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Short title: *Arabidopsis* SUMO isoforms

Synopsis

Protein modification by SUMO has emerged as an essential regulatory mechanism in eukaryotes. Even though the molecular mechanisms of SUMO conjugation/ deconjugation are conserved, the number of SUMO machinery components and their conservation degree are specific to each organism. Here, we present data contributing to the notion that the four expressed *Arabidopsis* SUMO paralogs, AtSUMO1, 2, 3 and 5, have functionally diverged to a higher extent than their human orthologs. We have explored the conservation degree of these paralogs and found that the surfaces involved in E1 activating enzyme recognition and E2 conjugating enzyme and SIM (SUMO Interacting Motif) non-covalent interactions are well conserved in AtSUMO1/2 isoforms, whereas AtSUMO3 shows a lower conservation degree and AtSUMO5 is the most divergent isoform. These differences are functionally relevant since AtSUMO3 and 5 are deficient in establishing E2 non-covalent interactions, which has not been reported for any naturally occurring SUMO ortholog. In addition, AtSUMO3 is less efficiently conjugated than AtSUMO1/2 and AtSUMO5 shows the lowest conjugation level. A mutagenesis analysis revealed that decreases in conjugation rate, and thioester bond formation, are the result of the non-conserved residues involved in E1 activating enzyme recognition that are present in AtSUMO3 and 5. Our results support a role for the E1 activating enzyme in SUMO paralog discrimination, providing a new mechanism to favor conjugation of the essential AtSUMO1/2 paralogs.

Keywords

SUMO, non-covalent interactions, conjugation rate, E1 activating enzyme, E2 conjugating enzyme, polySUMO chains

Abbreviations footnote

SUMO, Small Ubiquitin-like MOdifier; SAE, SUMO Activating Enzyme; SCE, SUMO Conjugating Enzyme; At, *Arabidopsis*, Hs, Homo sapiens; y, yeast

Introduction

In plants, regulation of protein activity by SUMO (Small Ubiquitin-like Modifier) attachment is a posttranslational modification that has been shown to be essential during seed development and to have a major role in abiotic and biotic stress responses [1]. A common property between plants and animals is that the SUMOylation system appears to be a target for pathogenic effectors [2-5], as well as the accumulation of SUMO conjugates in response to heat and oxidative stresses [6, 7]. But there are also biological processes specific to plants in which SUMO has a relevant role such as flowering [8], phosphate starvation [9], drought responses [10], and abscisic acid signaling pathway [11, 12], hormone that mediates plant responses to environmental stresses and a key regulator of plant growth and development.

SUMO is covalently attached to target proteins by the sequential action of E1 activating, E2 conjugating and E3 ligase enzymes [13]. SUMO activation is mediated by a heterodimeric enzyme consisting in a large subunit, SAE2, and a small subunit, SAE1. The SAE2 subunit contains the adenylation, catalytic cysteine, ubiquitin fold (UFD) and C-terminal functional domains [14]. The adenylation domain is responsible for SUMO recognition and adenylation of SUMO C-terminus. After adenylation, the catalytic cysteine sulphydryl attacks the SUMO C-terminal adenylate resulting in the formation of a thioester bond between the E1 and SUMO, in a mechanism that involves a rotation of the Cys domain [15]. At this stage, SUMO

can be transferred to the E2-conjugating enzyme. SUMO-charged E2 is competent to transfer SUMO to the target lysine in the substrate, although this reaction is facilitated by E3 ligases enzymes *in vitro* and *in vivo* [16, 17].

During conjugation, SUMO molecules establish non-covalent interactions with the E1-activating and the E2-conjugating enzymes. Structural studies identified eleven residues in the human SUMO surface that establish contacts with the E1 activating enzyme and which, presumably, are responsible for conferring modifier specificity [14]. SUMO can be attached to the target protein as a monomer or polymer, and polySUMO chains have been shown to act as signals to promote ubiquitination of the SUMO-modified substrate targeting it for proteasomal degradation [18]. SUMO chain growth is dependent on the presence of a sumoylation consensus site at the SUMO N-terminal tail, and polymerization is facilitated by non-covalent interactions between SUMO and the E2-conjugating enzyme [19, 20]. *In vitro*, the chain length is modulated by the relative abundance of HsSUMO2/3, which can build SUMO chains, versus HsSUMO1, which does not have a SUMOylation consensus site and that could function as a chain terminator [21].

The consequences of covalent SUMO attachment to target proteins are very variable and include regulation of subcellular localization, protein activity and stability, and protein-protein interactions. At the molecular level, SUMOylation outcome is achieved through the interaction with specific effectors that contain a SUMO interaction motif (SIM). Most SIMs consist of a hydrophobic core of 3-4 aliphatic residues flanked by acidic residues [22, 23]. Structural and functional studies determined that a hydrophobic groove surrounded by basic residues in SUMO is crucial for SIM interaction. Interestingly, the relative position between the hydrophobic groove and the basic residues differ among human SUMO isoforms and this could confer SUMO-SIM interaction specificity [24, 25]. During conjugation, SUMO paralog selection can be mediated by SIM-dependent recruitment of targets to SUMO thioester-charged E2 and/or SUMO modified E2 [26, 27]. Moreover, SIMs have been identified in E3 ligases and shown to regulate ligase activity and localization [28-30].

In plants, much less is known about the molecular mechanisms that regulate sumoylation and the complexity of the sumoylation components is apparently higher when compared to other organisms. In *Arabidopsis*, expression has been detected for the four SUMO paralogs AtSUMO1, AtSUMO2, AtSUMO3 and AtSUMO5, being AtSUMO1 and 2 the most closely related isoforms. Previous studies showed that these SUMO paralogs do not serve as equivalent substrates of *Arabidopsis* SUMO proteases, referred as ULP. The four proteases AtULP1a, c, d and AtESD4 displayed similar peptidase and isopeptidase activities towards AtSUMO1 and AtSUMO2 isoforms, although none of them showed a significant activity towards AtSUMO5. Only AtULP1a exhibited a poor peptidase activity towards AtSUMO3 and no isopeptidase activity at all [31-33]. In addition, in non-quantitative assays, only AtSUMO1, 2 and 3 were shown to be conjugated to the yeast substrate PCNA and, the capacity to form polymeric chains was displayed exclusively by AtSUMO1 and AtSUMO2 [32]. At first glance, it seemed that the situation of the *Arabidopsis* SUMO1 and 2 isoforms resembles that of the human SUMO2 and 3 isoforms according to their capacity to form polymeric chains, versus the human SUMO1 and the *Arabidopsis* SUMO3 isoforms that are conjugated as monomers. But, in contrast, homology studies failed to cluster human and *Arabidopsis* isoforms according to their ability or inability to polymerize [11, 32]. In addition,

AtSUMO3 and 5 are not capable to complement the lethal double mutant *atsumo1atsumo2* plants [34], whereas SUMO1 knockout mice are viable. These results suggest that mammalian SUMO1 and SUMO2/3 have partially redundant functions [35, 36], in contrast to *Arabidopsis* SUMO paralogs that seem to have developed more divergent functions [34, 37].

To gain new insights into the complex *Arabidopsis* sumoylation system, we have assessed whether AtSUMO1, 2, 3 and 5 have distinct molecular properties that might influence their *in vivo* conjugation and biological function. We have found that *Arabidopsis* SUMO isoforms have heterogeneous properties at different molecular levels. AtSUMO1/2 were competent to interact non-covalently with their cognate E2-conjugating enzyme AtSCE1, whereas AtSUMO3 and AtSUMO5 did not conserve this property. Mutagenesis analysis revealed that the single residue Asp-63, conserved in the AtSUMO1/2 surface but not in AtSUMO3 and AtSUMO5, is essential for non-covalent interactions with E2 and that it is necessary for polySUMO chain formation. It is even more significant the fact that SUMO isoforms differed in their conjugation rate, being AtSUMO1/2 the most efficiently conjugated paralogs, AtSUMO3 was less efficiently conjugated and AtSUMO5 showed the lowest conjugation level. A mutagenesis analysis showed that the lower conjugation rate of AtSUMO3 and AtSUMO5 were related with changes in SUMO residues involved in E1 interaction, which also affected thioester bond formation. These results suggest that the first step in the SUMO conjugation cascade would have a regulatory role as SUMO paralog discrimination. Overall, our results suggest that AtSUMO1/2 might be the most efficiently conjugated SUMO isoforms *in vivo* and we postulate that this could constitute a molecular mechanism to assure conjugation of the essential AtSUMO1/2 paralogs versus the non-essential AtSUMO3 and 5.

EXPERIMENTAL

Cloning, expression and protein purification. Arabidopsis SUMO isoforms, AtSAE2, AtSAE1a/b, AtCAT3 were amplified by PCR from cDNA obtained from 2-week old plants grown on MS plates under LD at 22ºC (Superscript®III Reverse Transcriptase from Invitrogen and Pfu DNA polymerase from Stratagene). AtSAE1a/b were cloned into pET15b (Novagen) to encode a native polypeptide and, AtSAE2, AtSCE1[11], AtSUMO1 (1-93), AtSUMO2 (1-92), AtSUMO3 (1-93) and AtSUMO5 (1-103) were cloned into pET28a (Novagen) to encode an N-terminal hexahistidine fusion protein. AtCAT3Ct (419-472) was clone into pGEX-6P1 (Amersham) to encode an N-terminal GST fusion. Plasmids were transformed individually, or co-transformed in the case of AtSAE2 and AtSAE1a/b containing plasmids, into *E. coli* strain BL21 Codon Plus RIL (Stratagene). Cultures (1-4 L) were incubated at 37°C until they reached an A_{600} of 0.6-0.8 and protein expression induced by adding 0.1 mM IPTG for 4 h at 30ºC. Cells were harvested and resuspended in 50 mM Tris–HCl (pH 8.0), 20% w/v sucrose, 350 mM NaCl, 20 mM imidazole, 0.1% NP40, 1 mM PMSF, 1 mM β-mercaptoethanol (BME), 1 µg/mL leupeptin, 1 µg/mL pepstatin and 50 µg/mL DNAse. Protein extracts were prepared by sonication and clarified by centrifugation. Purification via IMAC-sepharose resin (GE Healthcare) or glutathione-sepharose (GE Healthcare) was performed according to manufacturer's recommendations. SDS-PAGE analysis of the purified proteins is shown in Supplementary Fig. 2.

Yeast Two-Hybrid Experiments. Yeast expression constructs pGBKT7:AtSCE1/ AtUBC10 and pGADT7:AtSUMO1 (1-93)/ AtUBI were previously generated [11]. AtSUMO3 (1-93) and AtSUMO5 (1-103) were cloned into pGADT7 AD (Clontech) to encode an N-terminal

GAL4 activation domain fusion. AtSUMO1 and AtSUMO3 mutant alleles were generated by QuickChange Site-Directed mutagenesis (Stratagene). Plasmids, as indicated, were co-transformed into the yeast strain HF7c by the lithium acetate method as described in the Clontech Yeast Protocols Handbook. Transformed yeast culture was plated on to permissive SD medium complemented with histidine. A single clone per transformation was selected, disaggregated by vortex agitation in SD medium without amino acids and serial dilutions performed (1, 1/8, 1/32 and 1/64). 5 µL aliquots of each dilution were sowed on non-selective (SD medium complemented with histidine) or selective (SD medium not complemented with histidine) plates. After 2-day incubations at 30ºC, protein interactions were analyzed using His auxotrophy as a selective marker.

In vitro pull-down assay. 100 µM His:AtSCE1 and 25 µM of AtSUMO1/D63N were incubated in 40 µL of binding buffer (20 mM Tris pH 8.0, 50 mM NaCl and 20 mM imidazol) for 5 hours at 4ºC. Next, 10 µL of Ni^{2+}-IMAC-sepharose resin were added to the binding mixture and incubated for 30 minutes at 4ºC. The binding mixture was transferred to micro bio-spin chromatography columns (BIO-RAD, 732-6203) and the resin washed four times with 20 µL of binding buffer. The proteins bound to the resin were eluted with 20 µL of binding buffer containing 300 mM imidazol. 0.8 µL and 3.5 µL of the input and eluate fractions, respectively, were separated by SDS-PAGE and either stained with Coomasie fluor orange (Molecular Probes C-33250) or subjected to immunoblot analysis with anti-SUMO1 antibodies, as indicated.

Polyclonal anti-AtSUMO1 antibodies production. Polyclonal antibodies were raised against purified N-terminal hexahistidine fusion AtSUMO1 (1-93). 1 mg of purified protein was resolved by SDS-PAGE, the gel stained with Coomasie Blue and the gel slice containing His-AtSUMO1 used to immunize rabbits (Cocalico Biological, Reamstown, PA). A 1/1000 dilution of the serum produced was used in the immunoblot analyses.

In Vitro SUMO conjugation, polymeric chain formation and E1-thioester assays. In conjugation assays, we used the C-terminal tail of the Arabidopsis catalase 3 (419-472) fused to GST, GST:AtCAT3Ct. Reactions were carried out at the indicated temperatures in 25 µL reaction mixtures containing 1 mM ATP, 50 mM NaCl, 20 mM Hepes, pH 7.5, 0.1% Tween 20, 5 mM $MgCl_2$, 0.1 mM DTT, 2 µM SUMO, 0.5 µM AtSAE2/AtSAE1a, 0.5 µM AtSCE1 and 5 µM GST-AtCAT3Ct. After the specified incubation time, reactions were stopped by the addition of protein-loading buffer, boiled for 5 min and 10 µL aliquots were resolved by SDS-PAGE. Polymeric chain formation reactions were performed at 37º C in the same reaction buffer as SUMO conjugation assays and in the presence of 100 µM AtSUMO1, 1 µM AtSAE2/AtSAE1a and 10 µM AtSCE1. Reaction products were detected by immunoblot analysis with anti-GST polyclonal antibodies (SIGMA, G7781) or with anti-AtSUMO1 polyclonal antibodies, as indicated. E1-thioester assays were performed at 30 ºC in 50 µL reaction mixtures containing 1 mM ATP, 50 mM NaCl, 20 mM Hepes, pH 7.5, 0.1% Tween 20, 5 mM $MgCl_2$, 0.1 mM DTT, 10 µM SUMO and 5 µM AtSAE2/AtSAE1a. At the indicated time points, 15 µL aliquots were removed and analyzed by SDS-PAGE followed by Coomasie Fluor Orange staining according to manufacturer's indications (Molecular Probes C-33250). As a thioester bond formation control, an aliquot of each raection was treated with 100 mM DTT previously to loading into polyacrylamide gel.

SUMO conjugation rate quantification. Reaction products were detected by using ECL plus western blot detection reagents (GE healthcare) and signal acquired with LAS-3000 imaging system and quantified with Multi Gauge V3.0 (Fujifilm). Signals were normalized against known amounts of GST included in each blot. When data is represented by relative units, SUMO conjugation or E1-thioester rates are referred to the rate average calculated using all the rates obtained in each independent experiment.

Bioinformatics. Sequence alignments were performed using the protein multiple alignment software MUSCLE [38] and alignments edited with GeneDoc software [39]. Protein structure models were generated by using the SWISS-MODEL workspace [40] on automated mode. AtSUMO1/3/5 and AtSCE1 models were generated by using 2PE6 (2.40 Å) or 2IY1D (2.46 Å) as templates. Models were assembled and images generated using PyMOL [41].

Accession numbers. Assigned accession numbers for the studied genes are as follows: At4g26840 (AtSUMO1), At5g55160 (AtSUMO2), At5g55170 (AtSUMO3), At2g32765 (AtSUMO5), At2g21470 (AtSAE2), At4g24940 (AtSAE1a), At5g50580 (AtSAE1b), At3g57870 (AtSCE1), At4g02890 (AtUBI), At5g53300 (AtUBC10), At1g20620 (AtCAT3).

RESULTS

Conservation of E1, E2 and SIM-interacting residues among Arabidopsis SUMO paralogs. The overall homology degree among SUMO paralogs ranges from an 83% of sequence identity between AtSUMO1 and AtSUMO2, to a 42% and 30% of sequence identity between AtSUMO1 and AtSUMO3 or AtSUMO5, respectively. Interestingly, these differences are also present in the conservation degree found between residues involved in E1, and E2 non-covalent interactions. AtSUMO1 and AtSUMO2 have identical amino acid residues at the positions involved in non-covalent interactions with the E1-activating and E2-conjugating enzymes. On the contrary, AtSUMO3 and AtSUMO5 have more divergent interacting surfaces. AtSUMO3 has a 55% and a 75% conservation degree of E1 and E2-interacting residues, respectively, whereas AtSUMO5 is the most divergent isoform showing a 36% and a 56% of E1 and E2-interacting residues conservation (Figure 1A and B; supplementary Figure 1). Previous structural studies identified eleven residues in the HsSUMO1 surface involved in E1 interactions [14]. Taken as reference positions in AtSUMO1, the four residues Gln-25, Gly-27, Gly-92 and Gly-93 are identical in all Arabidopsis paralogs. Among the others, two are divergent only in AtSUMO5 isoform, Arg-66 and Asp-85, and five are not conserved in both AtSUMO3 and 5, Asn-56, Met-87, His-89, Gln-90 and Thr-91 (Figure 1A and 5A). All of them are identical between AtSUMO1 and 2. Regarding the residues involved in E2 non-covalent interactions, those residues establishing lateral chain contacts with the E2 conjugating enzyme are identical across *Arabidopsis* AtSUMO1/2, human HsSUMO2/3 and yeast ySmt3, except for the residue Met-87 in AtSUMO1 that is highly variable (Figure 1B and 2A). The deduced consensus motif considering the most frequent residues would be Asp/Glu-63, Glu-79, Asp-82 and Asp/Glu-85 (residue position is referred to AtSUMO1). When we analyzed AtSUMO3, it is remarkable that the acidic Asp/Glu-63 residue in the consensus is changed by the polar asparagine. This change is also present in the most divergent SUMO isoform AtSUMO5 that, in addition, has substitutions in Asp/Glu-77 and Asp/Glu-85 for histidine and cysteine, respectively (Figure 1B and 2A).

Another important interacting surface on SUMO involves the second β sheet and the downstream α helix, which form a hydrophobic groove flanked by basic residues that accommodates SUMO Interacting Motifs or SIMs [42]. Structural studies determined that aliphatic and aromatic residues constitute this hydrophobic groove, HsSUMO1 Ile-34, His-35, Phe-36, Val-38, Leu-47 and Tyr-51 [23]. Whereas functional studies identified residues required for the role of HsSUMO2 in transcriptional inhibition, which include the hydrophobic Val-30 and Ile-34 (equivalent to Ile-34 and Val-38 in HsSUMO1), the polar Thr-38 (Thr-42 in HsSUMO1), and the four basic residues Lys-33, Lys-35, Lys-42 and Arg-50 (Lys-37, Lys-39, Lys-46 and Arg-54 in HsSUMO1) [24]. Among the basic residues, HsSUMO1 Lys-39 has been proposed to interact with phosphorylated residues located next to the hydrophobic core that is the essential component of SIMs [22]. The previous functional amino acids identified in human SUMO1 and SUMO2 are conserved in AtSUMO1/2, suggesting that these paralogs will share the molecular basis for SIM interaction as their human orthologs. On the contrary, major changes are present in AtSUMO3 and AtSUMO5. The hydrophobic Val-30 and Ile-34 residues shown to be necessary for transcriptional repression in human SUMO2 are changed by an acidic residue in AtSUMO5 (Asp-40) and a polar residue (Asn-34) in AtSUMO3, respectively. In addition, the polar Thr-38 in human SUMO2, which is also necessary for transcriptional repression, is substituted by the hydrophobic alanine in both AtSUMO3 and AtSUMO5 (positions 38 and 48, respectively). Finally, the basic Lys-39 in human SUMO1 proposed to interact with phosphorylated residues in the target is substituted by an uncharged glycine residue in AtSUMO5 (Figure 1C). Considering that no SIM containing targets have been identified in *Arabidopsis*, we have focused in the functional analysis of E1 and E2 non-covalent interacting residues in *Arabidopsis* SUMO paralogs according to their role in conjugation.

E2 non-covalent interactions with SUMO isoforms. In order to assess the effect of changes in residues involved in SUMO-E2 non-covalent interactions, we performed yeast two-hybrid assays. In these experiments, the capacity of the yeast strain HF7c to grow in the absence of histidine was used as a marker for the interaction between proteins. Previous studies showed that AtSUMO1 and 2 were capable to interact with AtSCE1 in similar assays [11]. We found that histidine auxotrophy was restored only when AtSCE1a was co-transformed with AtSUMO1, but not with AtSUMO3 or AtSUMO5. In these experiments, AtSUMO1 was used as a positive control and *Arabidopsis* ubiquitin as a negative control. When AtSCE1 was replaced by the ubiquitin conjugating enzyme AtUBC10, we observed that histidine auxotrophy was conferred only when ubiquitin was co-expressed, consistently with the specificity of the system (Figure 2B). Our results demonstrate that AtSUMO3/5 are not competent to interact with AtSCE1, suggesting the existence of a change in the SUMO interacting surface that might be common to both paralogs. As described above, the central aspartate in AtSUMO1 and 2 (Asp-63 and Asp-62) is replaced by an asparagine at the equivalent position in AtSUMO3 and 5 (Asn-63 and Asn-73) (Figure 1B and 2A). To test the role of this divergent residue, we generated the mutants AtSUMO1 Asp-63Asn and AtSUMO3 Asn-63Asp and assessed their capacity to interact with AtSCE1. None of these mutant isoforms interacted with AtSCE1 in yeast two-hybrid assays (Figure 2C), indicating that the presence of aspartate residue at position 63 is essential but not sufficient for SUMO-E2 non-covalent interactions. To further evaluate the essential role of Asp-63 in non-covalent interactions with E2, we performed in vitro pull-down assays. In these assays, His:AtSCE1

was incubated in the presence of AtSUMO1 or AtSUMO1 Asp-63Asn. After binding to a Ni^{2+}-charged resin, His:AtSCE1 was eluted with imidazol and we observed that AtSUMO1 was co-eluted with His:AtSCE1. On the contrary, a small amount of AtSUMO1 Asp-63Asn was present in the elution fraction to the same extent as in the negative control, where His-AtSCE1 was omitted.

Identification and validation of Arabidopsis catalase 3 as a bona fide SUMO target in vitro. In order to reconstitute a complete *Arabidopsis* sumoylation system that allowed us the biochemical characterization of the SUMO paralogs, we aimed to identify an endogenous SUMO target. Since SUMOylation is involved in oxidative stress responses, we analyzed whether oxidative stress scavengers could be SUMO targets. We found that the isoform 3 of the *Arabidopsis* catalase contained a SUMOylation consensus site at its C-terminal domain (Supplementary Figure 3A). Previous studies determined that SUMO conjugation sites are located in an extended structure on the surface of the target protein in order to be accessible to the sumoylation machinery [43]. To determine the position of the putative AtCAT3 sumoylation site on the quaternary structure we performed the AtCAT3 structure prediction by using the *Exiguobacterium oxidotolerans* catalase structure as a template (2j2mC). The generated model indicated that the putative SUMO acceptor lysine, K423, in AtCAT3 is fully exposed in the protein surface (Supplementary Figure 3B and C). Validation of AtCAT3 as a SUMO substrate was performed by *in vitro* sumoylation reactions containing the AtCAT3 C-terminal domain, which includes the predicted sumoylation site (GST:AtCAT3Ct; Supplementary Figure 3D), in the presence of the reconstituted *Arabidopsis* sumoylation system, AtSAE2, AtSAE1a, AtSCE1 and AtSUMO1. As a result, we detected SUMO conjugation to AtCAT3Ct in an ATP dependent manner. In addition, the mutant AtCAT3Ct K423R was unable to accept SUMO (Figure 3E). These results validate AtCAT3 as a SUMO target and identify K423 as the acceptor site for SUMO modification. The advantage of using AtCAT3Ct as a substrate for *in vitro* reactions as opposed to other targets described in the literature is that it does not require the presence of an E3 ligase in order to be modified, which simplifies the biochemical analysis of SUMO conjugation.

AtSUMO1 Asp-63 is necessary for efficient polySUMO chain formation. Since AtSUMO1 Asp-63Asn prevented AtSCE1 non-covalent interactions, we tested whether this mutation affected polySUMO chain formation. *In vitro* polySUMO chain formation assays were performed in the presence of AtSAE2/AtSAE1a, AtSCE1 and the native or mutated SUMO form. Under these conditions, native AtSUMO1 isoform built efficiently polymeric chains and it was also conjugated to AtSCE1, on which polySUMO chains were also formed. When Asp-63 was substituted by Asn, reduction of polySUMO chain formation was observed. This defect was more evident from the second conjugation cycle, independently on whether polySUMO chains *sensu stricto* or polySUMO chains built on AtSCE1 were analyzed (Figure 3A and B). These results indicate that the mechanism to build polySUMO chains is conserved in *Arabidopsis* and that, presumably, the naturally occurring E2-non interacting SUMO isoforms, AtSUMO3 and AtSUMO5, will not interfere with polyAtSUMO1/2 chain formation *in vivo*. Consistently with a main role of SUMO-E2 non-covalent interactions in polySUMO chain formation, the mutant AtSUMO1 Asp-63Asn was conjugated to the target AtCAT3Ct with the same efficiency as the native AtSUMO1 (Figure 3C).

SUMO isoforms display distinct conjugation rate in vitro. Since *Arabidopsis* SUMO isoforms show differences in their capacity to establish non-covalent interactions with their cognate E2 conjugating enzyme, we explored the possibility that they could also differ in their conjugation rates. First, we chose a short incubation time, 10 minutes, in order to compare the first conjugation cycle. In this way, we avoided conjugation rate underestimation of the isoforms competent for polySUMO chain formation. We were also interested in analyzing the effect of the incubation temperature on conjugation rate, which could be biologically relevant since SUMO conjugates accumulate massively upon heat stress. Reactions were incubated at 22, 37, 42 and 48 ºC and we observed that the highest reaction rate was achieved at 42 ºC (Figure 4A). Under these experimental conditions, we did not observed conjugation of AtSUMO5. All other isoforms were conjugated to AtCAT3 at different rates. In general, AtSUMO1 and 2 were better conjugated than AtSUMO3, being the highest difference observed at 42 ºC. At this temperature, AtSUMO1 and AtSUMO2 conjugation rate is 2.4 and 3.2-fold higher than AtSUMO3, respectively (Figure 4B). Next, we performed a time course analysis that allowed us to detect AtSUMO5 conjugation after 60 minutes incubation at 37 ºC. At 42 ºC, AtSUMO5 conjugation was very weak and differences in conjugation level between AtSUMO1 and AtSUMO5, as well as between AtSUMO1 and AtSUMO3, were more pronounced than at 37 ºC (Figure 4C).

Non-conserved residues involved in E1 interaction are responsible for differences in SUMO paralogs conjugation rate. As it was described above, SUMO residues involved in E1 recognition are not evenly conserved between *Arabidopsis* SUMO isoforms. In order to evaluate the effect of these changes in conjugation rate we focused in those positions that were not conserved only in the less conjugated isoform AtSUMO5, which correspond to AtSUMO1 Arg-66 and Asp-85 residues. We also focused in AtSUMO1 His-89 that is not conserved neither in AtSUMO3 nor AtSUMO5 and whose equivalent position in Nedd8 was previously reported to confer modifier specificity [44]. We mutated AtSUMO1 Arg-66 and Asp-85 and His-89 residues to those present in AtSUMO3 and AtSUMO5 at the equivalent positions and analyzed their conjugation rate (Figure 5B and C). At 42 ºC, the most dramatic effect was observed in Asp-85 Cys followed by His-89 Ala and Arg-66 Glu mutants, which showed a 24%, 40% and 50% of the native AtSUMO1 rate, respectively. The His-89 Glu substitution had a minor effect and showed a 90% of the native AtSUMO1 activity. Similarly to what we observed during the conjugation analysis of SUMO isoforms, at 37 ºC differences in conjugation levels were minor, although their behavior was similar and Asp-85 Cys mutation was the most affected. These mutants, with the exception of the His-89 Glu substitution, also showed SUMO-E1 thioester bond formation defects, being the Asp-85 Cys mutation the most affected. These results suggest that conjugation level reduction of these mutants was the result of E1 interaction defects.

DISCUSSION

The molecular consequences of protein modification by SUMO will be dependent on the specific SUMO paralog that is attached to the target, and major effort has been put into elucidate the mechanisms involved in SUMO paralog specificity. Among these mechanisms, ULP mediated deconjugation and SIM mediated conjugation have been proposed to facilitate SUMO paralog selection [45]. Here, we present data supporting a role of the E1 activating

enzyme in SUMO paralog discrimination in *Arabidopsis*. The analysis of the molecular properties of the four expressed *Arabidopsis* SUMO isoforms indicates that they have diverged in a higher degree than their human orthologs, being the essential AtSUMO1 and AtSUMO2 the most functionally conserved isoforms.

Non-covalent interactions between SUMO and its cognate E2 conjugating enzyme is an intrinsic property of the system. Initial studies showed that this property is conserved in AtSUMO1 and 2 [11] but, surprisingly, our results showed that AtSUMO3 and 5 do not retain the capacity to interact with their cognate E2, AtSCE1. This situation is unique to the *Arabidopsis* system since all human SUMO isoforms and yeast Smt3 interact efficiently with their cognate E2 conjugating enzyme. We have also identified AtSUMO1 Asp-63 as an essential residue for establishing E2 non-covalent interactions. But the fact that AtSUMO3 Asn-63 substitution by Asp did not confer competence for this interaction suggests that the inability of AtSUMO3/5 to interact with AtSCE1 could be the result of two types of amino acid changes: the loss of essential residues and the appearance of residues that would be detrimental for this interaction. SUMO-E2 non-covalent interactions have been proposed to be involved in SUMO chain formation of human SUMO2 and 3 isoforms, which also contain a SUMO attachment site in their N-terminal tail [19, 20]. In this model, human SUMO1 would function as a chain terminator since it does not comprise an acceptor lysine for another SUMO molecule, while it retains the capacity to interact non-covalently with E2 enzyme. In *Arabidopsis*, a sumoylation consensus site is only present in those paralogs capable to interact non-covalently with AtSCE1, which are AtSUMO1/2, correlating with their ability to form polymeric chains. On the other hand, considering their inability to interact with AtSCE1, it is not clear whether AtSUMO3/5 would have a role as polyAtSUMO1/2 chain terminator, as it has been proposed for the human SUMO1 isoform, suggesting that other molecular mechanisms might regulate polySUMO chain length in *Arabidopsis*. Supporting this, recent proteomic studies have failed to identify AtSUMO3/5 peptides in purified AtSUMO1 conjugates [46].

Another remaining question to be addressed was whether *Arabidopsis* SUMO paralogs also differed in their conjugation levels. In addition, since SUMO conjugates accumulate dramatically upon heat stress, we were interested in studying the effect of the temperature in conjugation reactions. In previously reported assays, experiments were designed in such a manner that non-quantitative results were obtained (in most cases incubation time ranged from few hours to overnight) [32, 47]. Our results, using a quantitative sumoylation assay, showed that AtSUMO1 and 2 isoforms were more efficiently conjugated in comparison to AtSUMO3, whereas AtSUMO5 showed the lowest conjugation level. For the tested SUMO isoforms, with the exception of AtSUMO5, conjugation rate increased with temperature and it was striking to observe that the highest activity occurred at 42 ºC. Even tough this temperature is higher than standard environmental conditions, it highlights the robustness of the SUMO conjugation system. At the same time, it suggests that the massive and rapid SUMO conjugation observed in plants upon heat shock treatments could be mediated, at least in part, by the increasing activity of the conjugation system with temperature. In addition, this effect is more pronounced in the case of AtSUMO1 and AtSUMO2 conjugation, which are the isoforms that are greatly conjugated under heat stress.

Differences in conjugation efficiency among SUMO paralogs are also specific to the *Arabidopsis* sumoylation system since all three human SUMO isoforms have been shown to form E1 and E2-thioester bonds, and be conjugated to the substrate RanGAP with the same efficiency [14], suggesting that, in this case, SUMO paralog selection will be depending on availability or SIM-mediated interactions. The conservation analysis of the eleven SUMO residues involved in E1 adenylation domain interaction [14] showed that 5 and 7 residues are not conserved in AtSUMO3 and AtSUMO5, respectively, suggesting that adenylation could be deficient for these paralogs. A mutagenesis analysis revealed that some of these substitutions were responsible for a lower conjugation rate, although the effect was depending on the nature of the substitution. The most deleterious was the Asp-85 Cys substitution present in AtSUMO5, supporting the previous result indicating that AtSUMO5 was the less conjugated isoform. Interestingly, this position in the human SUMO1, Glu-89, was previously shown to be crucial for E1 thioester formation [19], supporting our results pointing to a critical role of this residue in SUMO-E1 recognition. As to AtSUMO3, the only tested substitution occurs at a position previously proposed to contribute to modifier discrimination [44] and, when introduced in AtSUMO1, it reduced the conjugation rate to a value equivalent to that of AtSUMO3, suggesting that this substitution has a major contribution in AtSUMO3 conjugation rate reduction. Conjugation defects in these mutants were more pronounced when reactions were incubated at higher temperatures, similarly to what we observed when comparing SUMO paralogs. Moreover, the reduction in conjugation correlated with a reduction in E1-thioester formation, supporting a function of the tested residues in SUMO recognition by the E1. As to our knowledge, this natural occurring SUMO paralog discrimination by the E1 activating enzyme has not been reported previously in any other system.

Among the different studies that have addressed the characterization of *Arabidopsis* sumoylation system, *in vitro* AtSUMO5 conjugation has only been detected to a mammalian substrate, RanGAP, in the presence of the mammalian conjugation system [31]. It is well established that SUMO conjugation is highly regulated by specific protein–protein interactions and using heterologous systems to test SUMO conjugation might give results that could be not relevant *in vivo*. This could explain that AtSUMO5 conjugation was facilitated by the SUMO mammalian system but when we have used the *Arabidopsis* system, which presumably is more selective, AtSUMO5 was very poorly conjugated. Similarly, a previous report showed that human SUMO1 was able to interact with the *Arabidopsis* ubiquitin conjugating enzyme AtUBC10 in yeast two-hybrid assays. On the contrary, in the same assays, neither AtSUMO1 nor AtSUMO2 interacted with AtUBC10, suggesting that selective interactions within the SUMO pathway are more permissive when heterologous sumoylation components are used [11], and highlighting the importance of using homologous systems in biochemical studies.

In vivo, different evidences point to a predominant role for AtSUMO1/2 paralogs. Early studies showed that endogenous AtSUMO1 and 2 [7, 11] and AtSUMO3 [7] were conjugated *in planta*, although AtSUMO3 conjugate levels were lower than for AtSUMO1/2. Instead, endogenous AtSUMO5 conjugation has not been observed. In addition, according to Genevestigator database, AtSUMO3 and AtSUMO5 mRNA levels are in average 10-fold lower than AtSUMO1/2. Furthermore, none of the identified SUMO specific proteases are competent to process AtSUMO5 and only AtULP1a displays an inefficient peptidase activity

towards AtSUMO3, suggesting that, even if expressed at lower levels, it would have to be determined which fraction is present in its mature/conjugable form [31, 32]. Finally, the fact that double *atsumo1atsumo2* mutant plants are not viable [34] suggests a biological specialization and, regardless of AtSUMO3 and 5 *in vivo* function, it seems clear that they can not compensate AtSUMO1/2 loss. A recent study suggests that AtSUMO3 function might differ from AtSUMO1 and AtSUMO2 in flowering and salicylic acid dependent responses, although homozygous *atsumo3* mutant plants showed normal plant development [48]. Interestingly, a more recent report described the identification of proteins that are specifically conjugated by AtSUMO3 and not AtSUMO1, although the molecular mechanism of this specificity remains elusive. [49]. Surprisingly, almost half of the substrates analyzed were exclusively modified by AtSUMO3 in contrast to the low levels of AtSUMO3 conjugates previously detected in crude plant extracts.

Overall, different molecular mechanisms seem to converge in order to assure a proper conjugation of the essential *Arabidopsis* SUMO1/2 isoforms versus the non-essential AtSUMO3/5. These mechanisms comprise regulation of expression levels, maturation and release from targets, being AtSUMO1/2 the highest expressed isoforms and the most efficient substrates of the characterized endogenous proteases [31, 32], suggesting that most of the endogenous pool of mature SUMO will be constituted by these two isoforms. Here, we provide evidence for the existence of a preferential conjugation of AtSUMO1/2 versus AtSUMO3/5, which is determined by a role of the E1 activating enzyme in SUMO paralog discrimination.

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Figure 1. Conservation analysis of residues on Arabidopsis SUMO paralog surfaces involved in non-covalent interactions. Arabidopsis SUMO paralog structures, as predicted by the SWISS-MODEL comparative protein modeling server, are shown as a ribbon diagram. Residues contained in the N-terminus tail and those absent in the mature forms were not included in the modeling. Residues involved in E1 (**A**) and E2 (**B**) non-covalent interactions are shown in stick representation. Residues corresponding to AtSUMO1, AtSUMO2, AtSUMO3 and AtSUMO5 are shown in black, green, yellow and blue, respectively. (**C**) Same structure model as in A but showing residues involved in SIM interactions in surface representation. Basic residues and the groove-forming residues are colored cyan and yellow, respectively. Side chain N, O atoms are colored blue and red, respectively.

Figure 2. Non-covalent E2 interaction properties of *Arabidopsis* SUMO isoforms. (**A**) Sequence alignment of *Arabidopsis* (At), human (Hs) and yeast (y) SUMO residues involved in SUMO-E2 non-covalent interaction through their lateral chain contacts. Non-conserved residues are in grey background and residues exclusively non-conserved in AtSUMO3/5 are in bold. (**B**) Yeast two-hybrid assay to study interactions between *Arabidopsis* SUMO1, 2, 3, 5 or ubiquitin and AtSCE1 SUMO conjugating enzyme or AtUBC10 ubiquitin conjugating enzyme. (**C**) Interaction analysis between the mutant AtSUMO1 D63N (S1*) or AtSUMO3 N63D (S3*) and AtSCE1 as in B. Native SUMO isoforms were also included as a control. (**D**) PolyHis pull-down assay of AtSUMO1 or AtSUMO1 D63N mutant variant using His:AtSCE1 as a bait. Incubations in the absence of the bait or the prey were used as negative controls. Aliquots of input and eluate fractions were resolved by SDS-PAGE. AtSUMO1/D63N and AtSCE1 were analyzed by immunoblotting or Coomasie fluor orange staining (C-orange), respectively.

Figure 3. *PolySUMO chain formation is dependent on AtSUMO1 Asp-63 residue. In vitro* sumoylation assays were performed in the presence of AtSAE2/AtSAE1a, AtSUMO1, or AtSUMO1 Asp-63 Asn and AtSCE1. Reaction mixtures were incubated at 37ºC and stopped at the specified time points. Reaction products were resolved by SDS-PAGE and examined by immunoblot analysis with anti-AtSUMO1 (**A**) or AtSCE1 (**B**) antibodies. (**C**) *In vitro* sumoylation assays were performed in the presence of AtCAT3-Ct as a substrate. Reaction mixtures were incubated at 37ºC, aliquots removed at the specified time points and reaction products analyzed by immunoblot analysis with anti-GST antibodies.

Figure 4. *In vitro* conjugation properties of *Arabidopsis* SUMO isoforms. (**A**) *In vitro* sumoylation assays were performed in the presence of AtE1a, AtSUMO1, AtSUMO2, AtSUMO3 or AtSUMO5, AtSCE1a and GST:AtCAT3Ct as a substrate. Reaction mixtures were incubated at the indicated temperature and stopped after a 10-minute incubation. Reaction products were resolved by SDS-PAGE and examined by immunoblot analysis with anti-GST antibodies. (**B**) Reactions were performed in triplicates and GST:AtCAT3Ct sumoylation rate quantified. Average values and standard deviation bars were plotted on the graph. The table containing the data plotted into the graph is shown on the right of the panel. (**C**) Time course of SUMO conjugation *in vitro* reactions were performed at 37 ºC (top) and 42 ºC. Reaction mixtures were set up as in (A). The asterisk indicates a contaminating protein.

Figure 5. Mutations in the AtSUMO1 residues involved in E1 recognition resulted in SUMO conjugation defects. (**A**) Sequence alignment of *Arabidopsis* (At), human (Hs) and yeast (y)

SUMO residues involved in SUMO-E1 non-covalent interaction. Non-conserved residues are in grey background and residues exclusively non-conserved in AtSUMO3/5 are in bold. (**B**) *In vitro* sumoylation assays were performed in the presence of AtE1a, AtSUMO1 or the indicated mutant, AtSCE1a and GST:AtCAT3Ct as a substrate. Reaction mixtures were incubated at 37ºC and stopped at the indicated time point. Reaction products were resolved by SDS-PAGE and examined by immunoblot analysis with anti-GST antibodies. (**C**) Same as in (**B**) but reactions were incubated at 42ºC. (**D**) Time course for AtSAE2-thioester formation using Arabidopsis E1 (AtSAE2:AtSAE1a) and AtSUMO1 and AtSUMO1 mutant variants as substrates. Reaction mixtures were incubated at 30ºC and aliquots removed at the specified time points. In each case, a 10-min aliquot was treated with DTT as a control for thioester bond formation and it is indicated as 10^{+}. Reaction products were separated by SDS-PAGE and stained with Coomasie fluor orange. Asterisk indicates a contaminating protein. (**E**) Reactions were performed in triplicates and GST:AtCAT3Ct sumoylation and AtSAE2-thioester formation rates quantified. Average values of the relative activity among AtSUMO1 variants and the corresponding standard error bars were plotted on the graph.

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Figure 1

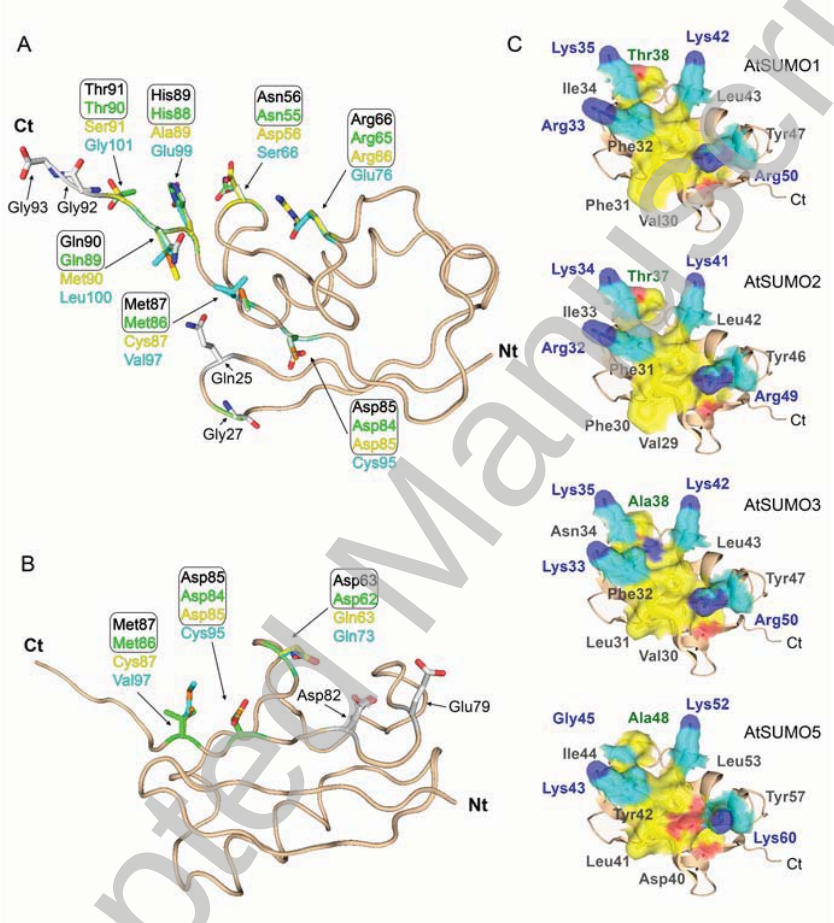

Figure 2

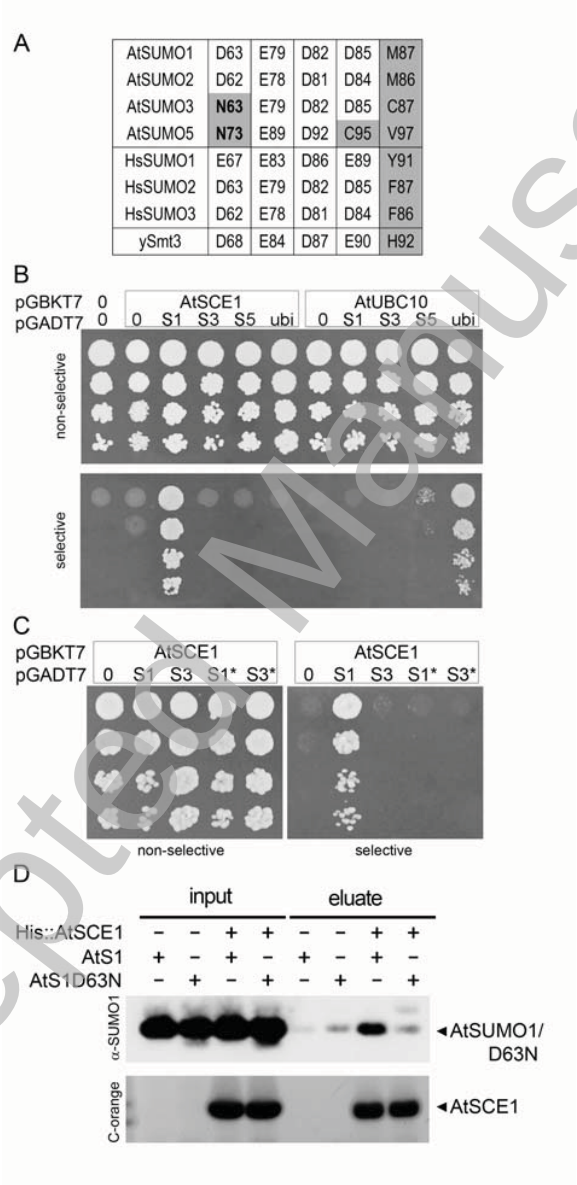

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Figure 3

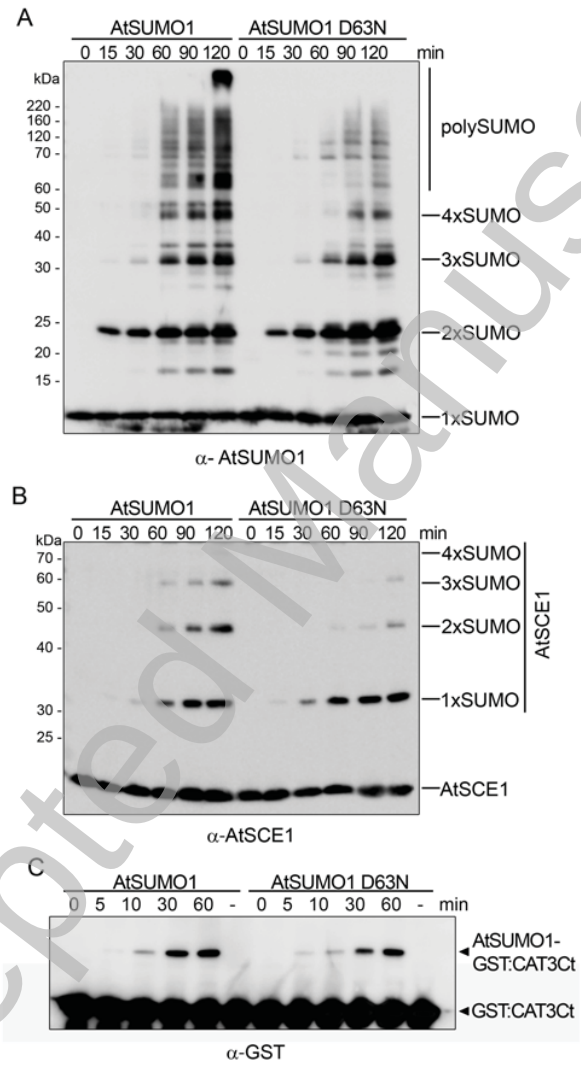
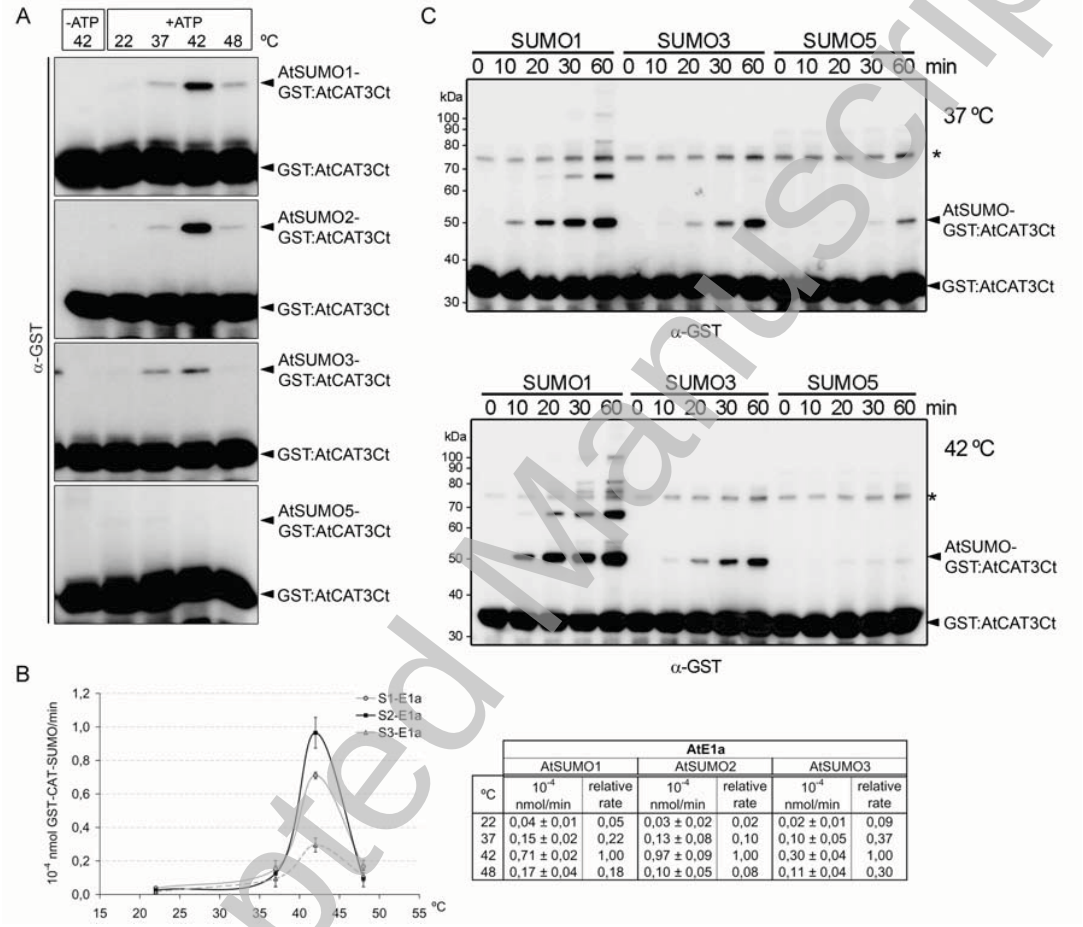


Figure 4



	AtE1a					
	AtSUMO1		AtSUMO2		AtSUMO3	
°C	10^{-4} nmol/min	relative rate	10^{-4} nmol/min	relative rate	10^{-4} nmol/min	relative rate
22	0,04 ± 0,01	0,05	0,03 ± 0,02	0,02	0,02 ± 0,01	0,09
37	0,15 ± 0,02	0,22	0,13 ± 0,08	0,10	0,10 ± 0,05	0,37
42	0,71 ± 0,02	1,00	0,97 ± 0,09	1,00	0,30 ± 0,04	1,00
48	0,17 ± 0,04	0,18	0,10 ± 0,05	0,08	0,11 ± 0,04	0,30

Figure 5