and acquired thermotolerance (Larkindale et al., 2005). The latter phenomenon is an acclimation that occurs in response to brief exposure to high temperature or longer-term exposure to increasing temperature and facilitates survival of thermal extremes that previously were lethal (Hong and Vierling, 2000; Hong et al., 2003). siz1 mutations cause heat sensitivity in assays that assess basal thermotolerance (Fig. 3B; Col-0 and siz1-2, P < 0.01; Col-0 and siz1-3, P < 0.05); consequently, their effects on acquired thermotolerance were examined. siz1-2 and siz1-3 seedlings exhibited a similar capacity for acquired thermotolerance as wild type, as indicated from viability and hypocotyl elongation assays (Fig. 3, A and B). An exposure to 39°C for 90 min is a sublethal heat shock treatment for all the genotypes compared in this experiment. hot1-3 seedlings did not acclimate in response to the sublethal temperature pretreatment stress, because the mutation abrogates capacity for acquired thermotolerance (Hong and Vierling, 2001). By inference, SIZ1 function in high temperature adaptation seems restricted to basal thermotolerance.
SIZ1 and SA was assessed by genetic analysis of heat shock effects on siz1-2 and NahG siz1-2 seed germination and seedling growth. NahG transgenic plants express the Pseudomonas putida salicylate hydroxylase that catabolizes SA and effectively prevents accumulation (Delaney et al., 1994). NahG expression in wild type caused thermosensitivity (Figs. 2 and 3), which is consistent with data of others and supports the notion that SA facilitates basal high temperature tolerance (Clarke et al., 2004; Larkindale et al., 2005). NahG expression in siz1-2 caused additive hypocotyl elongation thermosensitivity (Fig. 3B; NahG siz1-2 and siz1-3, P < 0.01; NahG siz1-2 and NahG, P < 0.01), and this correlated with SA levels that are comparable to wild type (Fig. 4; Lee et al., 2006b). NahG siz1-2 seedlings exhibited impaired development after heat shock treatment during seed imbibition (Fig. 2A). NahG siz1-2 also resulted in hyperthermosensitivity during germination (Fig. 2B; NahG siz1-2 and siz1-2, P < 0.01; NahG siz1-2 and siz1-3, P < 0.01; NahG siz1-2 and NahG, P = 0.12). Catechol is a product of SA degradation by NahG and itself causes a loss of nonhost pathogen resistance in Arabidopsis (van Wees and Glazebrook, 2003). However, catechol treatment does not affect thermotolerance in Arabidopsis (Clarke et al., 2004), suggesting that thermosensitivity caused by NahG is due specifically to decreased SA levels. Together, these results indicate that SIZ1-mediated sumoylation positively affects basal thermotolerance independent of SA. SIZ1 also negatively regulates SA accumulation (Fig. 4; Lee et al., 2006b), yet the positive affect of the SUMO E3 ligase on high temperature adaptation supercedes that of SA.

SIZ1 Controls Heat Shock-Induced SUMO Conjugation

Heat shock induced an increase in conjugation of SUMO1/SUMO2 to substrate proteins at 34°C to 43°C (Figs. 5 and 6; Kurepa et al., 2003; Miura et al., 2005). A 39°C heat shock treatment induced sumoylation in wild type but to a lesser extent in siz1 seedlings (Figs. 5 and 6). Immunoblots were probed with anti-SUMO1 that detects both SUMO1 and SUMO2, and heat shock does not induce SUMO3 conjugation in Arabidopsis (Kurepa et al., 2003). Increased SUMO conjugation and deconjugation rates were different in NahG and hot1-3 seedlings (Fig. 7). NahG seedlings exhibited more rapid SUMO conjugation in response to heat shock and delayed SUMO deconjugation after return to ambient temperature (Fig. 7, center). SUMO deconjugation of
hot1-3 seedlings was impaired during the recovery period. siz1-2 suppressed induction of sumoylation that is associated with NahG expression (Fig. 6). This is indicative that SIZ1-mediated SUMO conjugation may be a biochemical process by which the E3 ligase regulates thermotolerance responses independently of SA.

SIZ1-Mediated Thermotolerance Is Independent of HSF Regulon Expression

HSF complexity in plants is predicted to be substantially greater than in other organisms. Yeast, Drosophila, and C. elegans have one HSF and vertebrates have four (Morimoto, 1998; Nakai, 1999; Nover et al., 2001; Baniwal et al., 2004), while bioinformatic analyses predict 21, 18, and 23 HSFs in Arabidopsis, tomato, and rice (Oryza sativa), respectively, based on sequence similarity (Baniwal et al., 2004). Transcript abundance of HSF1, 3, 4, and 7 was similar in wild-type and siz1-2 seedlings, indicating that SIZ1-dependent sumoylation does not regulate expression of these genes (Fig. 8A). HSF1 and HSF3 are major regulators of high-temperature-induced HSP expression (Lohmann et al., 2004). Expression of heat shock-induced genes was also similar in wild-type and siz1-2 seedlings (Fig. 8B). Included in the survey were genes that encode ascorbate peroxidase (APX1) and APX2, and HSFs, all of which are regulated by HSF1 and HSF3 and implicated to facilitate thermotolerance (Storozhenko et al., 1998; Hong and Vierling, 2000; Panchuk et al., 2002; Lohmann et al., 2004). APX1 and APX2 function as antioxidant enzymes that detoxify hydrogen peroxide, which is produced in response to heat stress and is presumed to be a major effector of cellular dysfunction (Panchuk et al., 2002). HSFs, APXs, and HSPs expression patterns were similar in siz1-2 and wild-type seedlings after heat shock treatment at 39°C (data not shown). Furthermore, heat shock-induced expression of these genes in NahG siz1-2 was similar to siz1-2 seedlings (data not shown), indicating that SIZ1

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DISCUSSION

The results of this study implicate a function for SIZ1 SUMO E3 ligase in basal thermotolerance of Arabidopsis. siz1-2 and siz1-3 cause thermal hypersensitivity (Figs. 1–3) that is manifested during seed germination, hypocotyl elongation, and seedling survival, inferring that SIZ1-mediated sumoylation is necessary for heat shock tolerance at numerous developmental stages. SUMO conjugation/deconjugation, in other organisms, is necessary for both ambient and high temperature-dependent HSF interactions with heat shock elements that regulate HSP expression (Goodson et al., 2001; Hong et al., 2001; Hilgarth et al., 2003, 2004; Hietakangas et al., 2006). However, the biological role of sumoylation in heat stress responses is unresolved, because it is not evident if SUMO conjugation to HSFs positively or negatively regulates thermotolerance (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2006). The biological role of sumoylation in heat stress responses is unresolved, because it is not evident if SUMO conjugation to HSFs positively or negatively regulates thermotolerance (Goodson et al., 2001; Hong et al., 2001; Hietakangas et al., 2006). SUMO1/2 conjugates accumulation is an early plant response (within minutes) to high temperature stress, implicating a function for sumoylation in thermotolerance (Kurepa et al., 2003; Miura et al., 2005). The MYB transcription factor PHR1 that is a controller of phosphate starvation-induced gene expression is a sumoylation substrate of SIZ1 (Miura et al., 2005). SIZ1 is implicated also in both thermal adaptation (herein) and pathogen defense (Lee et al., 2006b). Together, these results indicate that sumoylation/desumoylation is a key control process in the regulation of signal networks in plants. It is still to be resolved in any organism how sumoylation/desumoylation regulates diverse biological processes. However, emerging evidence implicates a function in gene expression regulator that involves chromatin remodeling (Gill, 2005; Hay, 2005).

AtSIZ1 is, by domain composition and functional data, a member of the PIAS family of SUMO E3 ligases (Kurepa et al., 2003; Miura et al., 2005). AtSIZ1 regulates gene expression and root architecture responses that are caused by phosphate deprivation (Miura et al., 2005). The Myb transcription factor PHR1 that is a controller of phosphate starvation-induced gene expression is a sumoylation substrate of SIZ1 (Miura et al., 2005). SIZ1 is implicated also in both thermal adaptation (herein) and pathogen defense (Lee et al., 2006b). Together, these results indicate that sumoylation/desumoylation is a key control process in the regulation of signal networks in plants. It is still to be resolved in any organism how sumoylation/desumoylation regulates diverse biological processes. However, emerging evidence implicates a function in gene expression regulator that involves chromatin remodeling (Gill, 2005; Hay, 2005).

PIAS family members are implicated to function as transcriptional regulators independent of the SP-RING domain that facilitates E3 ligase activity (Lee et al., 2006a; Sharrocks, 2006). The SAP domain of PIAS proteins is associated with transcriptional regulation resulting from chromatin remodeling (Shuai and Liu, 2005; Sharrocks, 2006). Consequently, there is a foundation to support the notion that SIZ1 may regulate basal thermotolerance through a sumoylation-independent process. Alternatively, it is also possible that SIZ1 is a determinant of thermotolerance through sumoylation-dependent and -independent processes.
SIZ1 Controls Basal Thermotolerance through a SA-Independent Process(es)

A genetic analysis of basal and acquired thermotolerance in Arabidopsis implicated the involvement of numerous heat shock-response pathways that do not involve a HSF regulon (Larkindale et al., 2005). Evidence implicates SA, ethylene, and ROS as possible intermediary signal molecules (Dat et al., 1998a; Cronje and Bornman, 1999; Larkindale and Knight, 2002; Clarke et al., 2004; Larkindale et al., 2005). Understanding of the biological role of SA in thermal adaptation is rudimentary, but SA regulates HSP17.6 expression in Arabidopsis and HSP70 in tomato (Cronje and Bornman, 1999; Clarke et al., 2004). However, HSP expression (particularly that of HSP101 and Hsa32) is presumed to be associated with acquired thermotolerance, and SA is not considered to be a principal regulator of this process (Hong and Vierling, 2002; Clarke et al., 2004; Larkindale et al., 2005).
SIZ1 Facilitates Basal Thermotolerance in Arabidopsis

Although SIZ1 is apparently not essential (Miura et al., 2005), it is evident that the SUMO E3 ligase is necessary for sumoylation that is associated with plant stress responses, as are PIAS and Siz orthologs in yeast, Drosophila, C. elegans, and vertebrates (Schmidt and Muller, 2002; Garcia-Estrada et al., 2003; Takahashi and Kikuchi, 2005). HSF-controlled gene expression is critical for high temperature tolerance in these organisms, and sumoylation of Xenopus and human HSFs regulates transactivation of HSP expression (Hong et al., 2001; Hilgarth et al., 2004). Although sumoylation of hHSF1, hHSF2, and hHSF4b likely result in transcriptional repression, it is postulated that SUMO conjugation and deconjugation are dynamic regulatory processes that are necessary for fine tuning regulation of basal and acquired thermotolerance (Anckar et al., 2006; Hietakangas et al., 2006).

At present, there is no evidence that Arabidopsis HSF family members are substrates for SUMO conjugation or that sumoylation/desumoylation of HSF is necessary for regulation of high temperature-induced HSP expression or thermotolerance in plants. It is important to note that redundant regulatory effect of AtHSF1 and AtHSF3 on HSP expression does not markedly influence thermotolerance (Lohmann et al., 2004) as does LeHSF1 in tomato (Mishra et al., 2002). Also, we detected no difference in high temperature-induced mRNA expression patterns of HSPs in siz1 and wild-type seedlings (Fig. 8), indicating that SIZ1-dependent sumoylation does not regulate acquired thermotolerance in plants (Fig. 3). Attempts in vitro sumoylation of AtHSF1 or AtHSF3 were inconclusive, although the assay does mediate SUMO conjugation to the transcription factor PHR1 (Miura et al., 2005).

Together, our results establish that SIZ1 facilitates basal thermotolerance in Arabidopsis through a SA-independent process(es). SIZ1, independent or dependent of sumoylation function, does not regulate acquired thermal adaptation responses in plants, unlike in other organisms as diverse as human, yeast, and Xenopus. The protein targets of SIZ1-dependent sumoylation that mediate thermotolerance remain to be identified as do the processes that control basal thermotolerance. Determinants that control high temperature signaling that regulate sumoylation/desumoylation and control thermal adaptation await identification. However, the results herein indicate that SUMO conjugation is a necessary process for basal thermotolerance at different plant developmental stages.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Col-0 ecotype genetic resources for this research were siz1-2, siz1-3 (Miura et al., 2005), hot1-3 (kindly provided by Dr. Elizabeth Vierling, University of Arizona, Tucson), NahG (Delaney et al., 1994), NahG siz1-2 (Lee et al., 2006b), and snc1 (kindly provided by Dr. Xin Li, University of British Columbia, Vancouver). Seeds were stratified for 3 d at 4°C and then sown onto a medium in petri plates containing 1× Murashige and Skoog basal salt mixture, 2% Suc, 2.5 mM MES, pH 5.7, and 0.8% agar. Seeds and seedlings, unless otherwise noted, were incubated under a 16-h-light (100 μmol m⁻² s⁻¹)/8-h-dark photoperiod at 22°C/18°C. For the hypocotyl elongation assay, seeds were sown onto agar (1.2%) medium and plates were placed in a vertical position in the dark under conditions as described above.

Thermotolerance Assays

Seedlings were subjected to heat shock in the dark at 60% relative humidity in a plant growth chamber (E-30B, Percival Scientific) with the capacity to control temperature fluctuation control by ±1°C. These conditions were used to minimize photooxidative and high humidity stresses (Larkindale and Knight, 2002; Zhou et al., 2004; Larkindale et al., 2005). Survival was monitored daily beginning 4 d after heat shock treatment. Stratified seeds were subjected to heat shock treatment in a temperature-controlled water bath.
Total RNA Isolation and Semiquantitative Reverse Transcription-PCR Analysis

Total RNA from 10-d-old seedlings grown at 22°C or heat shock treated for the indicated time was isolated by using PureLink Micro-to-Midi Total RNA Purification system (no. 12183–018, Invitrogen). Two micrograms of total RNA measured after 2.5 d.

In Vivo Analysis of Sumoylation Profiles

Total protein was extracted from 10-d-old seedlings grown on medium under conditions described above. Plant tissues (0.2 g) were extracted with a mortar and pestle in grinding buffer (100 mM Na-MOPS, pH 7.5, 10 mM NaCl, 1 mM EDTA, pH 8.0, 10% Suc, 5% β-mercaptoethanol, and 4% SDS) at room temperature. Protein concentration was measured by using Bio-Rad Protein Assay (no. 500–0006, Bio-Rad), and protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (no. 162–0177, Bio-Rad), probed with anti-SUMO1 antibody (ab5516, Abcam), and detected by using ECL plus Western Blotting Detection system (Amersham Biosciences).

SA Quantification

Shoots of 10-d-old seedlings that were grown on medium under conditions described above were harvested and frozen in liquid nitrogen. Tissue (0.2 g fresh weight, without roots) was extracted in 4 mL of methanol for 24 h at 4°C and then in a solution of 2.4 mL of water plus 2 mL of chloroform with 40 mL of 5 mM 3,4,5-trimethoxy-trans-cinamic acid (internal standard) for 24 h at 39°C or 45°C in dark for the indicated time. Plasmule position of each seedling was recorded by marking the plate, and the plate was rewrapped and incubated at 22°C/18°C (16 h/8 h) in dark. Hypocotyl growth after heat shock treatment was measured after 2.5 d.

Statistical Analysis

All data except germination rates were analyzed by Student’s t test for pairwise comparison. Germination was compared at different time points described above were harvested and frozen in liquid nitrogen. Tissue (0.2 g fresh weight, without roots) was extracted in 4 mL of methanol for 24 h at 4°C and then in a solution of 2.4 mL of water plus 2 mL of chloroform with 40 mL of 5 mM 3,4,5-trimethoxy-trans-cinamic acid (internal standard) for 24 h at 39°C or 45°C in dark for the indicated time. Plasmule position of each seedling was recorded by marking the plate, and the plate was rewrapped and incubated at 22°C/18°C (16 h/8 h) in dark. Hypocotyl growth after heat shock treatment was measured after 2.5 d.

Supplemental Data

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

Supplemental Table S1. Gene-specific primer sequences used to detect heat shock-related genes by RT-PCR.

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LITERATURE CITED


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