

Identification and Molecular Properties of SUMO-Binding Proteins in *Arabidopsis*

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Reversible conjugation of the small ubiquitin modifier (SUMO) peptide to proteins (SUMOylation) plays important roles in cellular processes in animals and yeasts. However, little is known about plant SUMO targets. To identify SUMO substrates in *Arabidopsis* and to probe for biological functions of SUMO proteins, we constructed 6xHis-3xFLAG fused AtSUMO1 (HFAtSUMO1) controlled by the *CaMV35S* promoter for transformation into *Arabidopsis Col-0*. After heat treatment, an increased sumoylation pattern was detected in the transgenic plants. SUMO1-modified proteins were selected after two-dimensional gel electrophoresis (2-DE) image analysis and identified using matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We identified 27 proteins involved in a variety of processes such as nucleic acid metabolism, signaling, metabolism, and including proteins of unknown functions. Binding and sumoylation patterns were confirmed independently. Surprisingly, MCM3 (At5G46280), a DNA replication licensing factor, only interacted with and became sumoylated by AtSUMO1, but not by SUMO1ΔGG or AtSUMO3. The results suggest specific interactions between sumoylation targets and particular sumoylation enzymes.

INTRODUCTION

Eukaryotic proteins are regulated by many types of post translational modifications, and in some case the modifying group is itself a protein. Ubiquitin, a small, highly conserved polypeptide, provides the prototypical example. It is reversibly linked to target proteins in the cell in a process termed ubiquitination (Hochstrasser, 2009). As another example, the small ubiquitin-like modifier (SUMO) resembles ubiquitin in its structure and mechanism. Activation and conjugation reactions involving SUMO have much in common with what we know about ubiquitin (Johnson, 2004; Melchior, 2000; Miura and Hasegawa, 2010).

SUMO attachment (sumoylation) to substrates depends on three components: heterodimeric SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2) and the substrate recognition factor (E3) that stimulates SUMO transfer from E2 to substrates (Johnson, 2004; Seeler and Dejean, 2003). Finally, sumoylated proteins can be removed from conjugates by SUMO proteases for SUMO recycling.

Although the sumoylation process resembles ubiquitination, unlike ubiquitin ligation, target proteins typically include several sumoylation sites consisting of a short consensus sequence, Ψ-K-X-E/D (Ψ, large hydrophobic amino acid; K, the acceptor lysine; X, any amino acid; E/D, glutamate or aspartate) (Schmidt and Müller, 2003). SUMO modification of substrate proteins in animals and yeasts participate in critical and diverse cellular processes, such as innate immunity, chromatin stability and cell division, DNA repair, nucleocytoplasmic trafficking, transcriptional regulation, and ubiquitination antagonism (Gill, 2005; Hay, 2005). Vertebrate RanGAP1, in which the modification by SUMO is required for its localization to the nuclear pore complex, has been identified as the first target of SUMO conjugation. To date, more than 50 mammalian proteins are known as sumoylation targets (Hannich et al., 2005; Matunis et al., 1996). Global analyses in yeast and metazoans indicated >100 target proteins (Denison et al., 2005; Panse et al., 2004; Wykoff and O'Shea, 2005; Zhao et al., 2004; Zhou et al., 2004).

In *Arabidopsis*, the SUMO gene family amounts to eight genes of which only four appear to be expressed (SUM1-3, and 5) (Budhiraja et al., 2009; Colby et al., 2006). Levels of SUMO1/2 conjugates are dramatically but transiently increased by various types of cellular stress, including exposure of the plants to heat shock, H₂O₂, ethanol, and canavanine, suggesting sumoylation as an important post-translational feature of the plant stress response. Indicating some specificity is the fact that levels and distribution of SUMO3 conjugates appear to be unaffected by any stress (Kurepa et al., 2003). Although our understanding of sumoylation remains limited in plants, the system appears to play an exceptionally important role in various plant environ-

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mental responses, including heat shock, pathogen defense, drought and cold tolerance, ABA signaling, flowering, and phosphate deficiency (Catala et al., 2007; Jin et al., 2008; Lee et al., 2007; Miura et al., 2005; 2007; 2009; Saracco et al., 2007; Yoo et al., 2006). Recently, van den Burg et al. (2010) reported that AtSUMO3 acted downstream of SA accumulation, while involvement of AtSUMO1 and AtSUMO2 has been reported in suppressing SA accumulation. Therefore, it implies that the *Arabidopsis* SUMO paralogs form a regulatory network that differentially modulates target modifications in a response to various environmental conditions. However, many aspects of biological function and regulation in plants, including the nature of SUMO target proteins, remain poorly defined. To date, a small number of SUMO target proteins have been uncovered in the form of seven genuine *Arabidopsis* proteins, MYB30, GTE3/5, PHR1, ICE1, ABI5 and FLD, that harbor sumoylation consensus motifs (Garcia-Dominguez et al., 2008; Jin et al., 2008; Miura et al., 2005; 2007; 2009; Okada et al., 2009). In addition, very recent are reports about systematic approaches using yeast two-hybrid assays and affinity enrichment methods with mass spectrometry for mapping SUMO target proteins (Elrouby and Coupland, 2010; Miller et al., 2010).

To extend our knowledge about biological implications of putative SUMO targets and SUMO binding proteins in plants, we have begun to screen *in-planta* for SUMO-binding proteins using by a mass spectrometry-based proteomics approach using a transgenic *Arabidopsis* line overexpressing AtSUMO1. Here we report the isolation of 27 proteins that fit the criteria. They include proteins with a variety of putative functions, in DNA or RNA-related metabolism, signaling pathway, general metabolism, and several functional unknown proteins. Specifically, based on a yeast split ubiquitin assay and *E. coli* sumoylation assay using SUMO1, SUMO1 Δ GG, or SUMO3 proteins in combination with MCM3 (At5G46280) protein, the functional properties of SUMO proteins detected *in vivo* might reflect a capability to conditionally differentiate sumoylation activities in plants.

MATERIALS AND METHODS

Plant materials and ABA treatment

Wild-type, *Arabidopsis thaliana* Columbia-0 (*Col-0*) was used in these experiments. Seeds were surface sterilized, stratified for three days at 4°C and then sown to a medium in petri plates containing 1 \times Murashige and Skoog basal salt mixture, 3% sucrose, 2.5 mM MES, pH 5.7, and 0.8% agar or soil. Plants were grown under a 16/8 h light/dark at 22°C, light intensity of 100 μ mol m⁻²s⁻¹ and relative humidity of 57-80%. To investigate the inhibition of root growth by ABA, 4-day-old seedlings were transferred onto plates supplemented with ABA. Root growth was measured as the difference in root length between the beginning and the end of the growth evaluation period. 14-day-old seedlings were photographed.

Plasmid constructs and plant transformation

To generate *Arabidopsis* transgenic plants over-expressing AtSUMO1, the protein coding region was amplified by RT (Reverse transcription)-PCR using *Arabidopsis thaliana* cDNAs. The 6xHis-3xFlag fused AtSUMO1 (HFAtSUMO1) was cloned into the *pGEM-T Easy* vector (Promega, USA), sequenced to verify the correct DNA sequence and then sub-cloned into the *pCAMBIA1300PT*-derived vector using the appropriate restriction enzymes. *Agrobacterium tumefaciens* GV3101 strains harbored the construct over-expressing HFAtSUMO1 were grown in LB liquid culture with 50 mg/L gentamycin, 50 mg/L rifam-

picin, and 50 mg/L kanamycin at 30°C. The *Col-0* plants were transformed by the floral deep method as previously described (Clough and Bent, 1998; Park et al., 2009). Hygromycin-resistant transgenic plants were selected on 1 \times MS medium containing 30 mg/L hygromycin.

Immunoblot analysis

Plant tissues were frozen and ground with mortar and pestle in liquid nitrogen. Protein extracts were prepared in protein extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride containing 1 \times Complete Protease Inhibitor (Roche)]. After centrifugation at 14,000 rpm for 20 min twice, the supernatant was used immediately or stored at -80°C. Protein concentration was determined using a protein assay kit (Bio-Rad), and 40 μ g of total protein was separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, probed with an anti-His antibody, and detected using the ECL Western blot detection system (Amersham Biosciences).

2-DE analyses and MALDI-TOF MS

SUMO conjugates were affinity purified using nickel-nitrilotriacetic acid agarose (Ni-NTA) resin according to the manufacturer's instruction (Qiagen, USA). Purified proteins were examined by immunoblot analysis using an anti HA-antibody. Quantified proteins (170 μ g) were mixed in sample buffer and then loaded onto IEF gel (18 cm tube gel) (O'Farrell, 1975). In the second dimension, proteins were separated in 12% SDS-polyacrylamide gels and visualized by silver staining with no glutaraldehyde (Blum et al., 1987). Gel images were scanned using a GS-800 Imaging Densitometer (Bio-Rad) and analyzed with the software PDQuest version 7.2.0 (Bio-Rad). For each sample, quantitation was performed with three analytical gels originating from three independent biological replicas. The silver-stained protein spots were excised from the gel, subjected to in-gel tryptic digestion (Promega, USA), and extracted as previously described (Kim et al., 2004). Peptide mass fingerprinting was carried out on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, USA) according to previously reported methods (Kim et al., 2004). For data processing, the software package PerSeptive-Grams was used.

Yeast split ubiquitin assay

The yeast split ubiquitin assay was performed as described previously (Laser et al., 2000; Yoo et al., 2005). *Saccharomyces cerevisiae* strain JD53 was used for all experiments. The putative SUMO1-binding cDNAs were cloned into *pMET-Ste14-C_{ub}-FURA3*, replacing yeast *Ste14*. *AtSUMO1*, *AtSUMO1 Δ GG*, and *AtSUMO3* cDNAs were cloned into modified versions of the *pCup-N_{ub}-Sec62* vector, replacing yeast *Sec62*, respectively (Staglar et al., 1998). Interactions between each pair of proteins were tested on selective medium containing 2 mg/ml 5-FOA and selective medium lacking uracil. Plates were incubated at 30°C for 3-5 days, unless specified otherwise.

Agrobacterium-mediated transient expression assay in tobacco plants

Seven-week-old *N. benthamiana* plants were used for *Agrobacterium*-mediated transient expression. AtSUMO1 was cloned into *pCAMBIA 1300-cLUC* and genes encoding putative SUMO binding proteins were into *pCAMBIA1300-nLUC* (Chen et al., 2008). The DNA constructs were introduced into *Agrobacterium tumefaciens* strain GV3101. The bacteria grown in Luria-Bertani medium supplemented with 10 mM MES buffer, 20 μ M acetosyringone, and appropriate antibiotics at 28°C were

washed with infiltration solution (10 mM MgCl₂, 10 mM MES buffer, and 100 μM acetosyringone) twice to avoid the toxicity of antibiotics. In addition, *Agrobacterium* transformed with p19, a suppressor of gene silencing, was cultured and prepared (Lakatos et al., 2004). For co-infiltration, each of *Agrobacterium* cultures was OD₆₀₀ = 0.5 in final infiltration solution and mixed in equal volumes. Bacterial suspensions were infiltrated into tobacco leaves using a needleless syringe. After infiltration, plants were immediately covered with plastic bags and placed at 23°C for 36 h. The tobacco leaves were sprayed three times with 1 mM luciferin in solution, and imaged by an EM CCD camera (iXon, Andor Technology plc, Ireland). Bioluminescence was recorded after quenching for 5 min in the dark.

Sumoylation assay in *E. coli*

The sumoylation assay in *E. coli* was carried out as described previously (Okada et al., 2009). *Escherichia coli* BL21(DE3) cells were transformed with *pACYCDuet-AtSAE1b-AtSAE2*, and *pCDFDuet-AtSUMO1/3* (AA or GG)-*AtSCE1a*. The transformed cells were used for the preparation of competent cells. For *in vivo* sumoylation reactions, the competent cells were transformed with *pET28a-AtMYB30* or *pET28a-At5g46280*, respectively. Transformed cells were cultured in 5 ml of LB medium at 37°C until the OD₆₀₀ was 1.0, followed by the addition of 0.4 mM IPTG. After an approximately 12 h induction at 25°C, 2 ml of the culture cells was harvested, washed with 1× PBS buffer and suspended with 200 μl of 1× PBS buffer. The samples were lysed by sonication, and boiled at 95°C for 5 min. 20 μl of total protein was loaded to 7.5% continuous gradient gel, transferred to a polyvinylidene difluoride membrane, probed with an anti-T7 antibody, and detected using the ECL Western blot detection system (Amersham Biosciences).

RESULTS AND DISCUSSION

Construction of AtSUMO1 transgenic *Arabidopsis* plants

To identify SUMO-modified proteins in *Arabidopsis*, we constructed AtSUMO1 fused to His₆-FLAG₃ (HFAtSUMO1) and generated transgenic *Arabidopsis* lines. The His₆-FLAG₃ was added to the N-terminal end, and expression of the construct was under control of the *CaMV35S* promoter (Fig. 1A). A free His₆-FLAG₃ construct was compared as the control vector. Twenty-five individual transgenic *Arabidopsis* lines were selected using hygromycin resistance and then further selected by protein gel blot analysis using anti-His tag Antibody. Figure 1B shows protein expression levels of five representative HFAtSUMO1 lines from transgenic plants expressing fusion protein. Western blot analysis using anti-His tag antiserum showed two different bands in HFAtSUMO1 transgenic *Arabidopsis* plants. These two bands represent precursor and mature forms with SUMOs covalently attached to lysine residues in target proteins *via* cleavage of the C-terminal GG motif (Meulmeester and Melchior, 2008).

To confirm whether HFAtSUMO1 protein was over-expressed in the transgenic plants, we performed peptide mass fingerprinting (PMF) using MALDI-TOF mass spectrometry. After purification of plant crude extracts by histidine affinity chromatography, the purified sample was applied to SDS-PAGE analysis and a band containing the putative HFAtSUMO1 was excised from the gels, digested with trypsin, and identified by MALDI-TOF mass spectrometry. Peptide mass fingerprinting of the purified protein showed a match with the SUMO1 peptide sequence, indicating that the protein was derived from the HFAtSUMO1 DNA sequence (Fig. 1C).

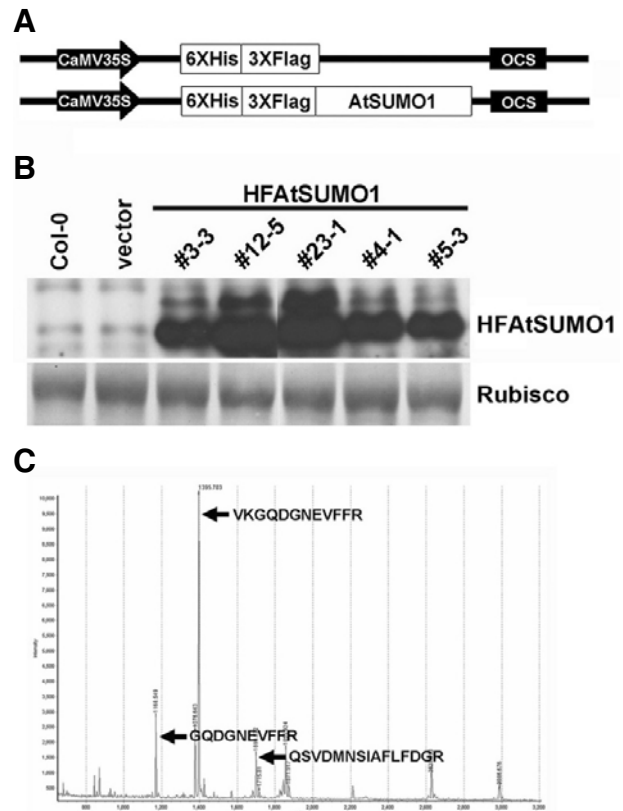


Fig. 1. Expression of AtSUMO1 in *Arabidopsis*. (A) Structure of the binary vector used for expression of AtSUMO1 in transgenic *Arabidopsis*. The 6xHis-3xFlag fused AtSUMO (HFAtSUMO1) were placed between the *CaMV35S* promoter and the *OCS* terminator of the *pCAMBIA1300PT*-derived binary vector. (B) Expression of HFAtSUMO1 in homozygous T3 over-expressing transgenic plants and control plants (*Col-0* and HF inserted vector). The resulting crude extracts (40 μg) were subjected to SDS-PAGE, and immunoblot analysis was carried out with anti-HA antibody. (C) Identification of AtSUMO1 protein by MALDI-TOF MS. MALDI-TOF mass spectrometry-based analysis identified the peptide sequence of the expressed protein as AtSUMO1.

Effect of ABA and accumulation of SUMO1 conjugates by heat stress in AtSUMO1 transgenic plants

Overexpression of SUMO1/2 had been reported to suppress abiotic stress responses mediated by abscisic acid (Lois et al., 2003). We also analyzed whether abscisic acid affected root growth in HFAtSUMO1 transgenic *Arabidopsis* plants. Fig. 2A showed that ABA-mediated root growth inhibition was reduced significantly in plants that overexpressed AtSUMO1 in comparison to *Col-0* and empty vector control plants. In addition, the transgenic plants that exhibited high transgene protein levels (#3-3, #12-5, and #23-1 lines) demonstrated approximately 33% reduction of ABA-mediated root growth inhibition, compared to those of *Col-0* and empty vector control plants (Fig. 2B).

Sumoylation in animals is activated by various environmental stresses and some SUMO targets are part of the stress response (Goodson et al., 2001; Hong et al., 2001; Mao et al., 2000; Melchior, 2000; Saitoh and Hinchey, 2000). In *Arabidopsis*, the levels of SUMO1 and -2 conjugates but not SUMO3 conjugates increased after exposing seedlings to stress conditions, such as heat shock, H₂O₂, ethanol, and the amino acid

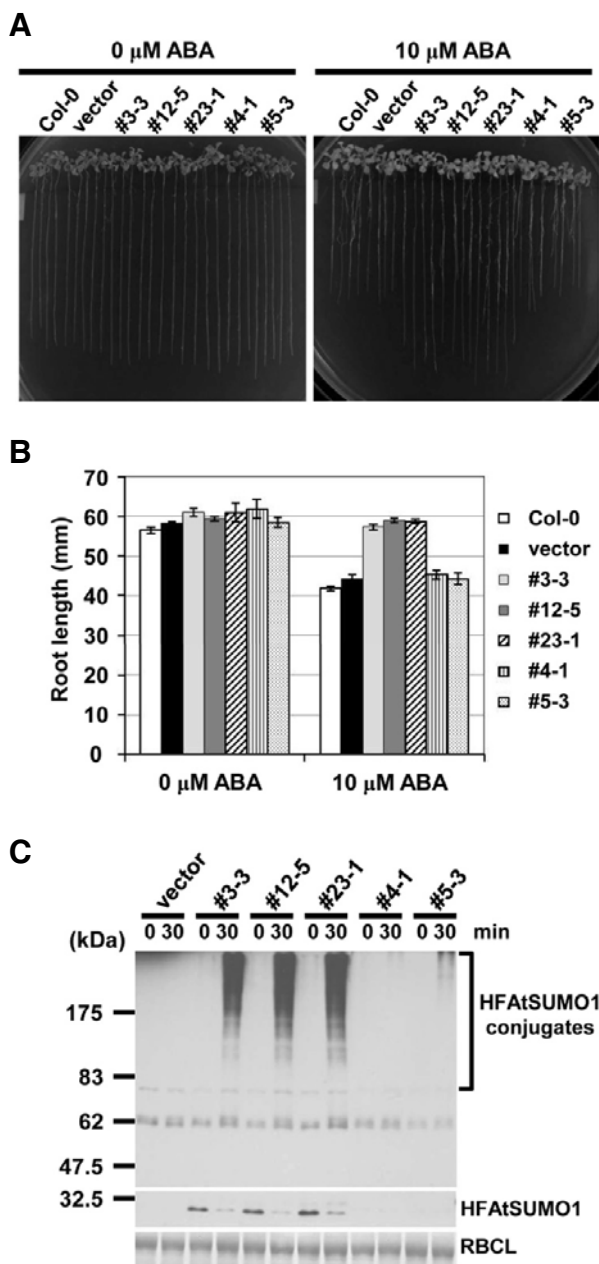


Fig. 2. Phenotype of transgenic plants expressing HFSUMO1 and heat shock-induced SUMO conjugation. (A) Root growth inhibition by ABA is decreased in HFSUMO1 over-expressing transgenic plants. Three-day-old seedlings were transferred to MS medium without or supplemented with 10 μM ABA and maintained under a 16-h-light/8-h-dark daily photoperiod at 24°C. Photographs were taken after 24 days. (B) Quantitative representation of the data in (A). Error bars represent standard deviations ($n = 15$). Mean values of over-expressing transgenic plants are significantly different from control plants at $*P < 0.01$. (C) SUMO conjugation patterns after heat treatment in HFSUMO1 over-expressing transgenic plants. Ten-day-old seedling of vector, and each of the SUMO over-expressing lines (#3-3, #12-5, #23-1, #4-1, and #5-3) were exposed to a 30 min-heat shock at 39°C in the dark. Total proteins were extracted from untreated ($t = 0$) or heat shock-treated ($t = 30$) seedlings, and 30 μg of proteins was subjected to SDS-PAGE. The immunoblot was probed with anti-His-tag antiserum.

analog canavanine. In addition, overexpression of SUMO2 enhanced both the steady state levels of SUMO2 conjugates under normal growth conditions and showed subsequent heat shock-induced accumulation in *Arabidopsis* plants (Kurepa et al., 2003). To examine endogenous SUMO conjugates in independent HFAAtSUMO1 transgenic *Arabidopsis*, we performed gel blot analyses using anti-His tag antiserum after heat shock treatment (Fig. 2C). In His₆-FLAG₃ fused AtSUMO1 (HFAAtSUMO1) controlled by the *CaMV35S* promoter, SUMO conjugates were not detected under no-stress conditions. This result is in agreement with SUMO conjugation data obtained in yeast, possibly because placement of the His₆-FLAG₃ tag at the N-terminus of AtSUMO1 reduced the efficiency of SUMO conjugations (Hannich et al., 2005). However, the conjugation pattern increased drastically over time and in amount in a protein expression-dependent manner (Fig. 2C). Thus, the #12-5 over-expression line was selected as a suitable candidate for screening of SUMO1-modified proteins by 2-DE analysis.

Identification of SUMO1-modified proteins by 2-DE analysis

In order to isolate *in vivo* SUMO-modified proteins using His₆-FLAG₃ fused AtSUMO1 (HFAAtSUMO1) overexpressing plant (#12-5 line), we established mass spectrometry-based screening combined with 2-DE. We first analyzed by performing Ni-NTA chromatography to purify the SUMO-modified proteins of AtSUMO1 overexpressing transgenic plants compared to the vector control plants after treatment with heat stress at 39°C for 30 min. Using 2-DE analysis, differences in the protein spots between the vector control and AtSUMO1 transgenic plants were compared after heat-shock treatment. Fig. 3 shows representative images for vector control (Fig. 3A) and HFAAtSUMO1 transgenic plants (Fig. 3B). New protein spots were detected in HFAAtSUMO1 transgenic plant, suggesting appearance of SUMO-modified proteins by HFAAtSUMO1 (arrow heads in Fig. 3). In total, 27 line-specific protein spots were excised from the gels and further analyzed by MALDI-TOF MS.

Excised protein spots were digested by trypsin, and the resulting peptides were extracted and identified by peptide mass fingerprinting (PMF) using MALDI-TOF MS. The list of SUMO1-modified proteins is compiled in Table 1. It shows that the putative SUMO targets are involved in DNA and RNA-related processes, signaling pathways, and in general metabolism. Generally, the SUMO proteins are predominantly nuclear proteins, but sumoylation also occurs in the cytoplasm, and the set of putative SUMO-modified proteins identified in this work encompasses both nuclear and cytoplasmic proteins. Recently, Budhiraja et al. (2009) reported that SUMO target proteins are involved in DNA-related or in RNA-dependent processes, such as the regulation of chromatin structure, splicing, or translation. We have as well obtained proteins involved in DNA and RNA metabolism, in particular MCM3 (At5G46280), UBA2A (At3G56860), an SMC-like protein (At3G54670), a zinc finger family protein (At3G08505), and RAD54 (At3G19210). Intriguingly, a disease resistance related protein (At5G46500) was also found. Together, these examples of SUMO-modified proteins indicated that SUMO modification is not limited to soluble proteins, and, like ubiquitin, might potentially have a membrane-based signal function. Indicating specificity in the putative target proteins, all SUMO1-modified candidate proteins contained predicted sumoylation sites with motifs of high probability (Table 1).

Interaction between SUMO1 and SUMO1-modified proteins *in vivo*

To verify that the SUMO1-modified proteins isolated were in-

Table 1. Identification of SUMO-modified proteins in HFAtSUMO1 *Arabidopsis* transgenic seedlings under heat stress

Gene name	AGI code	Predicted sumoylation site ^a (Motifs with high probability)	Description
DNA, RNA-related processes			
MCM3	At5G46280	3	Replication licensing factor
UBA2A	At3G56860	1	AU-rich element binding / RNA binding
SMC like protein	At3G54670	7	Structure maintenance of chromosomes
Zinc finger family protein	At3G08505	1	Zinc finger (CCCH-type/C3HC4-type RING finger) family protein
AtRAD54	At3G19210	3	ATP binding, DNA binding, helicase 1
Signaling pathway			
Transducin family protein	At2G37160	1	WD-40 repeat family protein
Transducin family protein	At3G53390	1	WD-40 repeat family protein
EIF4G	At3G60240	7	Eukaryotic Translation Initiation Factor 4G
Disease resistance protein	At5G46500	4	TIR-NBS-LRR class
Phospholipase C	At3G47290	3	Phosphoinositide-specific phospholipase C family protein
EF-Tu	At4G02930	3	Putative elongation factor Tu
RPT4a-like protein	At1G45000	3	26S proteasome regulatory complex subunit p42D
Metabolism			
MLS	At5G03860	2	Malate synthase
PYK10	At3G09260	2	Phosphate starvation response 3.1, hydrolase
Glucose-inhibited division family A	At2G13440	6	Glucose-inhibited division family A protein
SHM1	At4G37930	1	Serine hydroxymethyltransferase 1
Aminomethyltransferase	At1G11860	2	Putative aminomethyltransferase
BXL1	At5G49360	5	Beta-xylosidase 1
GAPA	At3G26650	2	Glyceraldehyde 3-phosphate dehydrogenase A subunit
GAPC-2	At1G13440	4	Glyceraldehyde 3-phosphate dehydrogenase C subunit
KPHMT1	At2G46110	3	Ketopantoate hydroxymethyltransferase 1
Aspartyl aminopeptidase	At5G60160	3	Putative aspartyl aminopeptidase
ASP5	At4G31990	3	Aspartate aminotransferase 5
LPD2	At1G48030	2	Lipoamide dehydrogenase 2
Unknown protein			
Unknown protein	At3G19870	4	Unknown function
Unknown protein	At2G16760	2	Unknown function
Unknown protein	At4G26920	2	Putative homeodomain protein

^aPredicted sumoylation sites were analyzed by "http://www.abgent.com/tools/sumoplot_login".

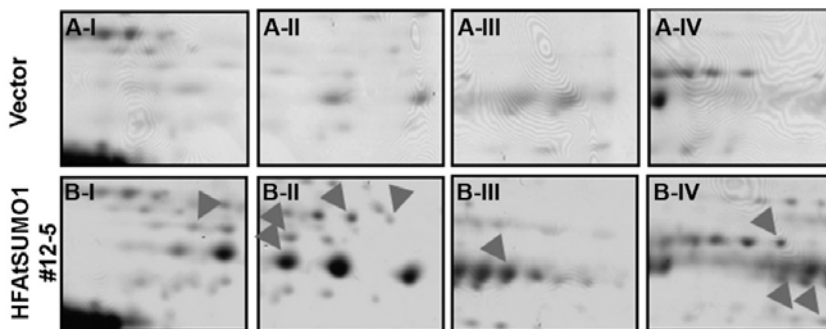


Fig. 3. Representative 2-DE gel images of vector control and AtSUMO1 (#12-5) over-expressing transgenic seedlings. The vector (A-I, II, III, and IV) and AtSUMO1 (B-I, II, III, and IV) over-expressing transgenic seedlings were grown for 7 days in liquid culture media under continuous light at 24°C, and exposed to a 30 min-heat shock at 39°C. For isoelectric focusing, a total of 170 µg proteins were loaded onto pH 4-7L IPG strips (18 cm) followed by SDS-PAGE on 12% gel and silver staining. Arrow heads indicate the various putative SUMO modified proteins.

deed SUMO-protein conjugates, we determined whether AtSUMO1 directly interacted with five representative SUMO1-modified proteins by using a yeast split-ubiquitin assay system, which is based on the reassembly of the N- and C-terminal halves (N_{ub} and C_{ub}) of ubiquitin (Ub). AtSUMO1 and SUMO1-modified proteins were fused to the C-terminus of N_{ub} and the N-terminus of C_{ub} , respectively (Fig. 4A). If AtSUMO1 directly interacted with SUMO1-modified proteins, N_{ub} and C_{ub} should reassemble into a native-like Ub (Varshavsky, 1996). Finally, the cells containing SUMO1-modified proteins- C_{ub} -RUra3p and

Nub-AtSUMO1 will be unable to grow on plates lacking uracil but will be able to grow on plates containing 5-FOA, which is converted into toxic 5-fluorouracil by RUra3p. In the opposite case, yeast cells are uracil prototrophs and 5-FOA sensitive (Fig. 4A). As shown in Fig. 4B, cells co-expressing SUMO1-modified proteins- C_{ub} -RUra3p and Nub-AtSUMO1 were unable to grow on plates lacking uracil, but grew on plates containing 5-FOA, indicating that AtSUMO1 effectively forms stable complexes with the SUMO1-modified proteins *in vivo*. In negative controls, no interaction was observed between Nub-AtSUMO1

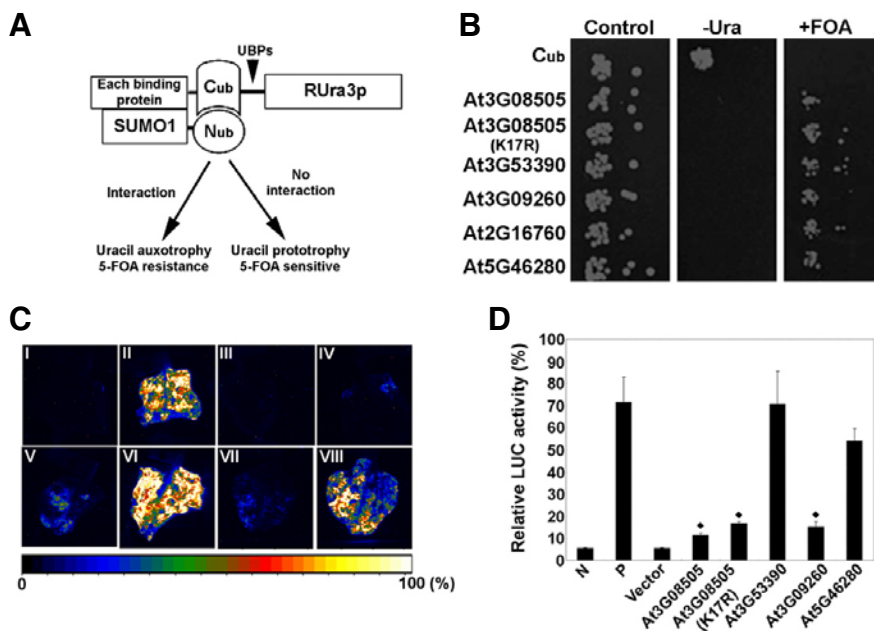


Fig. 4. Confirmation of the interaction between SUMO1 and putative SUMO1 binding proteins in yeast and tobacco expression systems. (A) Schematic diagram of the split ubiquitin system and its application to the interaction of SUMO1 with putative SUMO1 binding proteins. (B) Interaction between SUMO1 and putative SUMO1 binding proteins in the yeast split ubiquitin system. (C) Interaction between SUMO1 and SUMO1 binding proteins in *N. benthamiana* leaves. LUC image of *N. benthamiana* leaves co-infiltrated with the *Agrobacterium* strain containing the putative SUMO1 binding protein-NLuc and CLuc-SUMO1. (I, vector control; II, SGT1a-NLuc and CLuc-RAR1 Chen et al., 2007; III, NLuc; IV, At3G08505-NLuc; V, At3G08505^{K17R}-NLuc; VI, At3G53390-NLuc; VII, At3G09260-NLuc and VIII, At5G46280-nLuc). (B) Quantification of LUC activity in leaves expressing the putative SUMO1 binding protein-NLuc and CLuc-SUMO1.

(N; vector control, P; SGT1a-NLuc and CLuc-RAR1; see: Chen et al., 2007, Vector; NLuc). Data were collected 36 h after infiltration. The data are presented as mean \pm SE of three independent samples.

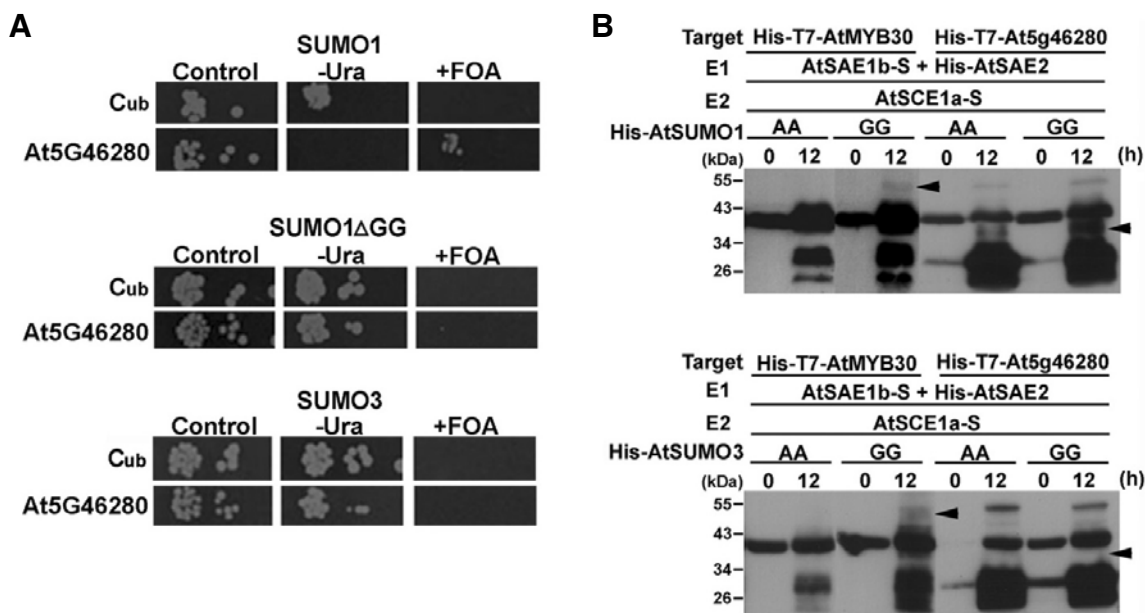


Fig. 5. Specific interaction and sumoylation pattern between SUMO1 and MCM3 (At5G46280) protein. (A) AtSUMO1 specifically interacts with MCM3 (At5G46280) protein in the yeast split ubiquitin system. Shown are interactions with MCM3 (At5G46280) protein and SUMOs (SUMO1, SUMO1 Δ GG, or SUMO3) by serial dilutions of cells co-expressing C_{ub} or C_{ub}-SUMOs (SUMO1, SUMO1 Δ GG, and SUMO3) fusions, respectively, on plates lacking tryptophan and histidine (control) additionally lacking uracil (-Ura) or containing 5-FOA (+FOA). Proteins were expressed from single copy vectors. (B) Specific sumoylation pattern of MCM3 (At5G46280) protein by AtSUMO1 using AtSAE1b as a subunit of the E1 heterodimer. *Escherichia coli* BL21(DE3) was transformed with each construct. Transformed *E. coli* cells were incubated at 37°C until the OD₆₀₀ reached 1.0, and the expression of tagged proteins and sumoylation was then induced overnight with 0.4 mM IPTG at 25°C. Cell lysates were prepared and Western blotting was carried out using an anti-T7-tag antibody. Sumoylated MCM3 (At5G46280) protein was observed as bands shifted to higher molecular weight in lanes using mature AtSUMO (GG) (indicated as GG), but not in lanes using mutated AtSUMO (AA) (indicated as AA). AtMYB30 was used as positive control for the sumoylation assay in an *E. coli* system. Arrow heads indicate sumoylated proteins.

and Cub-RUra3p.

In addition, we constructed a zinc finger family protein (At3G08505)^{K17R} carrying an amino acid substitution (lysine to arginine) at the indicated lysine residue of the putative sumoylation site. Interestingly, co-expression of AtSUMO1 and the zinc finger family protein (At3G08505)^{K17R} mutant, which lacks Lys residue required to form a covalent bond with Gly residues of SUMO protein did not influence 5-FOA resistance, suggesting that AtSUMO1 still interacted with the zinc finger family protein (At3G08505)^{K17R} mutant. It is speculated that the zinc finger family protein (At3G08505) may participate in non-covalent interaction with AtSUMO1 or that it may recognize and switch to other sumoylation sites. This may be similar to all finding in animals by Minty et al. (2000) who defined a Ser-Xaa-Ser motif surrounded by hydrophobic and acidic amino acids as a SUMO-interacting motif (SIM). Among SUMO-interacting proteins, several zinc finger-containing proteins have been determined to be involved in processes like DNA repair or transcriptional repression (Hecker et al., 2006).

Replacing the yeast assay, we next investigated whether these AtSUMO1-modified proteins interacted with AtSUMO1 under physiological conditions *in-planta*. Using an *Agrobacterium*-based tobacco LUC transient expression system, we tested the interactions between AtSUMO1 and AtSUMO1-modified proteins after the addition of luciferin, the substrate for firefly LUC. As shown in Fig. 4C, co-expression of CLuc-RAR1 and SGT1a-NLuc led to strong LUC activity in the tobacco leaf as positive control (Fig. 4C-II; Chen et al., 2008). In contrast, N-conjugated Luc coexpressed with C-attached Luc as vector control did not show LUC activity (Fig. 4C-III). Although LUC activity levels varied with respect to different targets (Fig. 4D), co-expression of CLuc-AtSUMO1 and AtSUMO1-modified proteins-NLuc showed LUC activity of higher levels compared to that of vector control. This result is in agreement with data obtained from the yeast split ubiquitin assays. Thus, the results imply that the SUMO-modified proteins isolated in this work appear to be *bona fide* SUMO binding proteins.

Specific interaction and sumoylation pattern between SUMO1 and MCM3 (At5G46280) protein

Arabidopsis contains eight SUMO genes (Kurepa et al., 2003; Novatchkova et al., 2004). Only four of these (SUMO1, SUMO2, SUMO3, and SUMO5) are highly expressed, functional and probably of major importance, although the other SUMOs could be expressed in very specific cells or under specific conditions only. SUMO1 and SUMO2 are most closely related, sharing 89% protein sequence identity, whereas SUMO3 shows 48% identity and SUMO5 only 35% identity to SUMO1. This suggests that individual SUMOs may have distinct targets and that functional diversification exists. Therefore, we investigated whether these SUMO-modified proteins bind preferentially to SUMO1, SUMO1 Δ GG, or SUMO3 by using MCM3, (Mini Chromosome Maintenance3; DNA replication licensing factor (At5G46280) protein, which has an important role in the initiation of replication (Stevens et al., 2002). As shown in Fig. 5A, SUMO1 specifically bound with MCM3 protein, whereas both SUMO1 Δ GG, or SUMO3 did not modify this protein as demonstrated by the yeast split ubiquitin assay. These results provide a first evidence that the MCM3 protein is covalently interacting with SUMO1 alone in sumoylation.

For a detailed examination of MCM3 (At5G46280) covalent modification by SUMO1, we investigated sumoylation patterns of AtSUMO1 and AtSUMO3 using a sumoylation assay system in *E. coli*. Recently, AtMYB30 has been used as a model SUMO target protein of AtSUMO1/2/3 and 5 for sumoylation

assays in *E. coli* (Okada et al., 2009). AtSUMO1 and AtSUMO3 were modified to expose the C-terminal Gly-Gly sequence, AtSUMO1 (GG) or AtSUMO3 (GG), which is necessary for covalent attachment to a target protein. As negative controls, AtSUMO1 or AtSUMO3 with the C-terminal Gly-Gly mutated to Ala-Ala, AtSUMO1 (AA) or AtSUMO3 (AA) were constructed, respectively. As shown in Fig. 5B, sumoylation reactions were performed in *E. coli* using AtSUMO1 or AtSUMO3 in combination with MCM3 protein (At5G46280) as a substrate. As a positive control, AtMYB30 was sumoylated by AtSUMO1 (GG) or AtSUMO3 (GG), respectively. Whereas the MCM3 protein (At5G46280) as a substrate was strongly sumoylated by AtSUMO1 (GG), AtSUMO3 (GG) was not recognized and modified. In addition, we also investigated the sumoylation pattern of UBA2A (At3G56860) with AtSUMO1 (GG) using the sumoylation assay system in *E. coli*. UBA2A (At3G56860) was sumoylated by AtSUMO1 (GG) as well (Supplementary Fig. S1). Thus, the results imply that proteins sumoylated in plants are specifically sumoylated by different SUMO proteins.

CONCLUSION

Sumoylation regulates various biological functions. Examples, mostly obtained with animals and yeasts, include cell division, and DNA repair and transcription. In contrast, only few plant SUMO targets have to date been identified and their functions analyzed at the molecular level. In a very general sense, plant SUMO proteins are involved in the regulation of flowering time, and in orchestrating biotic and abiotic stress responses. To better understand the significance of sumoylation in plants, we have screened SUMO-binding proteins using by a mass spectrometry-based proteomics approach for identifying such target proteins in transgenic *Arabidopsis* overexpressing AtSUMO1. Based on the results, SUMO-modified proteins are ubiquitous in plants, indicating that sumoylation represents an important factor in the modulation of protein location and activity in different compartments of the cell. In addition, the MCM3 protein (At5G46280) represents a specifically sumoylated target by SUMO1, but not SUMO3, suggesting that individual SUMO proteins recognize distinct targets. Our study extends the knowledge on the SUMO pathway complexity and provides further insights into the function of sumoylation in plants. Determining the exact mechanism(s) leading to sumoylation of the many proteins that are apparently condition-specific targets of SUMO modification will be an important area for further study.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Blum, H., Beier, H., and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8, 93-99.
- Budhiraja, R., Hermkes, R., Müller, S., Schmidt, J., Colby, T., Panigrahi, K., Coupland, G., and Bachmair, A. (2009). Substrates related to chromatin and to RNA-dependent processes are modified by *Arabidopsis* SUMO isoforms that differ in a conserved residue with influence on desumoylation. *Plant Physiol.* 149, 1529-1540.
- Catala, R., Ouyang, J., Abreu, I.A., Hu, Y., Seo, H., Zhang, X., and Chua, N.H. (2007). The *Arabidopsis* E3 SUMO ligase SIZ1 regulates plant growth and drought responses. *Plant Cell* 19, 2952-2966.
- Chen, H., Zou, Y., Shang, Y., Lin, H., Wang, Y., Cai, R., Tang, X., and Zhou, J.-M. (2008). Firefly luciferase complementation imaging assay for protein-protein interactions in plants. *Plant Physiol.* 146, 368-376.
- Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for *agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735-743.
- Colby, T., Matthai, A., Boeckelmann, A., and Stubble, H.P. (2006). SUMO-conjugating and SUMO-deconjugating enzymes from *Arabidopsis*. *Plant Physiol.* 142, 318-332.
- Denison, C., Rudner, A.D., Gerber, S.A., Bakalarski, C.E., Moazed, D., and Gygi, S.P. (2005). A proteomic strategy for gaining insights into protein sumoylation in yeast. *Mol. Cell. Proteomics* 4, 246-254.
- Elrouby, N., and Coupland, G. (2010). Proteome-wide screens for small ubiquitin-like modifier (SUMO) substrates identify *Arabidopsis* proteins implicated in diverse biological processes. *Proc. Natl. Acad. Sci. USA* 107, 17415-17420.
- García-Domínguez, M., March-Díaz, R., and Reyes, J.C. (2008). The PHD domain of plant PIAS proteins mediates sumoylation of bromodomain GTE proteins. *J. Biol. Chem.* 283, 21469-21477.
- Gill, G. (2005). Something about SUMO inhibits transcription. *Curr. Opin. Genet. Dev.* 15, 536-541.
- Goodson, M.L., Hong, Y., Rogers, R., Matunis, M.J., Park-Sarge, O.-K., and Sarge, K.D. (2001). SUMO-1 modification regulates the DNA binding activity of heat shock transcription factor 2, a promyelocytic leukemia nuclear body associated transcription factor. *J. Biol. Chem.* 276, 18513-18518.
- Hannich, J.T., Lewis, A., Kroetz, M.B., Li, S.-J., Heide, H., Emili, A., and Hochstrasser, M. (2005). Defining the SUMO-modified proteome by multiple approaches in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 18513-18518.
- Hay, R.T. (2005). Sumo: a history of modification. *Mol. Cell* 18, 1-12.
- Hecker, C.-M., Rabiller, M., Haglund, K., Bayer, P., and Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. *J. Biol. Chem.* 281, 16117-16127.
- Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* 458, 422-429.
- Hong, Y., Rogers, R., Matunis, M.J., Mayhew, C.N., Goodson, M., Park-Sarge, O.-K., and Sarge, K.D. (2001). Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J. Biol. Chem.* 276, 40263-40267.
- Jin, J.B., Jin, Y.H., Lee, J., Miura, K., Yoo, C.Y., Kim, W.-Y., Van Oosten, M., Hyun, Y., Somers, D.E., Lee, I., et al. (2008). 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.* 53, 530-540.
- Johnson, E.S. (2004). Protein modification by SUMO. *Annu. Rev. Biochem.* 73, 355-382.
- Kim, S.T., Kim, S.G., Hwang, D.H., Kang, S.Y., Kim, H.J., Lee, B.H., Lee, J.J., and Kang, K.Y. (2004). Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. *Proteomics* 4, 3569-3578.
- Kurepa, J., Walker, J.M., Smalle, J., Gosink, M.M., Davis, S.J., Durham, T.L., Sung, D.-Y., and Vierstra, R.D. (2003). The small ubiquitin-like modifier (SUMO) protein modification system in *Arabidopsis*: Accumulation of SUMO1 and -2 conjugates is increased by stress. *J. Biol. Chem.* 278, 6862-6872.
- Lakatos, L., Szittyá, G., Silhavy, D., and Burgyan, J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO* 23, 876-884.
- Laser, H., Bongards, C., Schuller, J., Heck, S., Johnsson, N., and Lehming, N. (2000). A new screen for protein interactions reveals that the *Saccharomyces cerevisiae* high mobility group proteins Nhp6A/B are involved in the regulation of the GAL1 promoter. *Proc. Natl. Acad. Sci. USA* 97, 13732-13737.
- Lee, J., Nam, J., Park, H.C., Na, G., Miura, K., Jin, J.B., Yoo, C.Y., Baek, D., Kim, D.H., Jeong, J.C., et al. (2007). Salicylic acid-mediated innate immunity in *Arabidopsis* is regulated by SIZ SUMO E3 ligase. *Plant J.* 49, 79-90.
- Lois, L.M., Lima, C.D., and Chua, N.H. (2003). Small ubiquitin-like modifier modulates abscisic acid signaling in *Arabidopsis*. *Plant Cell* 15, 1347-1359.
- Mao, Y., Desai, S.D., and Liu, L.F. (2000). SUMO-1 conjugation to human DNA topoisomerase II isozymes. *J. Biol. Chem.* 275, 26066-26073.
- Matunis, M.J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GAPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* 135, 1457-1470.
- Melchior, F. (2000). SUMO-nonclassical ubiquitin. *Annu. Rev. Cell Dev. Biol.* 16, 591-626.
- Meulmeester, E., and Melchior, F. (2008). Cell biology: SUMO. *Nature* 452, 709-711.
- Miller, M.J., Barrett-Wilt, G.A., Hua, Z., and Vierstra, R.D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 107, 16512-16517.
- Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000). Covalent modification of p73 α by SUMO-1. *J. Biol. Chem.* 275, 36316-36323.
- Miura, K., and Hasegawa, P.M. (2010). Sumoylation and other ubiquitin-like post-translational modifications in plants. *Trend Cell Biol.* 20, 223-232.
- Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A.S., Raghothama, K.G., Baek, D., Koo, Y.D., Jin, J.B., Bressan, R.A., et al. (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. USA* 102, 7760-7765.
- Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.-J., and Hasegawa, P.M. (2007). SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*. *Plant Cell* 19, 1403-1414.
- Miura, K., Lee, J., Jin, J.B., Yoo, C.Y., Miura, T., and Hasegawa, P.M. (2009). Sumoylation of ABI5 by the *Arabidopsis* SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. *Proc. Natl. Acad. Sci. USA* 106, 5418-5423.
- Novatchkova, M., Budhiraja, R., Coupland, G., Eisenhaber, F., and Bachmair, A. (2004). SUMO conjugation in plants. *Planta* 220, 1-8.
- O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250, 4007-4021.
- Okada, S., Nagabuchi, M., Takamura, Y., Nakagawa, T., Shimoyozu, K., Nakayama, J.-I., and Tanaka, K. (2009). Reconstitution of *Arabidopsis thaliana* SUMO pathway in *E. coli*: functional evaluation of SUMO machinery proteins and mapping of sumoylation sites by mass spectrometry. *Plant Cell Physiol.* 50, 1049-1061.
- Panse, V.G., Hardeland, U., Werner, T., Kuster, B., and Hurt, E. (2004). A proteome-wide approach identifies sumoylated substrate proteins in yeast. *J. Biol. Chem.* 279, 41346-41351.
- Park, H.C., Kim, M.L., Kang, Y.H., Jeong, J.C., Cheong, M.S., Choi, W., Lee, S.Y., Cho, M.J., Kim, M.C., Chung, W.S., et al. (2009). Functional analysis of the stress-inducible soybean calmodulin isoform-4 (*GmCaM-4*) promoter in transgenic tobacco plants. *Mol. Cells* 27, 475-480.
- Saitoh, H., and Hinchey, J. (2000). Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* 275, 6252-6258.
- Saracco, S.A., Miller, M.J., Kurepa, J., and Vierstra, R.D. (2007). Genetic analysis of SUMOylation in *Arabidopsis*: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. *Plant Physiol.* 145, 119-134.
- Schmidt, D., and Müller, S. (2003). PIAS/SUMO: new partners in transcriptional regulation. *Cell. Mol. Life Sci.* 60, 2561-2574.
- Seeler, J.S., and Dejean, A. (2003). Nuclear and unclear functions of SUMO. *Nat. Rev. Mol. Cell Biol.* 4, 690-699.
- Stagljär, I., Korostensky, C., Johnsson, N., and te Heesen, S.

- (1998). A new genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*. *Proc. Natl. Acad. Sci. USA* *95*, 5187-5192.
- Stevens, R., Mariconti, L., Rossignol, P., Perennes, C., Cella, R., and Bergounioux, C. (2002). Two E2F sites in the Arabidopsis MCM3 promoter have different roles in cell cycle activation and meristematic expression. *J. Biol. Chem.* *277*, 32978-32984.
- van den Burg, H.A., Kini, R.K., Schuurink, R.C., and Takken, F.L.W. (2010). Arabidopsis small ubiquitin-like modifier paralogs have distinct functions in development and defense. *Plant Cell* *22*, 1998-2016.
- Varshavsky, A. (1996). The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* *93*, 12142-12149.
- Wykoff, D.D., and O'Shea, E.K. (2005). Identification of sumoylated proteins by systematic immunoprecipitation of the budding yeast proteome. *Mol. Cell. Proteomics* *4*, 73-83.
- Yoo, J.H., Park, C.Y., Kim, J.C., Heo, W.D., Cheong, M.S., Park, H.C., Kim, M.C., Moon, B.C., Choi, M.S., Kang, Y.H., et al. (2005). Direct interaction of a divergent CaM isoform and the transcription factor, MYB2, enhances salt tolerance in Arabidopsis. *J. Biol. Chem.* *280*, 3697-3706.
- Yoo, C.Y., Miura, K., Jin, J.B., Lee, J., Park, H.C., Salt, D.E., Yun, D.-J., Bressan, R.A., and Hasegawa, P.M. (2006) SIZ1 small ubiquitin-like modifier E3 ligase facilitates basal thermotolerance in Arabidopsis independent of salicylic acid. *Plant Physiol.* *142*, 1548-1558.
- Zhao, Y., Kwon, S.W., Anselmo, A., Kaur, K., and White, M.A. (2004). Broad spectrum identification of cellular small ubiquitin-related modifier (SUMO) substrate proteins. *J. Biol. Chem.* *279*, 20999-21002.
- Zhou, W., Ryan, J.J., and Zhou, H. (2004). Global analyses of sumoylated proteins in *Saccharomyces cerevisiae*: induction of protein sumoylation by cellular stresses. *J. Biol. Chem.* *279*, 32262-32268.

