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Sumoylation of Arabidopsis heat shock factor A2 (HsfA2) modifies its activity during acquired thermotolerance

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Abstract Post-translational modification of target proteins by the small ubiquitin-like modifier protein (SUMO) regulate many cellular processes. In this work we show SUMOylation of the heat shock transcription factor, AtHsfA2, in connection with the plant's response to heat stress and acquired thermotolerance. Using the Yeast two hybrid and the bimolecular fluorescence complementation system, we have found that AtSUMO1 physically interacts with AtHsfA2. Further investigation allowed us to determine that Lys 315 of AtHsfA2 is the main SUMOylation site. Overexpression of AtSUMO1 led to a decrease in AtHsfA2 transcriptional activation of heat shock promoters. We have examined the effect of AtSUMO1 on AtHsfA2 during heat shock treatments. The phenotype of seedlings overexpressing AtSUMO1 resembled the phenotype of *AtHsfA2* knock out seedlings, which were more sensitive than wild type seedlings to repeated heat treatment. Furthermore, AtSUMO1 overexpressing seedlings exhibited lower expression levels of small heat shock proteins as compared with wild type seedlings after heat treatment. Based on our findings, we suggest that AtSUMO1 is involved in the regulation of AtHsfA2 in acquired thermotolerance.

Keywords Arabidopsis · Heat shock · HsfA2 · SUMO · Thermotolerance

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Abbreviations

HSP	Heat shock protein
HSF	Heat shock factor
SUMO	Small ubiquitin-like modifier protein

Introduction

Post-translational modifications of proteins have critical roles in many cellular processes owing to their ability to cause rapid changes in protein function. Modification by covalent linkage of two polypeptides was first established 28 years ago by finding Ubiquitin (Ub) (Hershko and Ciechanover 1998). Small ubiquitin-like modifier (SUMO), also known in humans as sentrin, GMP1, UBL1 and PIC1, is similar to Ub in its 3D structure but differs in the primary sequence (Kerscher et al. 2006). Unlike the Ub system, which primarily targets substrate proteins to the proteasome, SUMO conjugation has diverse cellular functions; SUMOylation is associated with cell cycle activity, DNA repair, subnuclear localization, enzymatic activity and stability, as well as regulation of gene expression (Anckar et al. 2006; Hay 2005; Hilgarth et al. 2004; Miura and Hasegawa 2010; Verger et al. 2003). SUMOylation is required for eukaryote viability in many species including yeast, nematodes, vertebrates, and plants (Fraser et al. 2000; Johnson and Blobel 1997; Nacerddine et al. 2005; Saracco et al. 2007). Typically, only a small fraction of a SUMO substrate is in the SUMOylated form at a given time point, suggesting that SUMO conjugation provides additional functionality required for non-standard tasks (Hilgarth et al. 2004; Johnson 2004). SUMO proteins are ubiquitously expressed throughout the eukaryotic kingdom. *Saccharomyces cerevisiae*, *S. pombe* and Drosophila have a

single SUMO gene, Smt3, Pmt3, and dSmt3, respectively (Johnson and Blobel 1997; Lehembre et al. 2000; Tanaka et al. 1999), mammalians have 4 SUMO genes SUMO1-4 (Guo et al. 2004; Melchior 2000), and plants have several SUMO genes (Colby et al. 2006; Kurepa et al. 2003). Conjugation of SUMO to the target proteins occurs through a series of biochemical steps (Hochstrasser 2009; Kerscher et al. 2006).

The discovery of SUMOylation led to the identification in mammalian of many different target proteins and SUMOylation has emerged as an important regulatory mechanism for protein function and localization in yeast, animals, and humans (Golebiowski et al. 2009; Kerscher et al. 2006). Several proteins were shown to undergo SUMOylation in Arabidopsis (Budhiraja et al. 2009; Jin et al. 2008; Miura and Hasegawa 2010; Miura et al. 2005, 2007a, b, 2009). In plants, SUMOylation has a role in stress responses (Kurepa et al. 2003; Miura et al. 2007a, b; Yoo et al. 2006), pathogen defense (Hanania et al. 1999; Hotson et al. 2003; Hotson and Mudgett 2004; Matarasso et al. 2005; Roden et al. 2004), abscisic acid signaling (Lois et al. 2003) the induction of flowering (Jin et al. 2008; Murtas et al. 2003; Reeves et al. 2002) and in regulation of gene expression (Miura and Hasegawa 2010; Miura et al. 2009).

Arabidopsis thaliana has eight SUMO genes encoding proteins significantly similar to animal and fungal SUMO proteins (Kurepa et al. 2003; Miura and Hasegawa 2010), while Maize and rice have 2 SUMO genes and tomato only a single SUMO gene (Miura et al. 2007a, b). This data is based on sequence similarity with proteins in animal and fungi which shows about 40% conservation. Antibodies directed against plant SUMO1/SUMO2 and SUMO3 (Kurepa et al. 2003; Lois et al. 2003; Murtas et al. 2003) indicate that these proteins form conjugates in vivo. In plants, the levels of SUMO1/2 conjugates are dramatically but transiently increased by various types of cellular stress, including heat shock. The increase and decrease in conjugates following heat shock are paralleled by a substantial decrease and increase in the free SUMO1/2 pool, suggesting that the heat induced conjugation is reversible (Kurepa et al. 2003).

Several of the mammalian SUMO targets are transcription factors, among them heat shock transcription factors (Anckar et al. 2006; Girdwood et al. 2004; Golebiowski et al. 2009; Tateishi et al. 2009). SUMOylation of the human heat-shock transcription factors Hsf1 and Hsf2 decrease their DNA-binding activity (Anckar et al. 2006; Tateishi et al. 2009). Furthermore, mutation of the Lys residue in the SUMOylation motif (KXE/D reduces the transcriptional activity of Hsf1 (Goodson et al. 2001; Hietakangas et al. 2006; Hong et al. 2001).

The heat shock response in eukayotic cells is initiated by the activation of the heat shock transcription factor (Hsf), a conserved protein present in all eukaryotic organisms studied to date (Nover et al. 2001; Wu 1995). The number of Hsf genes differs greatly among various eukaryotic organisms. *S. cerevisiae*, *Drosophila*, and *C. elegans* have a single Hsf gene (Hsu et al. 2003; Jedlicka et al. 1997; Sorger and Pelham 1988; Wiederrecht et al. 1988). The mammalian genome contains three Hsf isoforms, Hsf1, Hsf2, and Hsf4, each with a distinct biological function (Bu et al. 2002; Fujimoto et al. 2004; Xiao et al. 1999; Xing et al. 2005). In contrast to the low numbers of Hsf genes found in animals and yeast, the plant Hsf families shows striking multiplicity. Sequencing of the Arabidopsis genome revealed a unique complexity of the plant Hsf family with 21 members (Nover et al. 2001). Search of expressed sequence tag data bases indicated the presence of at least 18 Hsfs in tomato and 34 Hsfs in soybean (Baniwal et al. 2004).

The Arabidopsis HsfA2 seems to play a role in the transcriptional regulation of Hsp genes during prolonged heat stress or in recovery after heat shock, and it is important in the development of acquired thermotolerance (Baniwal et al. 2004). Arabidopsis transcriptome analyses revealed that at least 46 genes are controlled by HsfA2 during stress; among them Class I small heat shock proteins (sHSP-CI) (Schramm et al. 2006). Moreover, an HsfA2 knockout mutant displays reduced basal and acquired thermotolerance, while overexpression of HsfA2 enhances tolerance under these stress conditions (Li et al. 2005). HsfA2 is essential for acquired thermotolerance after a long recovery period (Charng et al. 2007).

In this work we show that AtSUMO1 binds to AtHsfA2. SUMOylation of HsfA2 by SUMO1 represses its transcriptional activity. Furthermore, we have found that SUMO overexpresion leads to decrease in sHSPs induction after heat shock.

Materials and methods

Plant material

Arabidopsis thaliana cv Columbia and *Nicotiana benthamiana* were grown from seeds under greenhouse conditions. The *Arabidopsis* HsfA2, T-DNA insertion line in Col-0 ecotype containing a T-DNA insertion in the *HsfA2* gene (Salk_008978) was provided by Dr. Pascal von Koskull-Doring (Schramm et al. 2006).

Chemicals and enzymes

Chemicals were obtained from Sigma and enzymes were obtained from Fermkentas or New England Biolabs unless otherwise stated.

Plasmid constructs and plant transformation

The coding region of *Arabidopsis thaliana* AtSUMO1 (NM_124898) was amplified by PCR and inserted into the vector pPILY (Ferrando et al. 2000) as a 311 bp *Bam*HI-*Pst*I fragment, allowing in frame cloning to an intron disrupted HA epitope tag. Subsequently a 1277 bp *Eco*RI-*Hind*III fragment including the Cam35S promoter, AtSUMO1-HA and NOS terminator was subcloned into the binary vector pBIN19plus (van Engelen et al. 1995).

Wild type Arabidopsis plants were transformed with *Agrobacterium tumefaciens* GV3101 harboring pBIN19-plus AtSUMO1-HA by the floral dip method (Clough and Bent 1998).

To generate the heat shock reporter construct, 1 kb upstream of the coding region of HsP 17.6 CI gene (At1g59860) or HsP 17.4 CI gene (At3g46230), or HsP 101 gene (At1g74310) were cloned independently in the binary vector pBIN19 plus (van Engelen et al. 1995) upstream of the uidA (GUS) reporter gene generating PHs17.6, PHs17.4, and PHs101, respectively.

Yeast two-hybrid analysis

The plasmids (pEG202, pJG4-5, pSH18-34, pRSHM1, and pJK101) and yeast strain EGY48 (ura3, his3, trp1, lexApo-leu2) were kindly provided by R. Brent (Massachusetts General Hospital, Boston, Massachusetts, USA). We performed the yeast two hybrid analysis as previously described (Gyuris et al. 1993; Hanania et al. 1999).

To create the in-frame LexA–AtSUMO1 fusion construct, we cloned the AtSUMO1 gene into the pEG202 vector. The bait vector construct was confirmed by DNA sequencing. The HsfA2 and HsfA2 mutant genes were cloned separately in the pJG4-5 vector.

Soluble -β-galactopyranosid assay

β-Galactosidase activity was measured essentially as described previously (Miller 1972) with minor modifications. In brief, yeast culture were resuspended in 0.8 ml Z buffer (5 mM $MgCl_2$, 250 mM β-mercaptoethanol, 50 mM KCl, 0.3 M Na_2HPO_4, 7 M H_2O, 0.2 M NaH_2PO_4) and incubated at 37 C for 30 min for lysis. Thereafter, lysates were incubated at 30 C after addition of 0.2 ml of o-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg/ml) in phosphate buffer. The reaction was stopped by the addition of 0.5 ml of 1 M sodium carbonate (Na_2CO_3) and appearance of yellow color due to release of o-nitrophenol was monitored at 420 nm spectrophotometrically. The β-galactosidase activity was calculated as described by Miller (1972). All experiments were repeated at least three times.

Statistical analysis

Analysis of variance (ANOVA) was applied to study the effects of the mutation in AtHsfA2 on the mean values of interaction strength (Sokal and Rohlf 1995). The Tukey HSD (honestly significant difference) test was used to compare differences of means among interaction strength for all combinations pairs ($P < 0.05$) (JMP 5.01 software, SAS Institute Inc. Tukey 1953).

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Quick-Change Site-Directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Mutations in the *AtHsfA2* gene were generated using the following primers: HsfA2_K5R (forward primer: 5′ctcgagttaaggttccgaaccaag; reverse primer 5′gaattcatggaagaactgcgcgtggaaatgg) HsfA2_K167R forward primer: 5′ gggaggttgagaggttgcgcagggatc atggtgtgc; reverse primer 5′ ccctccaactcttccaacgcgtccctag taccacacg) HsfA2_K269R (forward primer: 5′ gggaggttgag aggttgcgcagggatcatggtgtgc; reverse primer 5′ ccctccaactc ttccaacgcgtccctagtaccacacg) HsfA2_K315R forward primer: 5′ gcagcgttggatgtgcgcgtggaagatttggttgg; reverse primer 5′ cgtcgcaacctacacgcgcaccttctaaaccaacc).

BiFC—bimolecular fluorescence complementation analysis

HsfA2 and SUMO1 cDNA fragments were cloned into the SpeI site of pSY728 vector containing the N-terminal fragment of YFP (YN), and pSY738 vector containing the C-terminal fragment of the YFP (YC) (Bracha-Drori et al. 2004). The resulting constructs, pSY728-AtSUMO1 (YN-AtSUMO1), pSY738-AtHsfA2 (YC-AtHsfA2), pSY738-AtHsfA2_K5R (YC-AtHsfA2_K5R) were introduced into *Agrobacterium tumefaciens* strain GV3101 and then were co-transformed by injection into *N. benthamiana* leaves under various combinations. pSY728 and pSY738 plasmids were used as a control.

Confocal microscopy

Cells were analyzed using a 510 Zeiss or Meta confocal laser scanning microscope (Zeiss, http://www.zeiss.com/) with the following configuration: 30 mW Argon and HeNe lasers, 488 and 568 maximum lines. All images depict single sections.

Transient transformation

Transient expression was performed as previously described (Ron and Avni 2004). Briefly, *Agrobacterium* were

grown in LB medium overnight, diluted into an induction medium (50 mM MES pH-5.6, 0.5% (w/v) glucose, 1.7 mM NaH_2PO_4, 20 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$ and 200 μM acetosyringone) and grown for an additional 6 h until OD_{600} reached 0.4–0.5. The *Agrobacterium* culture was diluted to $OD_{600} = 0.05–0.2$, and the suspensions were injected into the *N. benthamiana* leaves or. Leaves were observed for protein expression 24–72 h after injection.

Fluorimetric assay and histochemistry assays

Excised leaf tissues were assayed for GUS activity as described previously (Jefferson et al. 1987). Leaf disk was homogenized in 100 μl extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM dithiothreitol, 1 mM Na_2EDTA, 0.1% Sodium Lauryl Sarcosine, 0.1% Triton X-100). 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) was added and incubated for 45 min at 37°C. The reaction was stopped by adding 0.2 M Na_2CO_3 and fluorescence was read in a Synergy plate reader at 460 ± 40 nm with 340 ± 11 nm as excitation amplitude. GUS activity was normalized against the protein concentration estimated by the Bradford assay. For each experiment the background GUS activity of each promoter was subtracted. Analysis of variance (ANOVA) was applied to study the effect of AtSUMO1 on GUS activity mean values ($n = 8$). For histochemical detection, tissues from transgenic plants were vacuum infiltrated for 5–15 min with 1 mM 5-bromo-4-chloro-3-indolyl 6-β-glucuronide, 25 mM sodium phosphate buffer, pH 7.0, and 1% DMSO followed by incubating for 360 min at 37°C in. To stop the reaction and to remove the chlorophyll, the tissues were treated with ethanol for 24 h. Stained leaves were bleached and scanned to obtain digital pictures.

Immuno blot analysis

Proteins extractions were performed as previously described (Aviezer-Hagai et al. 2007).

Antibodies against HsfA2 were provided by Dr. von Koskull-Doring (Goethe University Frankfurt, Germany). The antibodies against sHSPs-CI were provided by Dr. Adam Institute of Plant Genetics, Hebrew University Rehovot (Dafny-Yelin et al. 2008). Rat monoclonal anti-HA (Roche) and Mouse anti GFP (Santa Cruze) were used to detect HsfA2-AtSUMO1 conjugates.

Heat-stress conditions

For thermotolerance bioassays 5 days old seedlings were incubated for 3 h at 37°C, returned to recovery at 22°C in long-day conditions (16 h light, 8 h dark) for 48 h, followed by incubation for 60 min in a 42°C. After the treatment at 42°C the plates were returned to 22°C in long-day conditions (16 h light, 8 h dark) for an additional 6 days.

For protein extraction 10 day old seedlings were incubated for 3 h at 37°C and then return to recovery at 22°C in long-day conditions (16 h light, 8 h dark) for various times.

Total proteins were extracted at 0, 24 h and 48 h after the heat treatment, and analyzed by immunoblot with anti sHSPs-CI antibodies. Band intensities were quantified using ImageJ 1.42 software, using 12-bit images.

RNA isolation and reverse transcription polymerase chain reaction analysis

Total RNA from seedlings or leaves (100 mg) before and after heat-stress treatment was isolated as described previously(Aviezer-Hagai et al. 2007).

All RNA samples were quantified at 260 nm using a nano drop-ND-1000 spectrophotometer (Rockland, DE). First-strand cDNA was produced using 4 μg of total RNA, oligo (dT) 15 primer and M-MLV reverse transcriptase (Promega, USA). The resulting cDNA was diluted 40 times, and 5 μl were used as a template in 25-μl PCR reaction using gene specific primers (Supplemental Table 2). PCR amplification was carried out as follows : 94°C for 4 min followed by five cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, 19 (for ribosomal 40S) or 25 cycles (for all other genes used in this paper) at 94°C for 30 s, 55°C for 45 s and 72°C for 1 min. The reaction was terminated by 72°C for 5 min. The PCR reactions were performed using the PTC-200-PCR Peltier Thermal Cycler (MJ Research, USA). For semi-quantitative analysis, the intensity of the bands was determined by densitometry using ImageJ 1.42 software, using 12-bit images.

Results

AtSUMO1 interacts with AtHsfA2

To identify putative SUMO target proteins in Arabidopsis, the entire Arabidopsis transcriptome was analyzed by the $SUMO_{SP}$ 2.0 program (Xue et al. 2006). We have identified about 3,000 proteins harboring a putative SUMOylation site. About 300 proteins were suggested to have a role in stress responses and among them 16 were heat stress related proteins (Supplement Table I).

It has been reported that SUMO conjugation levels increase after heat shock treatment in mammalian cells (Golebiowski et al. 2009; Mao et al. 2000; Saitoh and Hinchey 2000). Given this observation we tested whether

SUMO1 modification levels would be affected by heat shock in Arabidopsis.

To search for SUMO modified protein in Arabidopsis, we generated Arabidopsis plants over expressing HA tagged AtSUMO1. Seven day old Arabidopsis seedlings overexpressing AtSUMO1-HA were exposed to a 42°C heat shock for 3 h. Total proteins were extracted and levels of free and conjugated SUMO were detected by western blot using anti-HA antibodies. The results showed that heat shock increased SUMO1 conjugation levels (Fig. 1). Our results support the data previously published (Kurepa et al. 2003).

Based on this result as well as mammalian findings (Golebiowski et al. 2009) we searched for a putative SUMO modification candidate among the Arabidopsis heat shock transcription factor family. The expression of HsfA2 is strongly induced during heat shock (Busch et al. 2005; Schramm et al. 2006). Using two bioinformatic programs $SUMO_{SP}$ 2.0 program (Xue et al. 2006) and SUMOplot (http://www.abgent.com) we identified 4 putative SUMOylation sites in AtHsfA2 (K5, K267, K269 and K315), while the PDSM motif (Hietakangas et al. 2006) is not present in the AtHsfA2. This observation suggests that phosphorylation is not involved in the SUMOyaltion of AtHsfA2.

We used the yeast two-hybrid system (Golemis et al. 2008) to test the possible interaction between AtHsfA2 and AtSUMO1. Yeast strains carrying the AtSUMO1 bait and AtHsfA2 prey grew in the absence of leucine, indicating LEU2 reporter gene activation. When grown on X-gal plates, these yeast cells were blue as a result of LacZ reporter gene activation (Fig. 2). In contrast, yeast strain carrying AtSUMO1 bait and a control pray TomWIPI (Acc K03290) or control bicoid bait and AtHsfA2 prey did not activate the LEU2 or LacZ reporter genes (Fig. 2a). Expression was dependent upon growth on galactose medium, indicating that expression of both AtHsfA2 and AtSUMO1 are required for expression of the reporter genes. Furthemore, the interaction was quantified using the substrate Ortho-nitrophenyl-β-D-galactopyranoside (ONPG). The results from the quantitative assay confirmed the plate assay results (Fig. 2b). We used the repression assay for DNA binding (Golemis et al. 2008) to confirm the expression of SUMO and its entrance to nucleus (Supplementary Fig. 1a). The expression of AtHsfA2 was tested by immuno blots (Supplementary Fig. 1b).

AtSUMO1 and AtHsfA2 interact in the plant nucleus

Interaction between AtHsfA2 and AtSUMO1 in planta was examined directly by bimolecular fluorescence complementation (BiFC) analysis, in which active YFP is reconstituted only when non-fluorescent N-terminal (YN) and C-terminal (YC) YFP fragments are brought together by protein–protein (AtHsfA2–AtSUMO1) interactions (Bracha-Drori et al. 2004). Reconstitution of YFP fluorescence was examined by transient co-expression of the tested protein pairs. Plants were transformed by infiltration of Agrobacterium tumefaciens harboring the appropriate plasmid to the abaxial side of *Nicotiana benthamiana* leaves. Cells co-expressing YN-AtSUMO1 and YC-AtHsfA2 showed clear YFP fluorescence localized primarily to

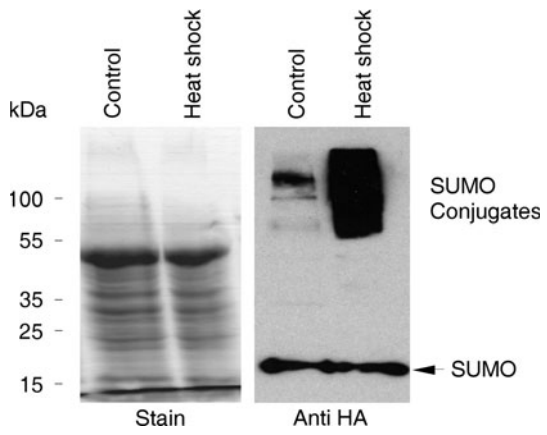

Fig. 1 Effect of heat shock on the accumulation of AtSUMO1 conjugates. Seven days old Arabidopsis seedlings overexpressing SUMO1-HA were grown at 22°C followed by heat shock treatment at 42°C for 3 h. Immediately after heat treatment total protein extracts (30 µg per lane) were separated on 12% acrylamide SDS–PAGE. SUMO1 conjugates were analyzed by immunoblotting using anti HA antibodies

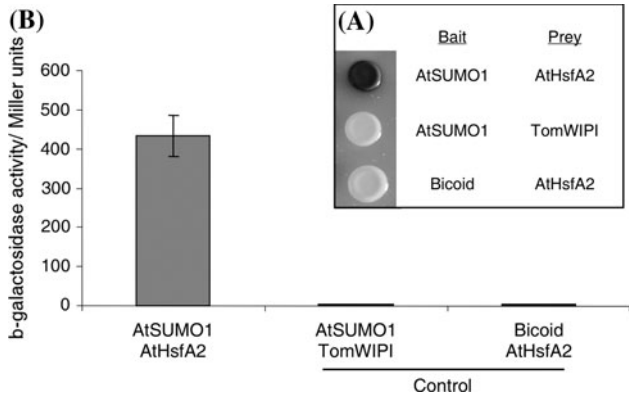

Fig. 2 Interactions between AtSUMO1 and AtHsfA2. **a** EGY48 yeast cells harboring AtSUMO1 (in pEG202), and AtHsfA2 (in pJG4-5) or appropriate controls as indicated, were grown on galactose medium lacking the amino acids uracil, histidine, and tryptophan, and supplied with X-gal. **b** Protein–protein interactions were quantified by ONPG assay as described in Methods on EGY48 yeast cells which were grown on liquid galactose medium lacking the amino acids uracil, histidine, and tryptophan with galactose as carbon source (mean ± 1SE $n = 5$)

the nucleus (Fig. 3). YC-AtHsfA2 and YN-AtSUMO1 were examined for fluorescence with the complementary half of the YFP protein and the results were negative (Fig. 3). The expressions of the different proteins were examined by immunoblots (Supplementary Fig. 2).

Our results demonstrate that AtSUMO1 is able to interact with AtHsfA2 in planta and that the complex is localized to the nucleus as seen in the DIC and fluorescence merge image (Fig. 3).

Lys 315 is the main AtSUMO1 interaction site in AtHsfA2

Four putative SUMOylation sites were identified in AtHsfA2 using SUMOplot (http://www.abgent.com), centered around Lys 5, 167, 269, and 315. Each Lys residue was changed to arginine using site directed mutagenesis. The resulting proteins (single or multiple mutations) were cloned in the yeast two hybrid system prey plasmid (pJG4-5) and their expression was tested by immuno blots (Supplementary Fig. 1b). Their interaction with AtSUMO1 (cloned in the bait plasmid) was examined as compared with the native AtHsfA2 protein using both qualitative and quantitative assays (Fig. 4). The interaction level of AtHsfA2 mutant proteins with AtSUMO1 varies (Fig. 4). AtHsfA2_K315R is the main target site for AtSUMO1 interaction as changing K315 to R315 is sufficient to completely abolish the interaction. Mutation K167R leads to a reduction in interaction level indicating its possible role as a secondary interaction target site. Mutation K269R resulted in an increased interaction level which could be due to a conformational change affecting interaction

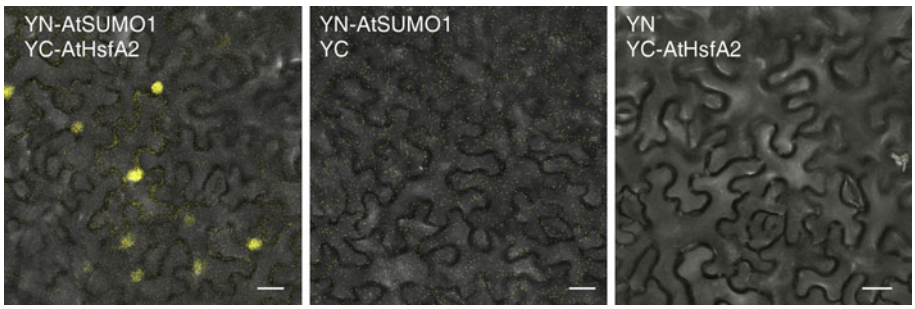

Fig. 3 Bimolecular fluorescence complementation (*BiFC*) visualization of the interaction between AtSUMO1 and AtHsfA2. *N. benthamiana* leaves transiently expressing YN-AtSUMO1 and YC-AtHsfA2 as indicated. Leaf sections were visualized 48 h after transformation under a laser-scanning-meta confocal microscope (Zeiss). Bars = 20 μm

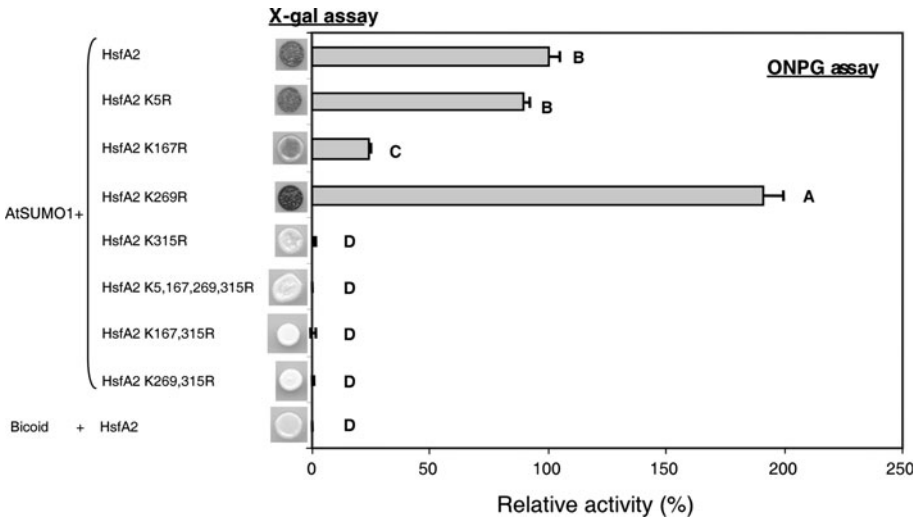

Fig. 4 Yeast two-hybrid assay for interactions between AtHsfA2 or mutated AtHsfA2 and AtSUMO1. EGY48 yeast cells harboring AtSUMO1 or Bicoid (in pEG202) and AtHsfA2 or mutated HsfA2 in which lysine residue was changed to arginine (in pJG4-5) were grown on galactose medium lacking the amino acids uracil, histidine, and tryptophan, with X-gal. Protein- protein interactions were quantified by ONPG assay on EGY48 yeast cells which were grown on liquid galactose medium lacking the amino acids uracil, histidine, and tryptophan with galactose as carbon source (mean ± 1SE $n = 5$). Different letters above error bars indicate significant differences (Tukey's multiple range test). Values were normalized to an activity of 100% native AtHsfA2

affinity. The K5R mutation does not alter the interaction of HsfA2 to AtSUMO1 indicating that this site is not involved in the AtSUMO1/AtHsfA2 interaction (Fig. 4).

We further examined the interaction between AtSUMO1 and AtHsfA2_K315R in planta using the BiFc system. Cells co-expressing YN-AtSUMO1 and YC-AtHsfA2 or YC-AtHsfA2_K5R showed YFP fluorescent signal localized to the cells nuclei, while no fluorescence signal was observed in the nuclei of cells co-expressing YN-AtSUMO1 and YC-AtHsfA2_K315R (Fig. 5). The in planta results indicate that K315 is the main AtSUMO1 interaction site in AtHsfA2. We confirm the expression of the different proteins by immunoblotting (Supplementary Fig. 2).

HsfA2 is SUMoylated

We used immuno-precipitation to demonstrate that AtHsfA2 undergoes a covalent modification by AtSUMO1, i.e. is SUMOylated. AtHsfA2-HA or AtHsfA2_K315R-HA and AtSUMO1-GFP were transiently co-expressed in *N. benthamiana* leaves. Co-expression of AtHsfA2 and AtSUMO1 yielded a migrating SUMO1-HsfA2 band which immuno reacted to both, HsfA2 (as seen by anti HA) and SUMO1 (as seen by anti GFP; Fig. 6). This band was absent in plants co-expressing AtHsfA2_K315R and AtSUMO1, as well as in cells expressing HsfA2 or SUMO1 alone (Fig. 6). These results indicate that native AtHsfA2 but not AtHsfA2_K315R is SUMOylated. The size of the SUMO-HsfA2 conjugate suggests that HsfA2 exist as a dimmer.

AtSUMO1 represses AtHsfA2 activity

The β-glucuronidase (GUS) reporter gene assay was employed to examine the effect of SUMOylation on AtHsfA2 activity.

To directly test the potential of HsfA2 to activate heat shock gene expression, we used reporter constructs containing 1 kb of upstream sequences from the HsP17.6 (At1g59860), HsP17.4 (At3g46230) or HsP101 (At1 g74310) genes (Kotak et al. 2007a, b) fused to β-glucuronidase (PHsf17.6:GUS; PHsf17.4:GUS; PHsf101:GUS) in transient assays in *N benthamiana* leaves. GUS activity was examined 48 h post transformation qualitatively by histochemical X-gluc staining and quantitatively by the 4-methylelumbelliferyl glucuronide (MUG) fluorimetric assay. Analysis of variance (ANOVA) was applied to study

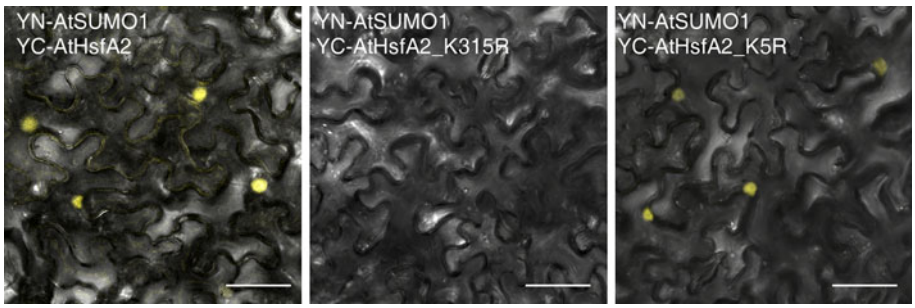

Fig. 5 Bimolecular fluorescence complementation (*BiFC*) visualization of the interaction between AtSUMO1 and mutant AtHsfA2. *N. benthamiana* leaves transiently expressing YN-AtSUMO1 and YC-AtHsfA2 or mutated HsfA2 in which lysine residue was changed to arginine as indicated. Leaf sections were visualized 48 h after transformation under a laser-scanning-meta confocal microscope (Zeiss). The experiment was performed three times independently. Bars = 50 μm

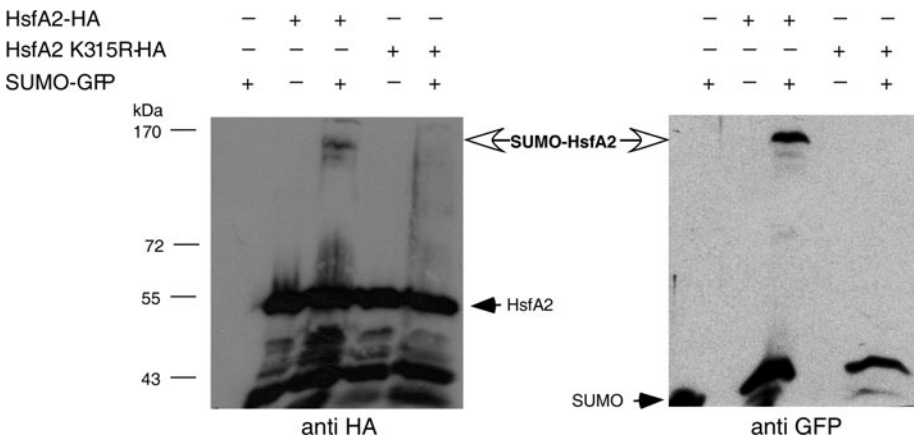

Fig. 6 *In planta* SUMOylation of AtHsfA2. HsfA2-HA or HsfA2 K315R-HA and SUMO-GFP were transiently expressed in *Nicotiana benthamiana* leaves. 48 h post transformation total protein extreacts (30 μg per lane) were separated on 8% acrylamide SDS–PAGE. Proteins were transferred to a nitrocellulose membrane probed with anti-HA antibodies, stripped and reprobed with anti-GFP antibodies

the effect of AtSUMO1 on GUS activity mean values ($n = 8$). AtSUMO1 led to a significant decrease in AtHsfA2 transcriptional activity for all analyzed promoters, approximately 80% for PHsf101 and PHsf17.6, and 40% for PHsf17.4. In the absence of AtSUMO1 the AtHsfA2 transcriptional activation was set to 100% for all target promoters (Fig. 7). These results clearly demonstrate that AtSUMO1 overexpression represses AtHsfA2 transcriptional activity for all analyzed heat shock promoters.

We used the AtHsfA2 mutants to confirm the above results. Pro_{35S}:AtHsfA2 or Pro_{35S}:AtHsfA2_K315R, and PHsf17.6:GUS were all co-injected into *N. benthamiana* leaves in the presence or absence of Pro_{35S}:AtSUMO1. GUS activity was examined 48 h post transformation quantitatively as described above. Both AtHsfA2 and AtHsfA2_K315R presented similar transcriptional activity levels (Fig. 8), indicating that the K315R mutation does not affect AtHsfA2 transcriptional activity. In the presence of AtSUMO1, the transcriptional activity level of AtHsfA2_K315R remained unchanged, while the transcriptional activity level of the native AtHsfA2 was repressed (Fig. 8). These results further validate Lys 315 as the main SUMOylation site in AtHsfA2.

Furthermore, we examined the transcriptional activation of the other AtHsfA2 mutants (AtHsfA2_K167R and AtHsfA2_K1269R) in the presence and absence of SUMO1 and found that their activity was repressed by the presence of AtSUMO1 (Supplement Fig. 3).

Arabidopsis SUMO1 overexpression leads to decreased acquired thermotolerance and decrease in sHSP levels

A significant heat-sensitive phenotype has been described for Arabidopsis *HsfA2* knockout mutant plants (Charng et al. 2007). *HsfA2* mutant seedlings collapse when exposed to a second heat shock after long recovery periods (24–72 h), suggesting that the HsfA2 transcription factor is associated with the development of acquired thermotolerance (Charng et al. 2007). To study the effect of SUMO1 on thermotolerance, 5 day old Arabidopsis AtSUMO1 overexpressing, *HsfA2* knockout or wild type seedlings were exposed to heat stress at 37°C for 3 h followed by 2 days recovery period at 21°C followed by exposure to a second heat stress at 42°C for 60 min. The response of the different seedlings was examined 6 days after the second heat treatment; a scheme of the heat shock treatment is shown in Fig. 9. Similar to the results described by Charng et al. (2007) most of the *HsfA2* knockout seedlings died during the second heat shock while wild type seedlings survived, demonstrating the effectiveness of the treatment

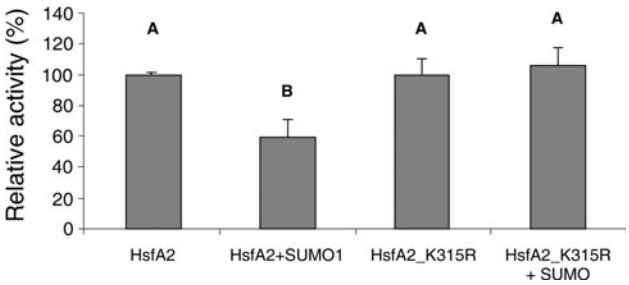

Fig. 8 The transcriptional activity level of AtHsfA2 K315R in the presence of AtSUMO1 in transient GUS reporter assays. Pro_{35s}-AtHsfA2 or Pro_{35s}-AtHsfA2 K315R, Pro_{35s}-AtSUMO1 and GUS reporter gene under the control of PHs17.6 were co-injected into *N. benthamiana* leaves. GUS activity was examined 48 h post injection by MUG fluorimetric assay (mean ± 1SE $n = 8$). Values were normalized to an activity of 100% in the absence of AtSUMO1. *Different letters* above error bars indicate significant differences (Tukey's multiple range test)

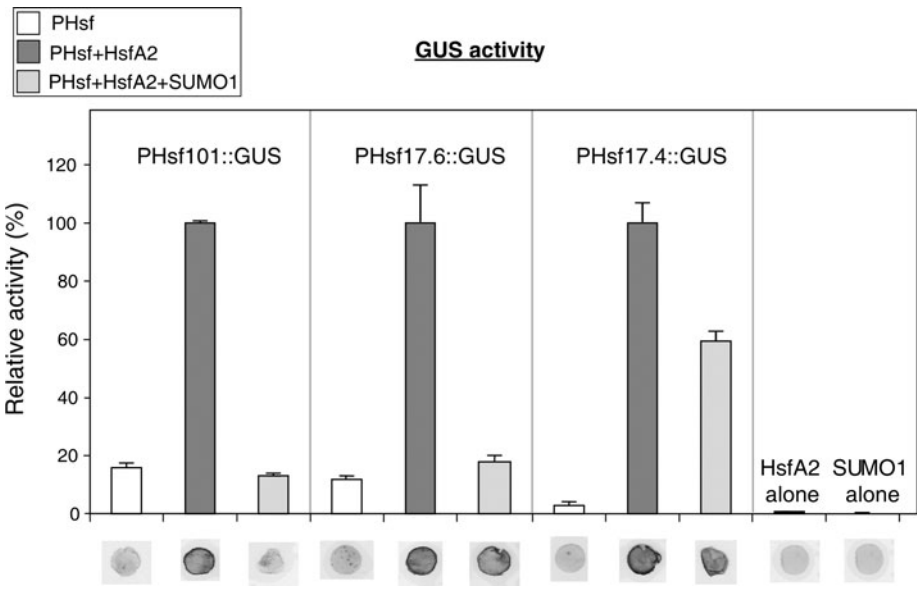

Fig. 7 AtSUMO1 repressed AtHsfA2 transcription activity on target promoters in transient GUS reporter assays. Pro_{35s}-AtHsfA2, Pro_{35s}-AtSUMO1 and GUS reporter gene under the control of different heat shock promoters (HsP, as indicated were co-injected into *N. benthamiana* leaves. GUS activity was examined 48 h post injection by MUG fluorimetric assay (mean ± 1SE $n = 8$), and qualitatively by histochemical X-gluc staining. Values were normalized to an activity of 100% in the absence of AtSUMO1

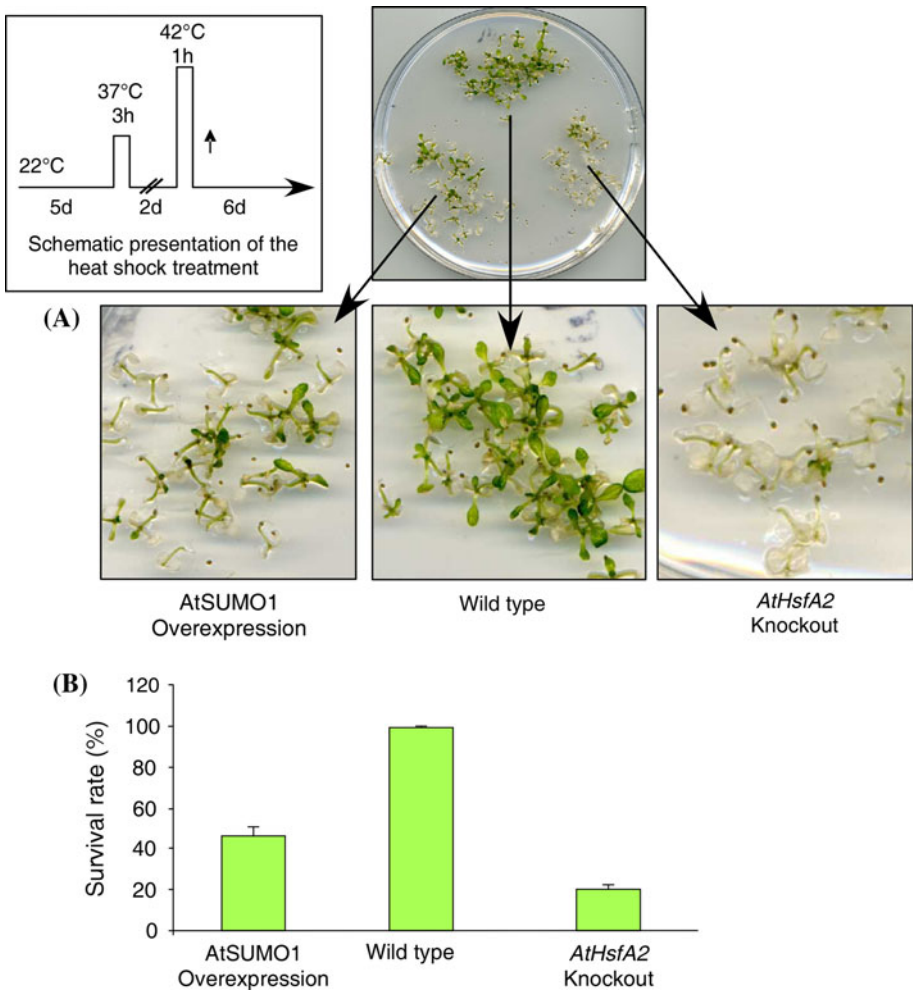

Fig. 9 The role of AtSUMO1 in the long term acquired thermotolerance. **a** Five days old Arabidopsis seedlings were exposed to heat stress at 37°C for 3 h followed by 2 days recovery period at 22°C followed by exposure to higher stress at 42°C for 60 min (see *Scheme*). Photographs were taken 6 days after second heat challenging. **b** Survival rates (survival seedlings divided by total seedlings) were calculated for all seedlings (mean ± 1SE $n = 3$)

(Fig. 9a). AtSUMO1 overexpressing seedlings were significantly less tolerant than wild type to the heat stress treatment although coped better than *HsfA2* knockout seedlings (Fig. 9a). The survival rate (surviving seedlings divided by total seedlings) of AtSUMO1 overexpressing seedlings significantly differed from the wild type. In AtSUMO1 overexpressing plants, 50% of the seedlings survived, less than 20% of the seedlings survived *HsfA2* knockout plants, while 100% of the wild type seedlings survived under the same treatment (Fig. 9b). These results suggest that the modification of AtHsfA2 by AtSUMO1 is involved in Arabidopsis long term acquired thermotolerance. In comparison 5 day old Arabidopsis AtSUMO1 overexpressing, *HsfA2* knockout or wild type seedlings were exposed just to a single heat stress at 42°C for 60 min. The seedlings were photographed 2 and 4 days after heat treatment (Suplementary Fig. 4). In this treatment no difference was observed between the different plants. Futhermore, we examined the expression of AtHsfA2 and AtSUMO1 and two heat shock genes Hsp17.6 and ascorbate peroxidase 2 (APX) during the same heat shock scheme (Fig. 10). AtSUMO1 uniformly expressed during the heat treatment and recovery period while AtHsfA2 is highly induced after the heat treatment, both in wild type and SUMO over-expressing plants. Hsp17.6 and APX show reduced levels of induction in SUMO overexpresing plants compared to wild type plants during the recovery period (Fig. 10). After the second heat shock the induction of Hsp17.6 and APX is similar in wild type and SUMO overexpressing plants suggesting that additional factors regulate the expression of those genes in the second heat stress period.

To further examine how AtSUMO1 affects AtHsfA2 in the recovery period we examined the expression of heat shock proteins. AtHsfA2 is a major transcription factor of heat stressed induced protein such as small heat shock proteins (sHSPs) (Schramm et al. 2006) which play an important role in the heat stress response (Larkindale and Huang 2004; von Koskull-Doring et al. 2007). We examined the expression level of sHSPs-class I(CI) in 10 days old AtSUMO1 overexpressing, *HsfA2* knockout and wild type Arabidopsis seedlings (Fig. 11) at different time

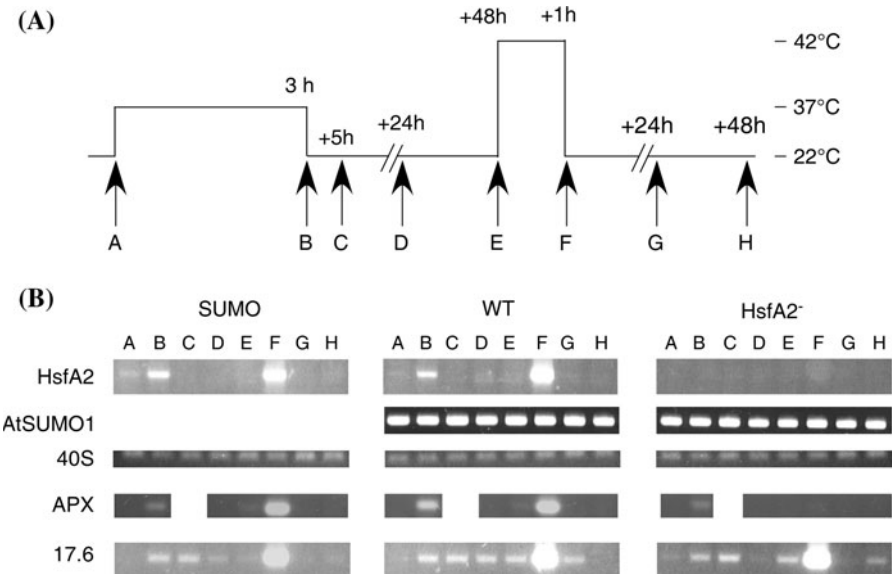

Fig. 10 Expression analysis of HsfA2 and Hsps in heat-stressed leaves from wt, HsfA2 knockout and SUMO over-expressing plants. **a** The pictogram shows the time course of the heat stress treatment. *Arrows* indicate the time points when the samples were harvested (*A–H*). **b** mRNA accumulation of the respective gene products was determined by semi-quantitative RT-PCR, as described in ''Materials and methods''. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Similar results were obtained from three independent experiments

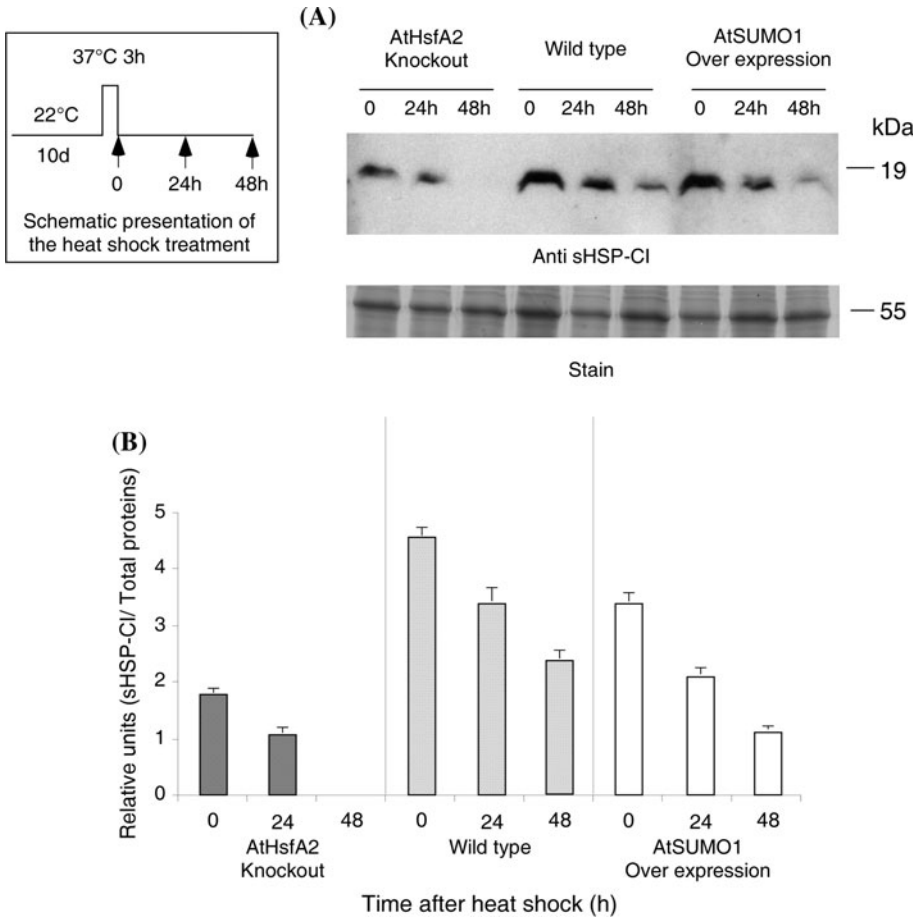

Fig. 11 Expression level of sHSP-CI in Arabidopsis seedlings during recovery period. Ten days old Arabidopsis seedlings were heat challenged (37°C for 3 h). **a** Total protein extracts of different time points after heat challenged (0,24 h and 48 h), were separated (30 µg per lane) on 16% acrylamide SDS–PAGE and analyzed by immunoblotting using anti sHSP-CI antibodies. **b** Quantification of immunoblot bands using ImageJ 1.42 software, using 12-bit images. The experiment was performed three times independently

points during the recovery period after 3 h heat treatment at 37°C. Whereas sHSPs-CI appeared in all seedlings immediately after heat challenge, their expression levels decreased after a recovery period of 24 h and 48 h. At all time points, seedlings overexpressing AtSUMO1 exhibited lower levels of sHSPs-CI than wild type and higher levels of sHSPs-CI than *HsfA2* knockout (Fig. 11). These results demonstrate that AtSUMO1 interferes with sHSP-CI expression after heat challenge, possibly due at least in part, to modification of AtHsfA2.

Altogether our results provide evidence for the modification of AtHsfA2 by AtSUMO1 leading to the repression of its transcriptional activity and ultimately disrupting the acquired thermotolerance pattern in Arabidopsis.

Discussion

SUMO was shown to be a reversible post-translational protein modifier a decade ago. During this time the search for SUMO substrates has produced a long list of mammalian protein targets, which appear to be involved in most cellular processes. Although SUMOylation has emerged as an important regulatory mechanism for protein function and localization in yeast, animals and humans, not much is known about its role in plants. Here we showed that the heat shock transcription factor HsfA2 undergoes SUMOylation which inhibits HsfA2 transcriptional activity.

We examined the effect of SUMOylation on the activity of HsfA2. We found that AtSUMO1 represses AtHsfA2 transcriptional activation. For all of the examined target genes, the presence of AtSUMO1 led to a decrease in GUS activity, indicating a decrease in AtHsfA2 transcriptional activation (Fig. 7). These results imply that SUMOylation represses AtHsfA2 activity. In mammalian HSF1 sumoylation was shown to be depends on phosphorylation of an adjacent site (Hietakangas et al. 2003; Hietakangas et al. 2006). Sumoylation was suggested to induce HSF1 and HSF2 activity (Goodson et al. 2001; Hietakangas et al. 2003; Hong et al. 2001). However, Hietakangas et al. (2006) suggested that SUMOylation repress the activity of HSF1 and HSF4b. We could not identify the PDSM motif in AtHsfA2 and we show that SUMOylation of AtHsfA2 inhibit its activity as suggested for the mammalian HSF1 and HSF4b.

To verify that AtSUMO1 modification represses AtHsfA2 transcriptional activation we examined the influence of AtSUMO1 on the mutated AtHsfA2_K315R, which cannot undergo SUMO modification, and found it to be unaffected. In the absence of AtSUMO1, both AtHsfA2 and AtHsfA2_K315R presented similar transcriptional activity levels (Fig. 8). Following the observation that AtHsfA2_K315R is the only mutant not affected by the presence of AtSUMO1, we suggest that Lys 315 is the major or single site in AtHsfA2 that undergoes SUMOylation in vivo.

The finding that AtSUMO1 represses AtHsfA2 transcriptional activation correlates with previously published data regarding the influence of SUMO on transcription factors. SUMO is known to regulate many transcription factors in mammalians, and in most cases SUMO modification leads to transcriptional repression (Girdwood et al. 2004). Furthermore, removal of SUMO by mutation of the SUMO acceptor Lys or by overexpression of a deSUMOylating enzyme, such as SENP1 (sentrin-specific protease 1) has been shown to increase activity of dozens of transcription factors, including the Androgen receptor, the CAAT/Enhancer-binding (C/EBP) proteins, Elk-1, Sp3 and Smad4 (Gill 2005).

It was demonstrated that *AtHsfA2* knockout mutant plants collapsed when challenged with repeated heat shock after prolonged recovery time pursuant to the original heat exposure treatment (Charng et al. 2007). This phenotype was correlated with reduced levels of sHSPs (Charng et al. 2007). Our data shows that when seedlings oversxpressing AtSUMO1 are challenged with repeated heat shock, their phenotype resembled the phenotype of the *AtHsfA2* knock out seedlings. This may indicate that AtSUMO1 interferes with the role of AtHsfA2 in acquired thermotolerance. Similarly, AtSUMO1 overexpressing seedlings exhibited lower expression levels of sHSPs as compared with wild type seedlings after heat shock treatment. We suggest that the lower levels of sHSP are due to SUMOylation of AtHsfA2 which leads to the repression of AtHsfA2 transcriptional activity.

Considering our results, we suggest that AtSUMO1 is involved in regulation of HsfA2 in the recovery phase after heat shock. SUMOylation is rapid and reversible, and therefore suitable for the function of a transcription factor regulator, requiring extremely rapid adaptation. The mechanism by which AtSUMO1 regulates AtHsfA2 is not clear. During the recovery period (after heat shock) AtSUMO1 modifies AtHsfA2. As a result, SUMO modified AtHsfA2 is inactivated and remains in an inactive state in the cell nucleus. Exposure to a new heat shock will lead to deSUMOylation of AtHsfA2 and result in an active protein. Schramm et al. (2006) demonstrated expression of the AtHsfA2 protein approximately 1 h after exposure to repeated heat shock. We suggest that the regulation of AtHsfA2 via SUMOylation/deSUMOylation in the recovery period provides initial protection against heat damage until the heat shock response is stabilized.

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