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Reconstitution of *Arabidopsis thaliana* SUMO Pathways in *E. coli*: Functional Evaluation of SUMO Machinery Proteins and Mapping of SUMOylation Sites by Mass Spectrometry

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Recent studies have revealed various functions for the small ubiquitin-related modifier (SUMO) in diverse biological phenomena, such as regulation of cell division, DNA repair and transcription, in yeast and animals. In contrast, only a limited number of proteins have been characterized in plants, although plant SUMO proteins are involved in many physiological processes, such as stress responses, regulation of flowering time and defense reactions to pathogen attack. Here, we reconstituted the *Arabidopsis thaliana* SUMOylation cascade in *Escherichia coli*. This system is rapid and effective for the evaluation of the SUMOylation of potential SUMO target proteins. We tested the ability of this system to conjugate the *Arabidopsis* SUMO isoforms, AtSUMO1, 2, 3 and 5, to a model substrate, AtMYB30, which is an *Arabidopsis* transcription factor. All four SUMO isoforms tested were able to SUMOylate AtMYB30. Furthermore, SUMOylation sites of AtMYB30 were characterized by liquid chromatography–tandem mass spectrometry (LC-MS/MS) followed by mutational analysis in combination with this system. Using this reconstituted SUMOylation system, comparisons of SUMOylation patterns among SUMO isoforms can be made, and will provide insights into the SUMO isoform specificity of target modification. The identification of SUMOylation sites enables us to investigate the direct effects of SUMOylation using SUMOylation-defective mutants. This system will be a powerful tool for elucidation of the role of SUMOylation and of the biochemical and structural features of SUMOylated proteins in plants.

Keywords: Arabidopsis • Post-translational modification • Reconstituted SUMOylation system • SUMO.

Abbreviations: EST, expressed sequence tag; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MCS, multicloning site; ORF, open reading frame; SBM, SUMO-binding motif; SIM, SUMO interaction motif; SUMO, small ubiquitin-related modifier.

Introduction

The small ubiquitin-related modifier (SUMO) is a reversible post-translational modifier, which is covalently conjugated to a lysine residue in a substrate protein. Since the discovery of SUMO (Meluh and Koshland 1995), information on the SUMOylation pathway and the functional consequences of SUMOylation has accumulated. SUMOylation leads to changes in protein–protein interactions, by which the activity, stability or localization of substrate proteins is altered (Melchior et al. 2003, Schmidt and Muller 2003, Johnson 2004).

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Moreover, in addition to SUMOylation, non-covalent interactions between SUMO and a target protein are also involved in changes in protein conformation (Hecker et al. 2006, Geiss-Friedlander and Melchior 2007, Kerscher 2007).

Covalent SUMO conjugation requires an E1/E2/E3 enzyme cascade similar to the ubiquitin-conjugating cascade. E1, the activating enzyme, facilitates the conjugation of SUMO to target proteins through adenylation, thioester formation and thioester transfer from E1 to E2 (Lois and Lima 2005). Unlike the ubiquitin E1 enzyme, which functions as a single subunit enzyme, the SUMO E1 enzyme consists of two subunits, SAE1 (Aos1) and SAE2 (Uba2). SAE1 contains a single domain that adenylates SUMO, whereas SAE2 contains three domains: the catalytic cysteine domain, the adenylation domain and the ubiquitin-fold domain (UFD) (Lois and Lima 2005). The E1–SUMO interaction is mediated exclusively by SAE2 (Lois and Lima 2005). In *Arabidopsis thaliana*, the smaller SAE1 subunit is encoded by two genes, *AtSAE1a* and *AtSAE1b*, which possess 81% amino acid sequence identity, whereas the larger SAE2 subunit is encoded by a single gene, *AtSAE2*. The activity of the E1 heterodimers, AtSAE1a–AtSAE2 and AtSAE1b–AtSAE2, has been demonstrated by in vitro SUMOylation assays (Colby et al. 2006, Budhiraja et al. 2009). According to the genetic analysis of *AtSAE1a* T-DNA mutants, both AtSAE1a and AtSAE1b are likely to share redundant functions (Saracco et al. 2007). The *Arabidopsis* E2 SUMO-conjugating enzyme is encoded by a single gene, *AtSCE1a*, which is essential for cell viability. The cysteine residue at position 94 in the catalytic domain of AtSCE1a is required for activity (Lois et al. 2003). To date, only one E3 SUMO ligase, AtSIZ1, has been identified in *Arabidopsis*. The deletion of this gene results in various phenotypes, including altered responses to nutrient deficiency, salicylic acid, cold stress and drought stress, as well as changes in flowering regulation (Miura et al. 2005, Yoo et al. 2006, Catala et al. 2007, Lee et al. 2007, Miura et al. 2007, Garcia-Dominguez et al. 2008, Jin et al. 2008).

The SUMO protein is expressed as a precursor and is processed by a SUMO-specific protease to expose the C-terminal Gly–Gly motif. Eight *SUMO* genes are encoded in the *Arabidopsis* genome, and four of them (*AtSUMO1*, *2*, *3* and *5*) are found in the expressed sequence tag (EST) database. The number of isoforms in *Arabidopsis* is relatively large compared with other organisms: a single SUMO gene is present in yeast, nematode and fly; four isoforms (SUMO1–SUMO4) are present in human (Guo et al. 2004); and three SUMO homologs (CrSUMO96, CrSUMO97 and CrSUMO148) are found in the complete genome sequence of *Chlamydomonas reinhardtii* (Wang et al. 2008). The large number of *Arabidopsis* SUMO isoforms implies the existence of SUMO isoform-specific features and functions. Indeed in human, while only a single SUMO1 is attached to a SUMOylation site, SUMO2/3 can form poly-SUMO chains, although the functional significance of this modification is still unknown (Tatham et al. 2001, Bylebyl et al. 2003). This feature is also observed in *Arabidopsis*: a single AtSUMO3 can be conjugated, but AtSUMO1/2 can form poly-SUMO chains (Colby et al. 2006). Furthermore, AtSUMO1/2 and AtSUMO3 exhibit different SUMOylation patterns in planta (Kurepa et al. 2003). Genetic analysis demonstrates that an AtSUMO1/2 double mutant is not viable (Saracco et al. 2007). The maturation of SUMO precursors and the deconjugation of SUMO from substrates are also regulated in a SUMO isoform-specific manner by distinct SUMO-specific proteases (Chosed et al. 2006, Colby et al. 2006, Chosed et al. 2007). However, the roles of individual SUMO isoforms are still unclear.

In animals, several hundred SUMO target proteins have been identified, and SUMOylation is involved in essential cellular processes, such as regulation of cell division, DNA repair and transcription (Johnson 2004). Most of the substrate proteins are nuclear proteins and SUMOylation was initially thought to occur in the nucleus. However, recent studies revealed the occurrence of SUMOylation throughout the cell, depending on the process being regulated, such as the regulation of ion channels or receptors on the plasma membrane, the regulation of tyrosine phosphatase-1B (PTP1b) on the endoplasmic reticulum or the control of mitochondrial fission (Geiss-Friedlander and Melchior 2007, Martin et al. 2007). In contrast to animals, little is known about the SUMOylation target proteins in plants, despite the involvement of SUMOylation in a wide range of physiological processes, such as stress responses, regulation of flowering and defense responses (Hotson et al. 2003, Kurepa et al. 2003, Lois et al. 2003, Murtas et al. 2003, Roden et al. 2004, Miura et al. 2005, Catala et al. 2007, Lee et al. 2007, Miura et al. 2007, Conti et al. 2008). Only five *Arabidopsis* proteins, AtMYB30, PHR1, ICE1, GTE3 and GTE5, and two SUMO machinery proteins, AtSIZ1 and AtSCE1, had been experimentally demonstrated to be SUMOylated (Miura et al. 2005, Colby et al. 2006, Miura et al. 2007, Garcia-Dominguez et al. 2008), until Budhiraja et al. (2009) very recently identified in vivo SUMOylation substrates by mass spectrometric analysis of the enriched SUMO conjugate from *Arabidopsis*. By this analysis, many proteins which are involved in the regulation of chromatin structure, splicing or translation were identified.

For functional analysis of SUMOylation in target proteins, identification of SUMOylation sites is indispensable for subsequent mutational assays. SUMO acceptor sites in substrate proteins are primarily found in the tetrapeptide consensus motif (ΨKXE), where Ψ is a hydrophobic residue and X is any amino acid residue. However, the verification of SUMOylation sites has been a laborious task, because of the relatively high frequency of SUMOylation at non-consensus sequences (26%) (Seeler and Dejean 2003), and the existence of multiple SUMOylation sites in a target protein.

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Here, we present a rapid method to check the SUMOylation of a potential SUMO target protein using a reconstituted SUMOylation system with *Arabidopsis* SUMO machinery proteins in *Escherichia coli*. In this system, the reconstitution of the mammalian SUMO pathway was modified using the Duet expression system (Novagen, Madison, WI, USA) (Uchimura et al. 2004a, Uchimura et al. 2004b). As a model target protein, AtMYB30, a positive regulator of programmed cell death (Daniel et al. 1999, Vailleau et al. 2002, Raffaele et al. 2006, Raffaele et al. 2008) was chosen to evaluate this system. The SUMOylation abilities of four SUMO isoforms, AtSUMO1, 2, 3 and 5, and the E1 enzyme activity of AtSAE1a and AtSAE1b were examined. Furthermore, we demonstrated that large amounts of SUMOylated recombinant proteins can be produced with this system, which enables us to perform biochemical analyses of SUMOylated proteins. We identified the SUMOylation sites in AtMYB30 using this reconstituted SUMOylation system in combination with liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Results

Reconstitution of the Arabidopsis thaliana SUMO pathway in E. coli

In order to perform SUMOylation reactions in *E. coli*, we constructed two types of plasmids carrying *Arabidopsis* SUMOylation machinery proteins: the first carried the two protein subunits of the E1 heterodimer, AtSAE1 and AtSAE2, and the second carried E2 (AtSCE1a) and an AtSUMO isoform (**Fig. 1A**). AtSUMO was modified to expose the C-terminal Gly–Gly sequence, AtSUMO(GG), which is necessary for covalent attachment to a target protein. As a negative control, AtSUMO with the C-terminal Gly–Gly mutated to Ala–Ala, AtSUMO(AA), was constructed. Since AtSAE1 and AtSUMO are encoded by gene families of two and eight members, respectively, we constructed plasmids for the AtSAE1a and b isoforms, and AtSUMO1, 2, 3 and 5, whose ESTs are found in TAIR (www.arabidopsis.org). AtSAE1 and AtSCE1a were fused to an S-tag at their C-termini, and AtSAE2 and AtSUMO were fused to a His-tag at their N-termini to enable their detection by Western blotting (**Fig. 1B**). Although the expression of AtSUMO5(GG) and AtSUMO5(AA) was lower than that of the other AtSUMOs, relatively equal amounts of the other components of the SUMOylation machinery were expressed from their respective constructs. As a model SUMO target protein, we chose the *Arabidopsis* transcription factor AtMYB30, a known SUMO target protein (Colby et al. 2006), which was cloned into the pET28a vector to fuse both a His-tag and a T7-tag at its N-terminus (**Fig. 1A**). E3 was not used, in accordance with previous reports (Uchimura et al. 2004a, Uchimura et al. 2004b).

AtSUMO1, 2, 3 and 5 can SUMOylate AtMYB30, and both AtSAE1a and AtSAE1b have E1 enzyme activity.

Initially, the possible occurrence of isoform-specific modification was investigated with this reconstituted system. To examine whether AtSUMO1, 2, 3 and 5 possess the ability to SUMOylate a target protein, the SUMOylation reaction was performed in *E. coli* using each SUMO isoform and AtMYB30 as a substrate. For SUMOylation reactions, freshly transformed *E. coli* were used since the protein expression levels decreased after storage at 4°C (data not shown). The molecular weight shift due to conjugation of a single SUMO was observed in all samples using AtSUMO(GG)s, but not in samples using AtSUMO(AA)s, which indicates that all of the tested SUMO isoforms were able to modify AtMYB30 (**Fig. 2A**). Poly-SUMOylation of AtMYB30 was not detected in this system. The SUMOylation of AtMYB30 was further confirmed using AtSCE1a point-mutated at Cys94 to serine (C94S) in the catalytic domain, which abolished the shift in molecular weight of AtMYB30 (**Fig. 2C**).

To test whether both AtSAE1a and AtSAE1b function as E1 enzymes, we performed in vivo SUMOylation with the constructs coding for AtSAE1a and AtSAE1b, respectively (**Fig. 2A, B**). Similar levels of AtMYB30 SUMOylation were observed in the reactions using both AtSAE1a and AtSAE1b with respect to all the tested SUMO isoforms, indicating that both AtSAE1a and AtSAE1b possessed approximately the same level of E1 enzymatic activity.

Evaluation of the in vivo SUMOylation system with potential SUMO target proteins

In order to evaluate the utility of this in vivo SUMOylation system, we checked the SUMOylation of two other potential SUMO target proteins, NAF (Budhiraja et al. 2009) and LAF1 (Ballesteros et al. 2001). The SUMOylation reaction was performed with AtSUMO1, 2, 3 and 5 to check the SUMO isoform specificity. All the tested SUMO isoforms could SUMOylate NAF, whereas the SUMOylation of LAF1 was not detected with any SUMO isoforms (**Fig. 2D, E**). Poly-SUMOylation of NAF was not detected.

Identification of SUMOylation sites in AtMYB30

Although many reports have demonstrated the reconstitution of SUMOylation in vitro or in vivo in various organisms, there are only a few examples of the application of these systems to identify the SUMOylation sites. In order to determine the SUMO target lysine residues in AtMYB30, three prediction programs, SUMOsp 2.0 (http://bioinformatics.lcd-ustc.org/sumosp) (Xue et al. 2006), SUMOplot™ (http://www.abgent.com/doc/sumoplot) and SUMOpre (http://spg.biosci.tsinghua.edu.cn/service/sumoprd/predict.cgi) (Xu et al. 2008) were used to identify putative SUMOylation

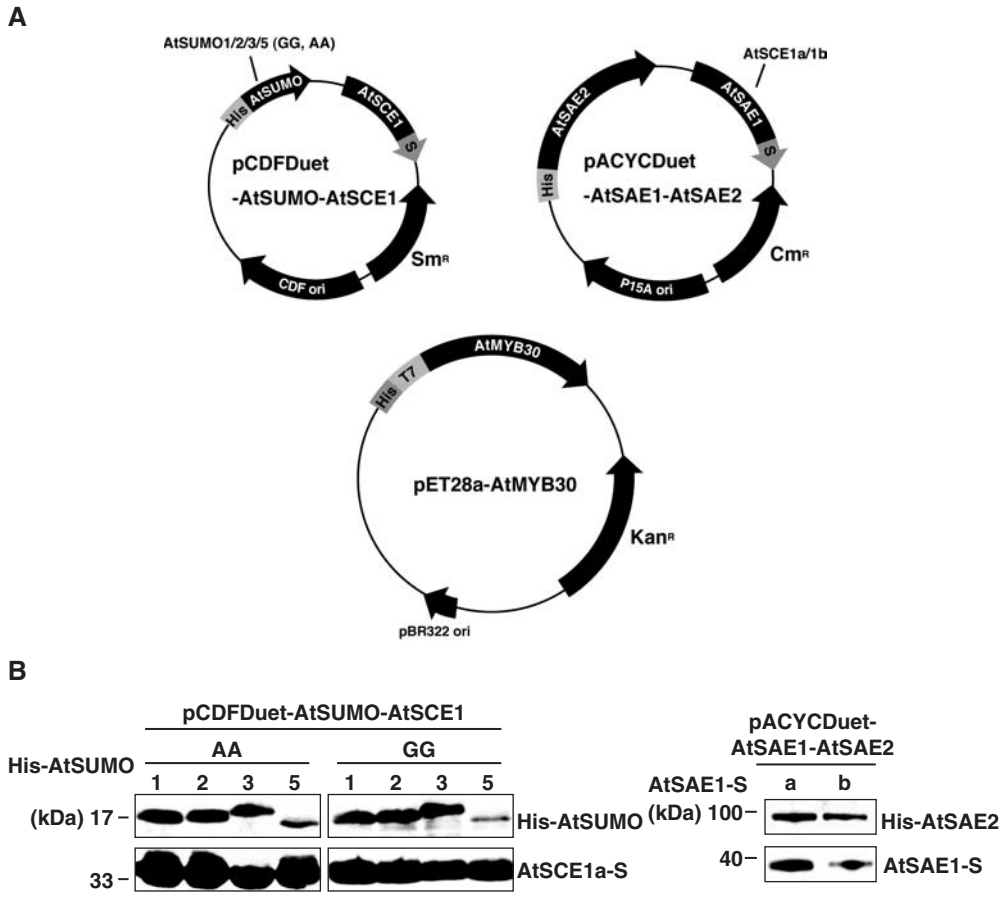

Fig. 1 Schematic representation of the plasmids used for reconstruction of *Arabidopsis* SUMOylation in *E. coli*. (A) Structures of pCDFDuet-AtSUMO-AtSCE1, pACYCDuet-AtSAE1-AtSAE2 and pET28a-AtMYB30. The *Arabidopsis* SUMO-related genes and *AtMYB30* gene, which encodes a model SUMO target protein, were cloned into three independent vectors, pACYCDuet-1, pCDFDuet-1 and pET28a, respectively. The mature forms of AtSUMOs, ASUMO1/2/3/5(GG) and AtSAE2 were His-tagged at their N-termini. AtSCE1 and AtSAE1 were S-tagged at their C-termini. The target protein, AtMYB30, was His- and T7-tagged at the N-terminus. The *E. coli* BL21(DE3) strain was transformed with these constructs for reconstitution of SUMOylation. As negative controls, modification-defective AtSUMOs, His-AtSUMO1/2/3/5(AA), were used. (B) Confirmation of expression of each SUMO-related protein by Western blot analysis. *Escherichia coli* BL21(DE3) was transformed with each construct, and tagged proteins were induced with 1 mM IPTG at an OD_{600} of 0.5. After a 2 h incubation at 37°C, cell lysates were prepared and subjected to Western blot analysis. His-AtSAE2 and His-AtSUMO1, 2, 3 and 5 were detected using an anti-His-tag antibody. For the detection of AtSAE1a-S, AtSAE1b-S and AtSCE1a-S an anti-S-tag antibody was used.

sites. No SUMOylation sites were predicted by SUMOpre, while one lysine, K246, was predicted as a non-consensus type of SUMOylation site at a low threshold (score = 2.353) by SUMOsp (**Table 1**). SUMOplot™ predicted five lysine residues as SUMOylation sites: three, K13, K66 and K250, were predicted with high scores (0.76, 0.77 and 0.94, respectively), and two, K9 and K14, with low scores (0.33 and 0.37, respectively) (**Table 1**). Altogether, no common lysine residues were predicted as SUMOylation sites by these three prediction programs.

We next used LC-MS/MS analysis to evaluate the utility of this reconstituted SUMOylation system for the identification of SUMOylation sites. AtSUMO3-modified AtMYB30 was overproduced using the reconstituted SUMOylation system and purified by His-tag affinity. This system enabled the production of large amounts of SUMOylated recombinant proteins that could be detected in Coomassie Brilliant Blue-stained gels (**Fig. 3A**). The SDS–PAGE-fractionated proteins were subjected to LC-MS/MS analysis. Since the target protein must be fragmented into short peptides for high resolution in the LC-MS/MS analysis, SUMOylated AtMYB30 was digested with proteases prior to LC-MS/MS (**Fig. 3B**).

Initially, SUMOylated AtMYB30 was digested with trypsin, which cleaves on the C-terminal side of lysine and arginine residues. Non-SUMOylated AtMYB30 was used as a negative control. Trypsin digestion of conjugated AtSUMO3 produces a specific tag of five amino acid residues (AMSGG)

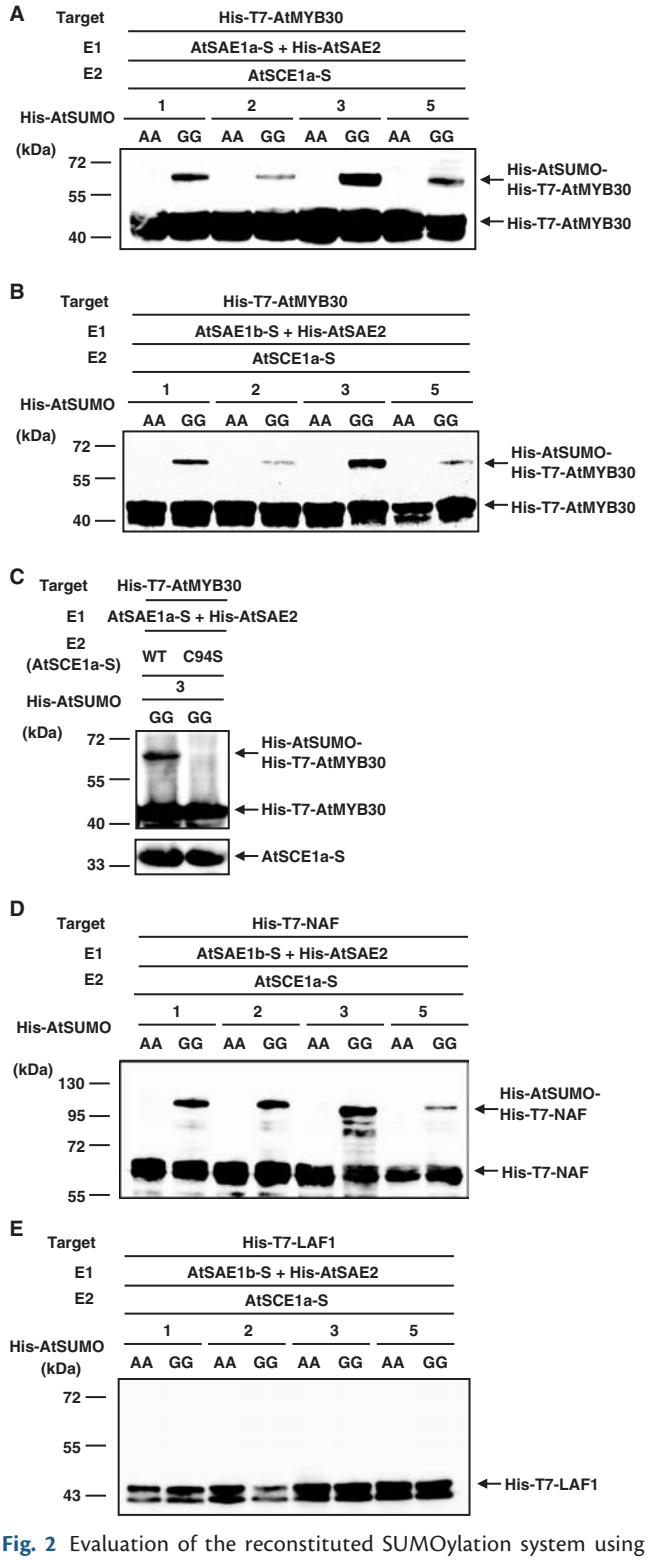

Fig. 2 Evaluation of the reconstituted SUMOylation system using AtMYB30 as a model substrate. (A) SUMOylation of AtMYB30 by AtSUMO1, 2, 3 and 5 using AtSAE1a as a subunit of the E1 heterodimer. *Escherichia coli* BL21(DE3) was transformed with different combinations of the three constructs which encode the tagged proteins indicated above each lane. Transformed *E. coli* cells were incubated at 37°C until the OD_{600} reached 1.0, and the expression of tagged proteins and SUMOylation was then induced overnight with 0.2 mM IPTG at 25°C. Cell lysates were prepared and Western blotting was carried out using an anti-T7-tag antibody. SUMOylated AtMYB30 was observed as bands shifted to higher molecular weight in lanes using mature AtSUMO(GG)s (indicated as GG), but not in lanes using mutated AtSUMO(AA)s (indicated as AA). (B) SUMOylation of AtMYB30 using AtSAE1b as a subunit of the E1 heterodimer. (C) Abolishment of SUMOylation in AtMYB30 with AtSCE1a point-mutated at Cys94 as an E2 enzyme. SUMOylation of AtMYB30 was detected using an anti-T7-tag antibody. Expression of wild-type (WT) and point-mutated (C94S) AtSCE1a was confirmed using an anti-S-tag antibody. (D) SUMOylation of NAF using AtSUMO1, 2, 3 and 5. Western blotting was carried out using an anti-T7-tag antibody. (E) SUMOylation of LAF1 using AtSUMO1, 2, 3 and 5. Western blotting was carried out using an anti-T7-tag antibody.

that remains covalently attached to the target lysine. Due to steric hindrance of the isopeptide bond, SUMOylation renders the target lysine residue resistant to efficient trypsin cleavage, which generates a longer fragment with a diagnostic tag derived from SUMO (**Fig. 3C**). In the initial LC-MS/MS analysis, we identified several candidate residues for SUMOylation, but very few fragments of AtMYB30 at the C-terminal region were detected, presumably due to the lack of lysine and arginine residues for trypsin cleavage. Therefore, we performed LC-MS/MS analysis using GluC-digested AtMYB30 (**Fig. 3B**). We also used AtSUMO3 with a single amino acid mutation of the serine next to the Gly–Gly C-terminus, RAMRGG, for higher resolution (Knuesel et al. 2005, Wohlschlegel et al. 2006). Although a background signal was frequently observed in the negative control, this approach also enabled us to identify additional candidate SUMOylation site residues (**Table 1**).

Based on the data from the three independent LC-MS/MS analyses, four lysine residues, K232, K246, K250 and K283, were substrate residues for SUMOylation (**Fig. 3D**, **Supplementary Fig. S1**). The other lysine residues, which were efficiently cleaved by trypsin digestion, seemed unlikely to be major substrate residues for SUMOylation. However, we could not exclude the four predicted lysine residues, K13, K115, K224 and K228, as substrate residues for SUMOylation because peptides containing these lysine residues were not detected in our LC-MS/MS analysis, which may be due to the peptide fragment length (**Table 1**).

Confirmation of SUMOylation sites using the in vivo reconstituted SUMOylation system

In order to verify the LC-MS/MS data, we introduced lysine to arginine mutations at the candidate SUMOylation sites in the target protein and evaluated the effect on SUMOylation using the reconstituted SUMOylation system. A lysine to

arginine mutation at a SUMOylation site abolishes SUMOylation at that site. We constructed AtMYB30$^{K13,\ 14,\ 232R}$, AtMYB30^{K246R}, AtMYB30^{K250R} and AtMYB30^{K283R}, which carry amino acid substitutions at the indicated candidate lysine residues. The SUMOylation level of AtMYB30^{K283R} was dramatically diminished (**Fig. 4A**), which indicates that K283 is the major SUMOylation site in AtMYB30. The triple mutations at K13, K14 and K232, and the single mutations at K246 and K250 had no effect on SUMOylation (data not shown). However, a low level of SUMOylation was still detected in AtMYB30^{K283R}, which may be derived from minor SUMOylation sites (**Fig. 4A**). To determine the minor SUMOylation sites, SUMOylation in the N-terminal region of AtMYB30 was tested using the N-terminal 163 amino acids of AtMYB30. Since no SUMOylation was observed in this region (data not shown), K224, K228, K232, K246 and K250 remained as the most probable minor SUMOylation sites. To examine whether these five lysine residues comprise all the minor SUMOylation sites, AtMYB30$^{K224,\ 228,\ 232,\ 246,\ 250,\ 283R}$ was constructed and the SUMOylation of this mutant was examined. No SUMOylation was detected in the AtMYB30$^{K224,\ 228,\ 232,\ 246,\ 250,\ 283R}$ mutant (**Fig. 4A**). To delineate

Table 1 Prediction and identification of SUMOylation sites with computational and LC-MS/MS analyses.

Position of lysine residues	Surrounding amino acids	Prediction software[a]		LC-MS/MS[b]			Probable SUMOylation sites[c]
		SUMOsp	SUMOplot	AtSUMO3(GG)	AtSUMO3(GG)	AtSUMO3R(GG)	
				Trypsin	Trypsin + GluC	Trypsin	
K9	D**K**GG		L	–	–	–	
K13	V**K**KG		H	n	n	n	±
K14	K**K**GP		L	n	–	n	
K51	S**K**SC			n	n	–	
K66	I**K**RG		H	–	–	–	
K76	E**K**MI			–	–	–	
K105	I**K**NY			–	–	–	
K113	L**K**KK			–	–	–	
K114	K**K**KL			–	n	n	
K115	K**K**LN			n	n	n	±
K118	N**K**VN			–	–	–	
K164	A**K**KA			–	–	–	
K165	K**K**AL			–	n	–	
K221	A**K**LL			–	–	–	
K224	L**K**GW			n	n	n	±
K228	V**K**NS			n	n	n	±
K232	P**K**TQ			S	–	S	+
K246	V**K**EV	L		S	–	–	+
K250	I**K**SD		H	S	n	–	+
K255	G**K**EC			n	–	n	
K283	T**K**PD			n	S	n	+
K301	E**K**WL			n	–	n	

[a]Prediction of SUMOylation sites with the computer programs, SUMOsp and SUMOplot. The lysine residues which were predicted as SUMOylation sites with low (L) and high (H) scores are indicated. The score values were stated in the text.

[b]Identification of SUMOylation sites with LC-MS/MS analysis. The native and mutated versions of AtSUMO3, AtSUMO3(GG) and AtSUMO3R(GG), respectively, were used for the SUMOylation reactions. The protein samples were digested with the proteases, trypsin and/or GluC. The LC-MS/MS results were categorized as follows: –, SUMO was not detected and the corresponding K was efficiently cleaved, or SUMO was detected in the negative control; n, corresponding fragments were not detected or only a few fragments were detected; S, SUMO was detected only in the SUMOylated protein sample.

[c]The summary of three LC-MS/MS analyses. The lysine residues, which could not be discriminated as SUMOylation sites (±), and which were detected as SUMOylation sites in at least one LC-MS/MS analysis (+) are indicated.

further the minor SUMOylation sites, a single arginine residue among the six substituted arginine residues in AtMYB30$^{K224, 228, 232, 246, 250, 283R}$ was back-mutated to lysine (5KR mutants). The recovery of SUMOylation was examined in each 5KR mutant. However, no recovery of SUMOylation was detected in any of the 5KR mutants, except for the 5KR mutant with the back mutation at K283 (**Fig. 4A**). Considering the fact that the substitution of a single or multiple lysine residues of these five sites into arginine residues in combination with the mutation at the K283 major SUMOylation site did not disrupt the SUMOylation completely (data not shown), several or all of these lysine residues, K224, K228, K232, K246 and K250, are likely to be minor SUMOylation sites. The SUMOylation of each single lysine residue at these minor sites may be below the detection limit of the Western blot analysis. These results indicate that SUMOylation sites in AtMYB30 are located at the C-terminal region of AtMYB30, where no known functional domains exist (**Fig. 4B**).

Discussion

We established a reconstituted SUMOylation system with *Arabidopsis* SUMOylation machinery proteins in *E. coli*. This system is a simple and convenient method for examining the SUMOylation of a protein of interest. Detection of SUMOylated proteins in eukaryotic cells is often difficult and requires

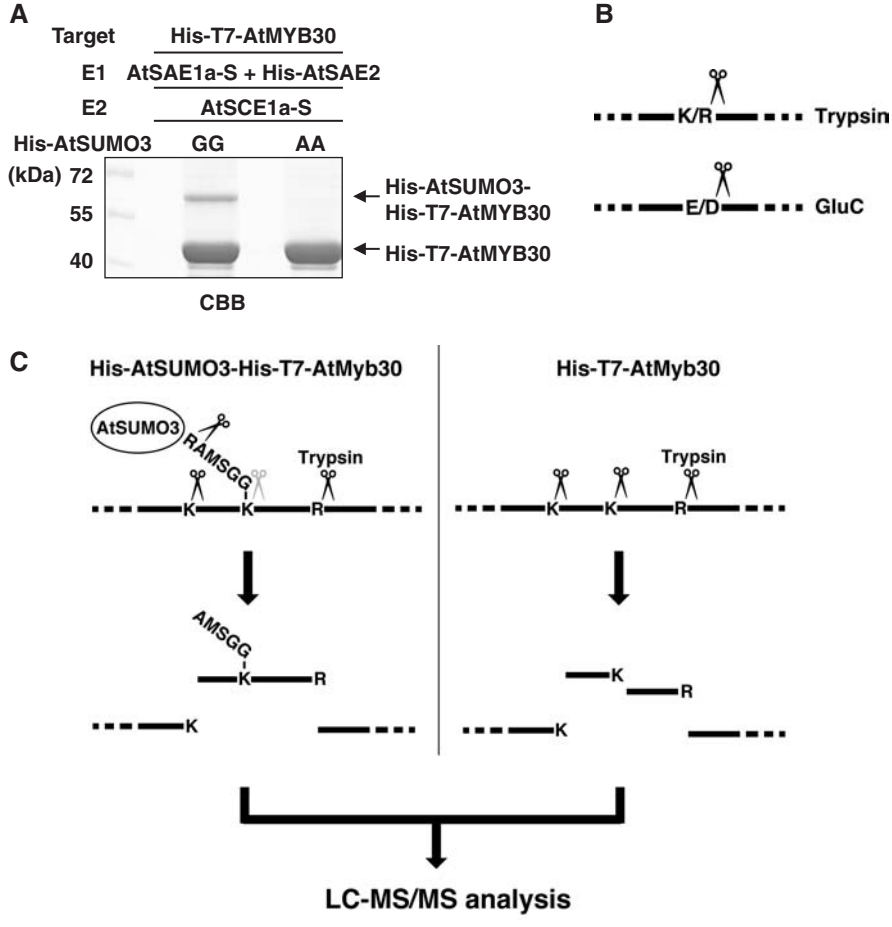

Fig. 3 Identification of SUMOylation sites of AtMYB30 by LC-MS/MS. (A) Purification of SUMOylated-AtMYB30 for LC-MS/MS analysis. *Escherichia coli* harboring pCDFDuet-AtSUMO3(GG)-AtSCE1a, pACYCDuet-AtSAE1a-AtSAE2 and pET28a-AtMYB30 were cultured in the presence of IPTG. Total lysates were incubated with HisLink resin, and the proteins bound to the resin were analyzed by 10% SDS–PAGE followed by Coomassie Brilliant Blue (CBB) staining. For a negative control, *E. coli* harboring pCDFDuet-AtSUMO3(AA)-AtSCE1a, pACYCDuet-AtSAE1a-AtSAE2 and pET28a-AtMYB30 was used. (B) Schematic illustration of the cleavage sites of trypsin and GluC, the two proteases used for fragmentation of AtMYB30. (C) Schematic illustration of fragmentation patterns of SUMOylated and non-SUMOylated AtMYB30. Fragmented proteins were subjected to LC-MS/MS analysis. (D) Representative MS/MS spectra of the peptide fragment spanning residues 282–307 for the control AtSUMO3-AA-incubated AtMYB30 (upper) and the AtSUMO3-GG-incubated AtMYB30 (lower). The observed *y* and *b* ions and the fragment map are shown.

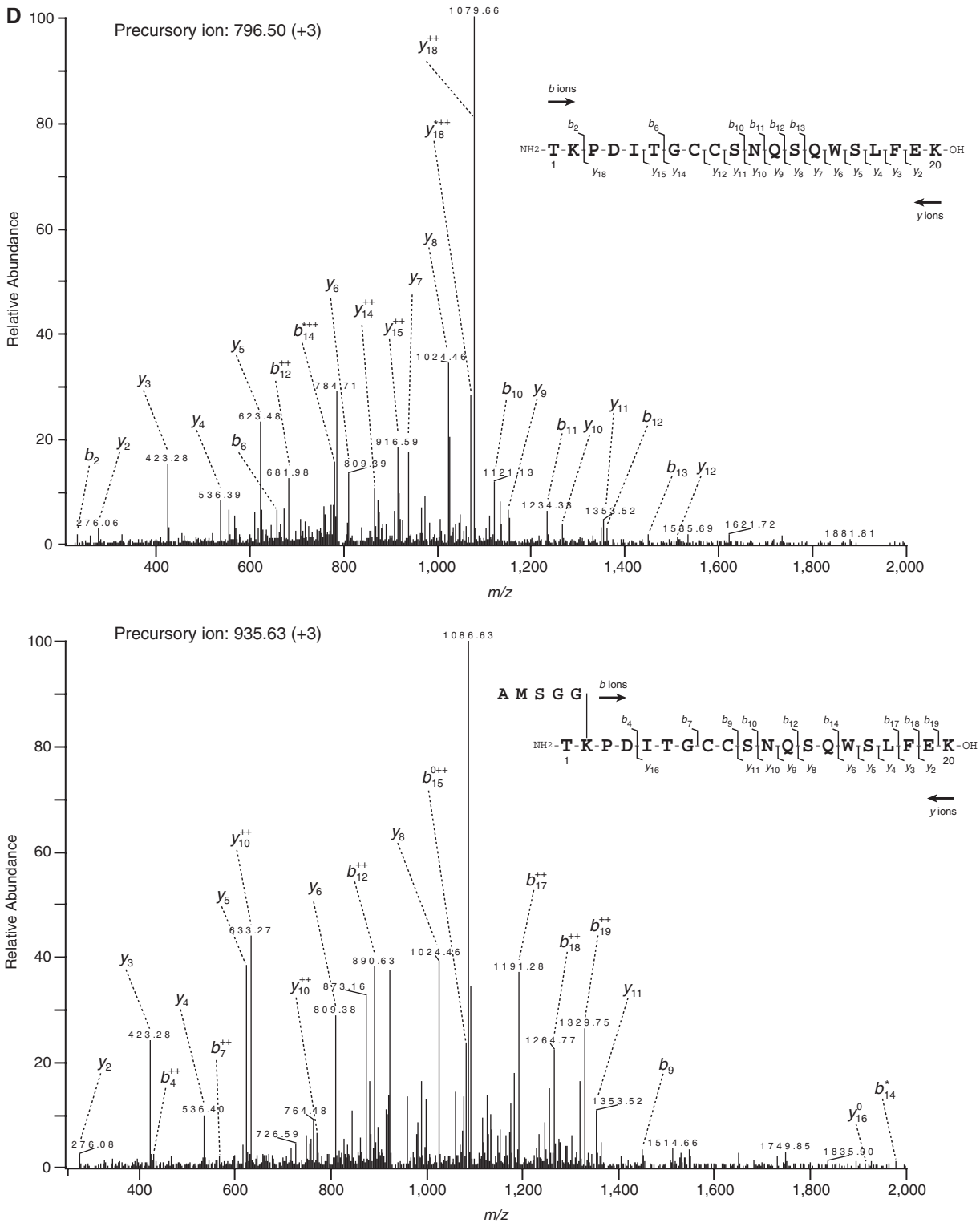

Fig. 3 Continued

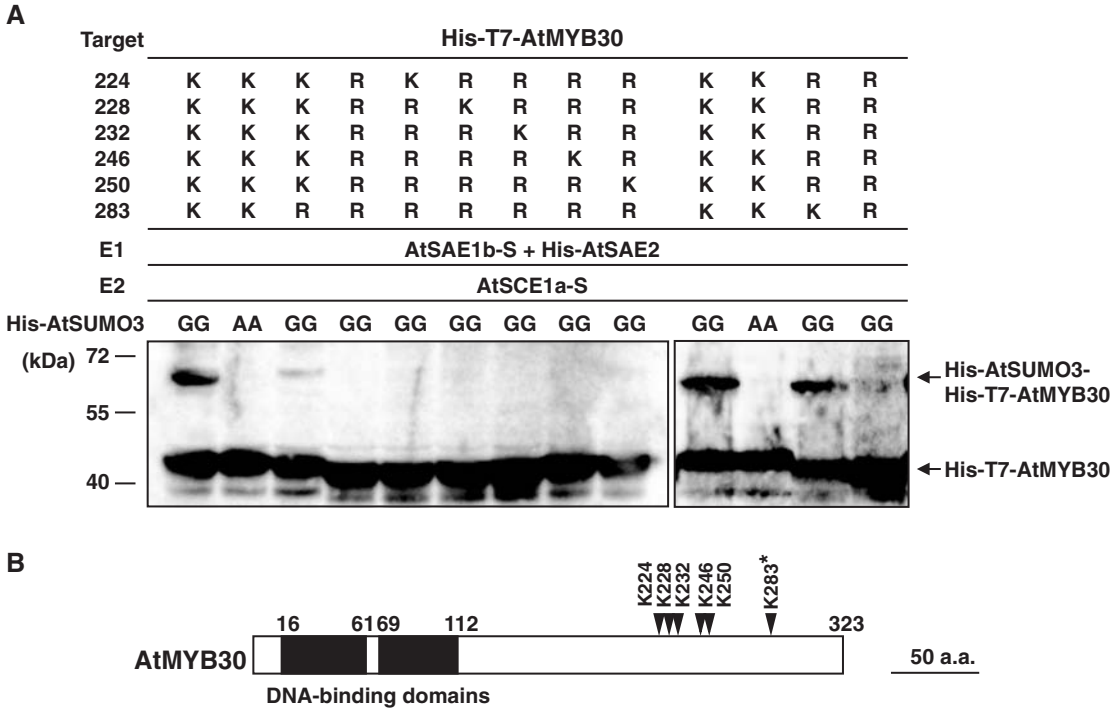

Fig. 4 Confirmation of SUMOylation sites using the reconstituted SUMOylation system. (A) Verification of SUMOylation sites with KR mutants. The in vivo SUMOylation reactions were performed as described above using AtMYB30 containing lysine to arginine substitutions at the putative SUMO target lysine residues. (B) Distribution of SUMOylation sites in AtMYB30. The main SUMOylation site, K283, is indicated by the asterisk.

special conditions due to the low level of modifications and the rapid deSUMOylation by SUMO-specific proteases. This reconstituted SUMOylation system takes advantage of the lack of SUMO-specific proteases in prokaryotic cells (Uchimura et al. 2004a, Uchimura et al. 2004b). We confirmed efficient SUMOylation in *E. coli* for the evaluation of substrate SUMOylation and biochemical analysis of SUMOylated proteins. No purification was required for the detection of SUMOylation prior to Western blot analysis. Purified SUMOylated proteins could be analyzed by LC-MS/MS to identify SUMOylation sites.

In this system, two Duet bacterial expression system plasmids (Novagen) were used for overexpression of SUMO machinery proteins: the pACYCDuet-1 plasmid which carries the E1 subunits with the chloramphenicol resistance gene as a selection marker and P15A ori as a replication origin, and the pCDFDuet-1 plasmid which carries E2 and one of several SUMO isoforms with a streptomycin resistance gene as a selection marker and CDF ori as a replication origin. To apply this system to additional possible SUMO target proteins, plasmids with other selection markers and other origins can be used. However, the tag sequences for substrate detection must be chosen carefully because lysine residues in some tag sequences can also be SUMOylated. For example, we found that the gluathione *S*-transferase (GST)-tag was SUMOylated at low levels in this system (data not shown).

With the reconstituted SUMOylation system, the function of the SUMO and E1 isoforms was evaluated. Little is known about isoform-specific SUMOylation of *Arabidopsis* SUMO target proteins. Previous studies have demonstrated that mammalian RanGAP (Ran GTPase-activating protein) and budding yeast PCNA (proliferating cell nuclear antigen) can be SUMOylated by AtSUMO1, 2, 3 and 5 in in vitro SUMOylation assays using human and *Arabidopsis* SUMOylation machinery proteins, respectively (Chosed et al. 2006, Colby et al. 2006, Chosed et al. 2007). As for the *Arabidopsis* SUMO target proteins, the number of known substrates is still limited compared with animals (Miura et al. 2005, Colby et al. 2006, Miura et al. 2007, Garcia-Dominguez et al. 2008, Budhiraja et al. 2009). The substrate specificity of SUMO variants has been analyzed with AtMYB30 (Colby et al. 2006) and SUMO target proteins identified by mass spectrometric analysis (Budhiraja et al. 2009) with AtSUMO1/2, and 3, excluding AtSUMO5. Using our in vivo SUMOylation system, AtSUMO5, as well as AtSUMO1, 2 and 3, were shown to modify AtMYB30 (**Fig. 2A, B**), which suggests that AtSUMO5 functions as a protein modifier in planta. Indeed, an AtSUMO5-overexpressing plant was shown to accumulate SUMO conjugates, as were AtSUMO1- and 3-overexpressing plants

(Budhiraja et al. 2009). This in vivo SUMOylation system will provides a tool for testing the isoform specificity of an increasing number of SUMO target proteins.

With regard to the modification pattern, AtSUMO1 and 2, but not AtSUMO3, possess self-SUMOylation sites (Colby et al. 2006), which allow the formation of poly-SUMO chains, similar to human SUMO2/3 and SUMO1, respectively (Saitoh and Hinchey 2000, Tatham et al. 2001). However, no poly-SUMOylation of AtMYB30 and NAF with any of the SUMO isoforms was detected, in contrast to the previous report (Budhiraja et al. 2009). Since we observed poly-SUMOylation of other *Arabidopsis* substrates by AtSUMO1 and 2, but not by AtSUMO3 (data not shown), poly-SUMOylation can be reconstituted in *E. coli* and there may be preferred substrates for poly-SUMOylation. Poly-SUMOylation by AtSUMO5 was not detected, as it has been reported to have lower tendency for chain formation (Budhiraja et al. 2009).

One of the subunits of the E1 heterodimer is encoded by two variants, *AtSAE1a* and *AtSAE1b*. Previously, the E1 activity of AtSAE1b, which is less similar to yeast SAE1 than AtSAE1a, was verified using an in vitro SUMOylation assay (Colby et al. 2006). Budhiraja et al. (2009) demonstrated that both AtSAE1a and AtSAE1b function as E1 enzymes in poly-SUMO chain formation. Here, we observed no SUMO isoform specificity between AtSAE1a and AtSAE1b in substrate SUMOylation, which is consistent with the absence of a direct interaction between SAE1 and SUMOs (Lois and Lima 2005). Since both *AtSAE1a* and *AtSAE1b* are expressed at similar levels in all organs, according to the genevestigator microarray database (http://www.genevestigator.ethz.ch/) (Zimmermann et al. 2004), and the *sae1a* alleles have no abnormal phenotype (Saracco et al. 2007), AtSAE1a and AtSAE1b are likely to function redundantly.

In this study, no isoform-specific modification pattern was observed in AtMYB30 and NAF SUMOylation. However, there may be specific functions of individual SUMO isoforms in planta, which could be determined by isoform-specific C-terminal processing by distinct proteases (Chosed et al. 2006, Colby et al. 2006, Chosed et al. 2007, Conti et al. 2008, Budhiraja et al. 2009) and/or temporal- and spatial-specific distribution of each SUMO isoform. Indeed, stress-induced conjugation of AtSUMO1/2, but not AtSUMO3, has been detected (Kurepa et al. 2003). In humans, HsSUMO4 is expressed in a tissue-specific manner in the kidney and immune tissues, whereas HsSUMO1–HsSUMO3 are constitutively expressed (Guo et al. 2004). Furthermore, many human target proteins are conjugated to distinct SUMO isoforms in vivo (Saitoh and Hinchey 2000, Rosas-Acosta et al. 2005, Vertegaal et al. 2006). Some additional components, such as a SUMO E3 ligase and SUMO proteases, may be required for isoform-specific SUMOylation. Alternatively, isoform-specific SUMOylation may be the result of non-covalent interactions between SUMO isoforms and their target proteins (Meulmeester et al. 2008). Further analyses with additional SUMO target proteins may identify the occurrence of isoform-specific SUMOylation in *Arabidopsis*.

In contrast to the previous prediction, LAF1 was proved not to be SUMOylated in this system (Ballesteros et al. 2001). The previously reported change in the cellular localization of LAF1^{K258R}–GFP (green fluorescent protein) mutant compared with that of wild-type LAF1–GFP was possibly caused by structural change, but not by SUMOylation.

To identify SUMOylation sites in AtMYB30, initial scanning of candidate SUMOylation sites was performed using three independent prediction programs, SUMOplot™, SUMOsp 2.0 and SUMOpre. SUMOylation occurs not only in the SUMOylation consensus motif (ΨKXE), but also in the non-consensus sequences, e.g. 26% (69/268) of the confirmed SUMOylation sites in mammals exist in non-consensus sequences (Seeler and Dejean 2003). With SUMOsp 2.0 and SUMOpre, which were trained with known SUMOylation sites, non-consensus-type SUMOylation sites can be predicted, as well as lysine residues in the SUMOylation consensus motif. In SUMOplot™, substitution of amino acid residues in the consensus sequence with those exhibiting similar hydrophobicity does not influence the potential of a SUMOylation motif. As a result of the computational analyses of AtMYB30 by these three programs, no consistent lysine residues were predicted as potential SUMOylation sites due to the differences in the algorithms on which these programs are based. Consequently, LC-MS/MS analysis was used to identify the major SUMOylation site, K283, in AtMYB30, which was located in a non-consensus sequence and was not predicted as a possible SUMOylation site by any of the three programs. Thus, the prediction programs were not effective tools for the identification of SUMOylation sites in AtMYB30. Analysis of more plant SUMO target proteins will be necessary to evaluate the existence of possible species-specific variation in SUMOylation motifs.

Although multiple SUMOylation sites were identified in AtMYB30, only one signal that corresponds to one SUMO addition to the substrate was observed (**Fig. 4A**). This signal could be a population of the protein substrate SUMOylated at different sites, which suggests that AtMYB30 could be SUMOylated by only one SUMO molecule. Alternatively, AtMYB30 may be initially SUMOylated at K283 prior to the additional SUMOylation at minor sites, which is undetectable by Western blot analysis.

Transcription factors are frequently targets of SUMOylation, which generally inhibits the activity of the factor due to steric hindrance in access to DNA. We examined the effect of a K283R mutation in AtMYB30 on its transcriptional activity using yeast one-hybrid analysis. In this experiment, the transcription activities of AtMYB30 and AtMYB30^{K283R} fused to the GAL4 DNA-binding domain were measured by using the *LacZ* reporter gene under the control of the GAL4

promoter. However, no obvious change in the transcriptional activity in mutated AtMYB30 was observed compared with that in wild-type AtMYB30 (data not shown). In order to verify the consistency of SUMOylation sites in *E. coli* and in plants experimentally, we tried to detect SUMOylation of AtMYB30 in *Arabidopsis*. Since the SUMOylation levels of target proteins in eukaryotic cells are generally very low, we tried to overexpress hemagglutinin (HA)-tagged AtMYB30 and AtMYB30^{K283R} stably in combination with FLAG-tagged AtSUMO1 protein in *Arabidopsis* cultured cells. However, we were not successful in detecting the overexpressed HA-tagged AtMYB30 by Western blot analysis possibly due to the toxicity of excess amounts of this protein (data not shown). Further detailed analysis should be done to reveal the significance of SUMOylation in AtMYB30 in planta.

To understand better the significance of SUMOylation in plants, the identification of SUMOylation sites in target proteins is essential, albeit laborious. LC-MS/MS analysis, in combination with the reconstituted SUMOylation system, is a powerful tool to survey and identify SUMOylation sites in a target protein. Furthermore, the comparison of the SUMOylation patterns among individual SUMO isoforms will provide a new paradigm for SUMO isoform preference in target proteins. This method will enable us to gain further insights into the function of SUMOylation in plants.

Materials and Methods

Plasmid construction

The open reading frames (ORFs) of mature AtSUMO1 (At4g26840), AtSUMO2 (At5g55160), AtSUMO3 (At5g55170) and AtSUMO5 (At2g32765), and the full-length ORFs of AtSAE1a (At4g24940), AtSAE1b (At5g50680), AtSAE2 (At2g21470) and AtSCE1a (At3g57870) were amplified from a MATCHMAKER *Arabidopsis* cDNA library (Clontech, Palo Alto, CA, USA). The mature AtSUMO1, 2, 3 and 5(GG) were generated using primers which introduce stop codons immediately after the sequences coding for the Gly–Gly at the C-terminus. For the generation of constructs which express each of the N-terminal His-tagged AtSUMO isoforms and the C-terminal S-tagged AtSCE1a (pCDFDuet-AtSUMO-AtSCE1), the *Bgl*II/*Xho*I fragment of AtSCE1a was cloned into the multicloning site 2 (MCS2) of the expression vector pCDFDuet-1 (Novagen, Palo Alto, CA, USA). Subsequently, the *Eco*RI/*Sal*I fragments of AtSUMO1, 2, 3 or 5(GG) were cloned into multicloning site 1 (MCS1). For the generation of constructs which express N-terminal His-tagged AtSAE1a (or AtSAE1b) and C-terminal S-tagged AtSAE2 (pACYCDuet-AtSAE1-AtSAE2), the *Bgl*II/*Xho*I fragments of the AtSAE1a or AtSAE1b ORF were cloned into the expression vector pACYCDuet-1 (Novagen), followed by cloning of the *Eco*RI/*Sal*I fragment of AtSAE2 into each construct. As a negative control for SUMOylation, the C-terminal Gly–Gly motif of each SUMO in the pCDFDuet vector was mutated to Ala–Ala by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA). For the additional negative control, Cys94 of AtSCE1a in the pCDFDuet vector was point-mutated to serine by QuikChange site-directed mutagenesis (Stratagene).

To construct the plasmid for substrate expression, the ORF of *AtMYB30* (*At3g28910*), *NAF* (*At2g19480*) and *LAF1* (*At4g25560*) was amplified from a MATCHMAKER *Arabidopsis* cDNA library (Clontech) by primers generating appropriate restriction sites. Subsequently, the digested fragments of *AtMYB30*, *NAF* and *LAF1* were cloned into the expression vector pET28a (Novagen) to express N-terminal His-T7-tagged AtMYB30, NAF and LAF1, respectively. For confirmation of SUMOylation sites, lysine residues at candidate SUMOylation sites were substituted with arginines by QuikChange site-directed mutagenesis (Stratagene).

To confirm expression of each SUMOylation factor, *E. coli* BL21(DE3) cells were transformed with the pCDFDuet-AtSUMO1/2/3/5(AA or GG)-AtSCE1a and pACYCDuet-AtSAE1a/b-AtSAE2 plasmids. Transformants were cultured in 5 ml of Luria–Bertani (LB) medium at 37°C to an OD_{600} of 0.5, followed by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After a 2 h induction, the cells were harvested from 500 µl of cell culture, and 50 µl of 1×SDS–PAGE sample buffer was added to each sample, followed by denaturation at 95°C for 5 min. The overexpressed proteins were detected by Western blot analysis.

Escherichia coli in vivo reconstituted SUMOylation assay

Escherichia coli BL21(DE3) cells were transformed with either pACYCDuet-AtSAE1a-AtSAE2 or pACYCDuet-AtSAE1b-AtSAE2, and were used for the preparation of competent cells. For in vivo SUMOylation reactions, the competent cells were transformed with two plasmids, pET28a-AtMYB30/pET28a-NAF/pET28a- pET28a-LAF1 and pCDFDuet-AtSUMO1/2/3/5(AA or GG)-AtSCE1a. Transformed cells were cultured in 5 ml of LB medium at 37°C until the OD_{600} was 1.0, followed by the addition of 0.2 mM IPTG. After an approximately 12 h induction at 25°C, cells were harvested from 500 µl of cell culture, and 100 µl of 1×SDS–PAGE sample buffer was added, followed by denaturation at 95°C for 5 min.

Western blot analysis

Equal amounts of *E. coli* protein extracts were loaded onto each lane of an SDS–polyacrylamide gel. Electrophoresed proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billeria, MA, USA), and incubated with anti-His (Qiagen, Tokyo, Japan), anti-S-tag [S-protein–horseradish peroxidase (HRP) conjugate, Novagen] or anti-T7 (Novagen) antibodies. Immunoblots were visualized by chemiluminescence (Millipore).

Purification of AtSUMO3-conjugated His-T7-AtMYB30

The SUMOylation reaction was performed in 200 ml of LB medium, as described above. Cells were harvested and AtSUMO3-conjugated His-T7-AtMYB30 was purified using the HisLink Protein Purification Resin (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Analysis of SUMOylated peptides by LC-MS/MS

After purification, in vivo SUMOylated substrate proteins were fractionated by one-dimensional gel electrophoresis. The substrate proteins, with or without SUMOylation, were excised from the gel and were subjected to reduction with 10 mM dithiothreitol (DTT), alkylation with 55 mM iodoacetamide and digestion with 10 μg ml^{-1} modified trypsin (Promega) or 20 μg ml^{-1} GluC (Sigma, St Louis, MO, USA) at 37°C for 16 h. After in-gel digestion, the peptides were extracted with 5% formic acid and 50% acetonitrile, dried under a vacuum, and dissolved in 2% acetonitrile and 0.1% formic acid. The digested peptides were fractionated by C18 reverse-phase chromatography (Paradigm MS4; Microm BioResources, Auburn, CA, USA) coupled directly to a quadrapole ion trap mass spectrometer (Finnigan LTQ; Thermo Fisher Scientific, Waltham, MA, USA) with a Fortis Tip mounted on a three-dimensional stage (AMR, Tokyo, Japan). The ion trap was programmed to carry out three successive scans consisting of: first, a full-scan MS over the range 450–2,000 *m/z*, and second and third, data-dependent scans of the two most abundant ions obtained in the first scan. Automatic MS/MS spectra were obtained from the highest peak in each scan by setting a relative collision energy of 35% and exclusion time of 15 min for molecules in the same *m/z* value range. The molecular masses of the resulting peptides were searched against the non-redundant NCBI database using the MASCOT program with an additional mass corresponding to the conjugated SUMO peptide.

Supplementary data

Supplementary data are available at PCP online.

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