Chapter 9

Kinetic Analysis of Plant SUMO Conjugation Machinery

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Abstract

Plants display a high diversification degree of the SUMO conjugation machinery, which could confer a biological specialization of the different isoforms. For instance, the two essential *Arabidopsis* SUMO isoforms, SUMO1/2, display the highest conjugation rate when compared to SUMO3 and 5, suggesting that their specific biochemical properties may be linked to their biological specialization. In order to study the biochemical properties of plant SUMO conjugation systems, quantitative biochemical assays must be performed. We will present a detailed protocol for reconstituting an in vitro SUMO conjugation assay covering all steps from protein preparation to assay development.

Key words SUMO, E1 SUMO-activating enzyme (SAE2/SAE1), E2 SUMO-conjugating enzyme, Catalase 3 C-terminal domain, In vitro SUMOylation assay, Thioester

1 Introduction

In eukaryotic cells, posttranslational modifications by SUMO (Small Ubiquitin-like MOdifier) modulates protein activity through regulation of subcellular localization, protein activity and stability, and protein–protein interactions [1]. SUMO conjugation initiates with SUMO activation, which is a two-step ATP-dependent reaction catalyzed by the heterodimeric E1-activating enzyme, SAE1/SAE2. SUMO activation is the first control point in the selection of the SUMO/Ubl (Ubiquitin-like) modifier to enter the conjugation pathway [2, 3]. SAE2 displays four functional domains: adenylation, catalytic cysteine, ubiquitin fold (UFD), and C-terminal domains and SAE1 contributes the essential Arg21 to the adenylation domain [4]. The adenylation domain is responsible for SUMO recognition and SUMO C-terminus adenylation. In a second step, the SUMO C-terminal adenylate establishes a thioester bond with the E1 catalytic cysteine. After the thioester bond is formed, SUMO can be transferred to the E2-conjugating enzyme in a reaction that requires E2 recruitment through the $SAE2^{UFD}$ domain [5] in collaboration with the $SAE2^{Cys}$ domain [6]. The E2-conjugating

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enzyme is competent for transferring SUMO to the substrate, although this reaction is facilitated by E3 ligases. SUMO conjugation is a reversible modification and the same proteases responsible for the processing of the SUMO immature form (peptidase activity) are also involved in its removal from the substrate (isopeptidase activity) [7].

In plants, SUMO conjugation controls plant development [8, 9] and it has a major role in the modulation of plant responses to hormones [10], development [11, 12], abiotic stress [13–16], and defense responses to pathogens [17, 18]. These plant biological processes regulated by SUMOylation have been uncovered by the analysis of proteases, ULP, and SUMO E3 ligase mutant plants [19]. Among them, the most studied mutants are the *siz1 and mms21* E3 ligases and the *esd4* ULP protease, which display pleiotropic growth defects and reduced viability. *siz1* and *mms21* null mutants display a reduction in endogenous SUMO conjugate accumulation [20–22], while an overaccumulation of SUMO conjugates is found in *esd4* mutant [8]. Even though they have opposite molecular effects, the physiological outcome of these mutations is very similar and, surprisingly, this pleiotropic phenotype is the result of an overaccumulation of salicylic acid in both *siz1* and *esd4* mutant plants [23]. These results indicate that SUMO conjugation homeostasis is under a tight control and over- or under-accumulation of SUMO conjugates results in a misregulation of essential processes.

The critical SUMO homeostasis in vivo can be achieved through regulation of SUMOylation machinery activity. Accordingly, biochemical studies have shown that SUMO conjugation machinery is a complex system in plants. In *Arabidopsis*, functional diversity has been found in SUMO proteases, SUMO isoforms, SUMO-activating enzyme E1, and SUMO E3 ligases. *Arabidopsis* SUMO proteases display distinct specific activities toward the existing SUMO isoforms, SUMO1, 2, 3, and 5 [24], which also display distinct biochemical properties that might influence their conjugation in vivo and biological function. The conjugation system seems to have evolved for assuring the conjugation of the essential SUMO1/2 isoforms. In this mechanism, the E1-activating enzyme would have a crucial role by conferring SUMO paralog specificity [3], in addition to a rate limiting role of SUMO activation during the conjugation cascade [25].

In order to understand the functional relevance of SUMO conjugation machinery diversification among plantae kingdom, performing accurate biochemical assays is a crucial approach. We provide a detailed protocol for performing SUMO conjugation assays, from protein preparations to quantification of kinetic data.

2 Materials

2.1 Expression and Purification of Enzymes

1. Plasmids: pET-15b (Novagen), pET-28a (Novagen), pGEX-6p (GE healthcare) (or similar).
2. LB agar plates: 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl in 1 L water, adjust to pH 7.5 with NaOH and add 15 g LB agar powder. After autoclave add the appropriate antibiotic and pour the LB agar into sterile Petri dishes.
3. Antibiotics stock solutions: 100 mg/mL ampicillin in water, 50 mg/mL kanamycin in water, and 34 mg/mL chloramphenicol in ethanol. Sterilize all antibiotics by filtration and store in 1 mL aliquots at −20 °C. Make a 1/1000 dilution for reaching the working concentration.
4. 2× TY medium: 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl in 1 L water and autoclave.
5. Isopropyl-β-D-thiogalactopyranoside (IPTG): 0.1 M IPTG in water, sterilize by filtration and store in aliquots at −20 °C.
6. Protease inhibitors stock: 0.1 M PMSF (phenylmethanesulphonylfluoride) in ethanol, 1 mg/mL pepstatin in ethanol, and 1 mg/mL leupeptin in ethanol. All solutions are stored at −20 °C in small aliquots.
7. Lysis buffer: 20% sucrose, 50 mM Tris–HCl pH 8.0, 350 mM NaCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1% NP40 (v/v), 50 μg/μL DNAsa, 1 mg/mL lysozyme, 1 mM PMSF, 1 μg/mL leupeptin, and 1 μg/mL pepstatin.
8. IMAC Sepharose 6 Fast Flow (17-0921-07, GE healthcare) or similar.
9. Equilibration buffer I: 50 mM Tris–HCl pH 8.0, 350 mM NaCl, 1 mM β-mercaptoetanol, and 20 mM imidazol.
10. Elution buffer I: 50 mM Tris–HCl pH 8.0, 350 mM NaCl, 1 mM β-mercaptoetanol, and 300 mM imidazol.
11. Dialysis membrane with a nominal 5 kDa molecular weight cut-off (MWCO).
12. Thrombin protease (27-0846-01, GE healthcare) prepare at 1 U/μL in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3).
13. Size exclusion buffer: 50 mM Tris–HCl pH 8.0, 100 mM NaCl, and 1 mM β-mercaptoetanol.
14. Glutathione Sepharose 4B (GE healthcare, 17-0756-01).
15. Equilibration buffer II: 50 mM Tris–HCl pH 8.0, 350 mM NaCl.

16. Elution Buffer II: 50 mM Tris–HCl pH 8.0, 350 mM NaCl, and 10 mM reduced L-glutathione (BioXtra, ≥98.0%, SIGMA-ALDRICH G6529).
17. Protein chromatography: AKTA-FPLC system with preparative gel filtration columns (HiLoad 16/60 Superdex 75 prep grade (120 mL) 17-1068-01 and HiLoad 16/60 Superdex 200 prep grade (120 mL) 17-1069-01; GE healthcare).
18. Centrifugal device concentrators: 10 and 50 kDa cut-off filters (Amicon).
19. Nylon or cellulose acetate membrane syringe filters 0.2 μm pore size.
20. PD-10 Desalting columns (GE Healthcare).
21. Bradford protein assay (Bio-Rad).

2.2 SUMO Conjugation Assays

1. Reaction Buffer 5×: 250 mM NaCl, 100 mM HEPES-NaOH pH 7.5, 0.5% Tween-20, and 25 mM $MgCl_2$.
2. ATP solution: 100 mM ATP dissolved in 1 M Tris–HCl pH 7.5.
3. SDS loading buffer 6×: 0.5 M Tris–HCl pH 6.8, 10% SDS, 30% glycerol, and 0.012% bromophenol blue. Store in 0.5 mL aliquots at −20 °C.
4. 4–12% gradient polyacrylamide Bis-Tris gels (Invitrogen).
5. Transfer buffer: 48 mM Tris–HCl, 39 mM glycine and 10% (v/v) methanol for semi-dry unit.
6. Blocking buffer: 3% (w/v) nonfat dry milk in TBST buffer.
7. TBST buffer: 20 mM Tris–HCl pH 7.5, 137 mM NaCl, and 0.1% (v/v) Tween 20.
8. Primary antibody: antibody anti-GST (Sigma, G7781) used at 1:2500 dilution in blocking buffer.
9. Secondary antibody: anti-rabbit IgG horseradish peroxidase linked whole antibody (GE healthcare, NA934) used at 1:5000 dilution in blocking buffer.
10. ECL Prime Western Blotting Detection Reagent (GE healthcare, RPN2232) or similar.
11. Chemiluminescence imaging system such as LAS4000 (Fujifilm).

3 Methods

Reconstituted SUMO in vitro reaction allows the study of the biochemical properties of SUMO machinery components. We use GST-*At*CAT3Ct as a substrate (Fig. 1), which is modified by SUMO at the Lys-423 leading to a detectable shift of 15 kDa. This posttranslational modification is visualized by SDS-PAGE

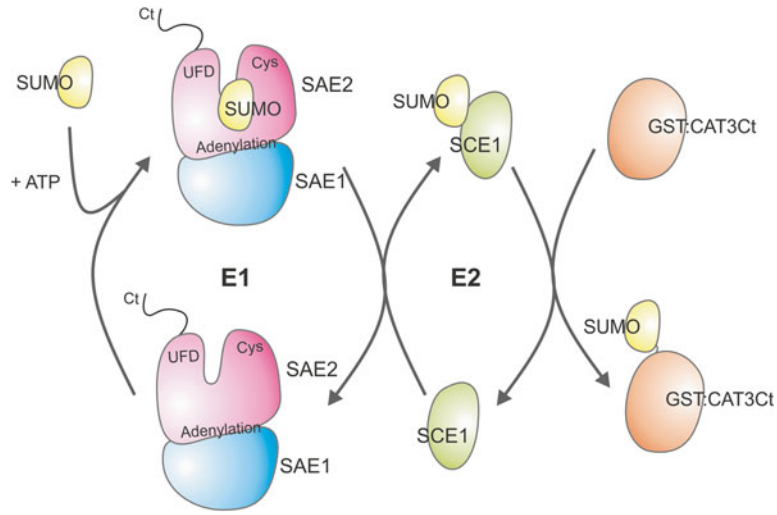


Fig. 1 SUMO conjugation assay. Kinetics analysis of SUMO isoforms and E1 isoforms was performed by monitoring SUMO conjugation to the C-terminal domain of catalase 3 (comprising amino acids 419–492), fused to GST, in the absence of SUMO E3 ligases

followed by gel Coomassie Blue staining or immunoblotting. The diversification of the SUMOylation system in plants is higher than in mammals, suggesting the existence of different molecular properties for each isoform that might affect their in vivo conjugation and biological function. To address this issue, we have developed an efficient time-course assay to facilitate analysis of SUMO conjugation in vitro. The assay is done with all the purified SUMOylation system components, as described through Subheadings 3.1–3.4, except for the E3 SUMO ligase enzyme.

3.1 Preparation of Recombinant SUMO Machinery Components: Expression and Purification of SUMO Isoforms and the SUMO-Conjugating Enzyme SCE1

cDNA-encoding SUMO proteins in their mature form *At*SUMO1, *At*SUMO2, *At*SUMO3, and *At*SUMO5 was obtained from 2-week-old plants and cloned into pET28a (Novagen) [3]. DNA encoding full-length *At*SCE1 was acquired from the ABRC (Ohio State University, Columbus) and cloned into pET28a [10]. All genes were cloned into pET28a to generate N-terminal thrombin-cleavage His_6-fusion proteins (Figs. 2 and 3).

1. Transform the plasmids encoding the HIS-tagged proteins into *E. coli* BL21 Codon Plus RIL (Stratagene) competent cells (*see* **Note 1**).
2. Transformed cells are selected on LB agar plates supplemented with 50 μg/mL kanamycin and 34 μg/mL cloramphenicol.
3. Pick a fresh single colony and inoculate 3 mL of 2× TY medium containing kan/chlr during 6 h at 37 °C with vigorous shaking (≈250 rpm).

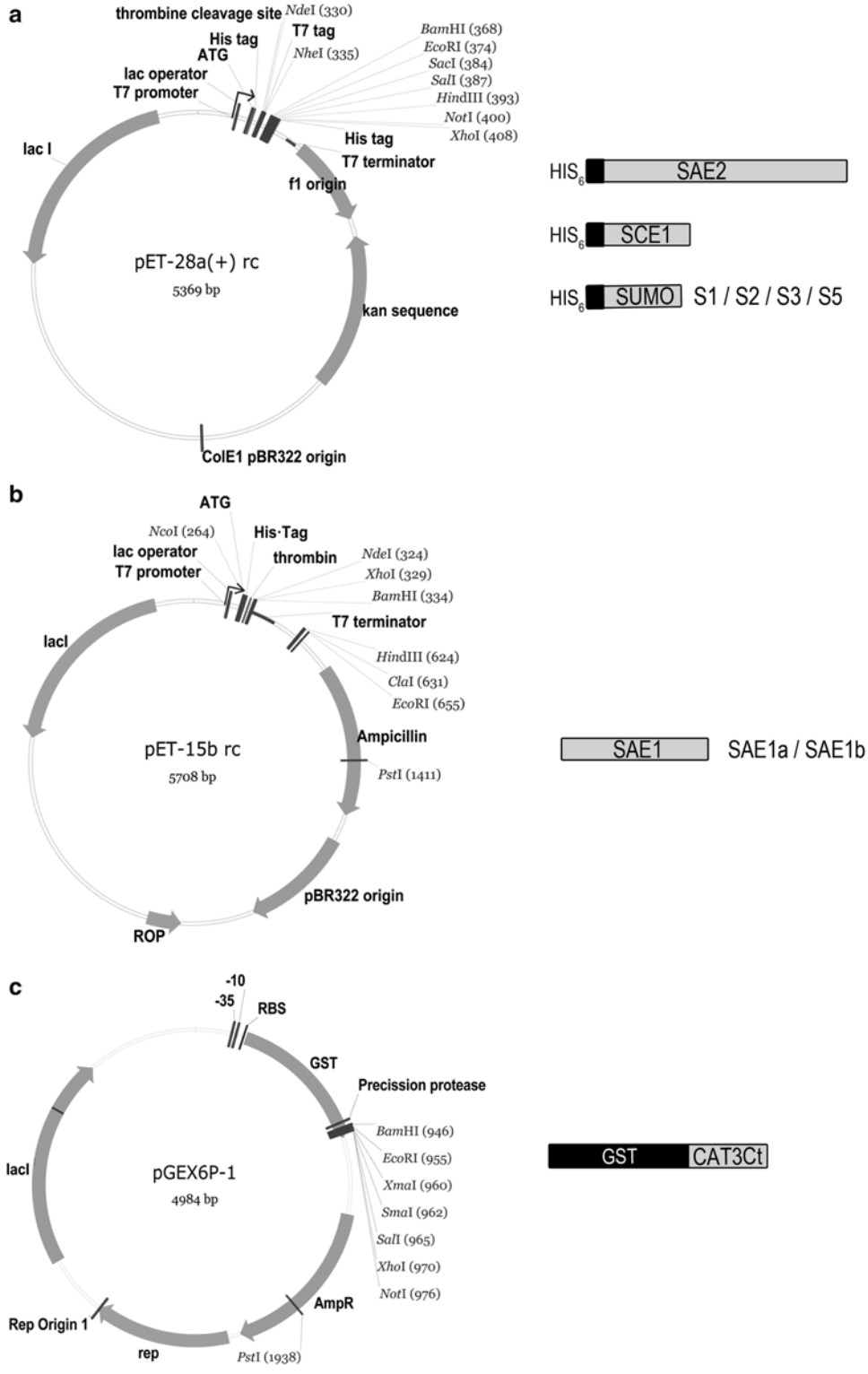


Fig. 2 DNA plasmids used for production of recombinant protein in *E. coli*. (**a**) SAE2, SCE1, and SUMO isoforms were cloned in the pET28a vector for generating HIS-tagged protein fusions. (**b**) SAE1a and b isoforms were cloned into the pET15b vector in the NcoI cloning site in order to produced untagged versions. (**c**) CAT3 C-terminal domain was cloned into the pGEX-6P vector for generating GST-protein fusions

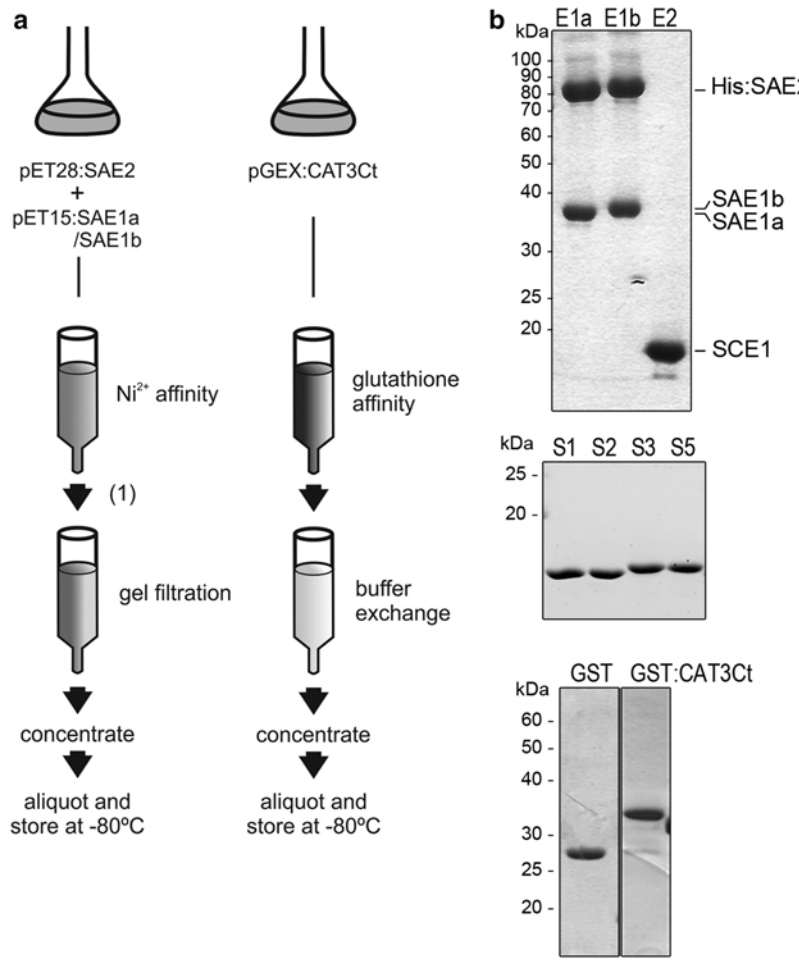


Fig. 3 Purification of SUMO conjugation assay components. (**a**) All proteins were expressed in independent *E. coli* BL21 cultures except for SAE2/SAE1a and SAE2/SAE1b that were coexpressed in order to purify the corresponding E1 heterodimer isoform. HIS-tagged fusion proteins were Ni^{2+}-affinity purified and eluted fractions were further purified through gel filtration chromatography. (1) After Ni^{2+}-affinity purification, HIS-tag was removed by thrombin digestion except for the E1 heterodimer sample. GST and GST-CAT3Ct were purified by glutathione-affinity chromatography followed by buffer exchange chromatography. For each purification experiment, fractions showing the highest purity degree were pooled together, concentrated, and aliquots stored at −80 °C. (**b**) Before storage, all samples were quantified by Bradford and purity analyzed by SDS-PAGE followed by Coomasie-Blue staining. Samples from representative purification experiments are shown

4. Dilute 1:50 the preculture into 60 mL of 2×TY with kan/chlr and grow overnight at 37 °C and 250 rpm.
5. Next morning, inoculate 0.5 L culture of 2×TY making a 1:50 dilution of the saturated overnight culture, and grow the bacterial culture at 37 °C and 250 rpm until an OD_{600nm} of 0.6–0.8 is reached. Induce protein expression by adding IPTG to final concentration of 0.5 mM. Cultures are grown for another 4 h at 28 °C and 250 rpm (*see* **Note 2**).

6. Harvest cells by centrifugation (6000 × *g* for 15 min at RT) and discard supernatant. At this point, cells can be extracted or kept it at −80 °C until use.
7. Thaw cell pellet and resuspend with 1/20 of the original culture volume in lysis buffer.
8. Sonicate the cell suspension in ice-water bath. Sonication cycle: 30 s ON, 30 s OFF at 10% amplitude. Repeat the sonication cycle six times (*see* **Note 3**).
9. Centrifuge the cell lysate at 39,000 × *g* for 1 h at 4 °C to remove cellular debris. Discard the pellet and retain the supernatant.
10. Pass the sample through 0.2 μm filter and add imidazole to a final 20 mM concentration (*see* **Note 4**).
11. Pre-pack a column with 3 mL of IMAC sepharose (50% slurry in 20% ethanol, which corresponds to 1.5 column volume, CV) (*see* **Note 5**). Add 7.5 mL (5 CV) of distilled water in order to eliminate the ethanol. To charge the column add 300 μL of 0.1 M $NiSO_4$ (0.2 CV) following by another wash with distilled water (5 CV). Equilibrate the column with 7.5 mL of equilibration buffer I (5 CV).
12. Pass the protein extract (input) through the column by gravity flow and collect the flow through (FT). Perform one wash with the equilibration buffer (5 CV). Elute the protein in fractions of 1 mL with Elution buffer I. Quantify elution fractions by Bradford assay and check purified proteins by 12% SDS-PAGE gel separation followed by Coomassie Blue staining.
13. Pool together elution fractions containing the desired protein (His_6-*At*SUMO1/2/3/5 or His_6-*At*SCE1), add thrombin, transfer to a dialysis membrane, and dialyze overnight at 4 °C against size exclusion buffer. Add 10 units of thrombin per 1 mg of the protein (*see* **Note 6**).
14. Concentrate the sample using 10 kDa cut-off filters (SUMO and SCE1 predicted MW are approximately 11 kDa and 18 kDa, respectively) to a final volume close to 1 mL. Filtrate the sample through 0.2 μm syringe membrane filter and apply to a gel filtration column, Superdex 75, equilibrated with size exclusion buffer. Fractions of 1 mL are collected and 10 μL aliquots corresponding to the eluted protein peak are analyzed by 12% SDS-PAGE. Those fractions containing the pure protein (SUMOs or SCE1) are pooled together and concentrated until 5–10 mg/mL using a centrifugal device (10 kDa cut-off). Freeze small aliquots in liquid nitrogen and store at −80 °C until use (*see* **Notes 7** and **8**).

3.2 Preparation of Recombinant SUMO Machinery Components: Expression and Purification of E1-Activating Enzyme Isoforms (SAE2/SAE1a and SAE2/ SAE1b)

The SUMO E1-activating enzyme is a heterodimer consisting of a large subunit, SAE2, and a small subunit, SAE1. *Arabidopsis* presents two isoforms of the SUMO E1 (E1a and E1b), which differ in the small subunit composition, SAE1a or SAE1b. In this case, purification of the dimeric E1 complex is performed by coexpression of His_6-tagged SAE2 and untagged SAE1a or SAE1b in *E. coli*, which were previously cloned in pET28a (SAE2) and pET15b (SAE1a/b). cDNA encoding SAE2 and SAE1a/b was obtained from 2-week-old plants and cloned into the expression plasmids [3] (Figs. 2 and 3).

1. For protein expression, cell lysis and protein purification through the IMAC, follow the steps described in **steps 1–12** in Subheading 3.1. Add the appropriate antibiotics in all steps of growing bacterial cell cultures: kanamycin (pET28a), ampicillin (pET15a), and chloramphenicol for the bacterial strain.
2. Analyze the elution fractions on Coomassie Blue stained 10% SDS gel. SAE2 migrates at 80 kDa while SAE1a/b migrates at 37 kDa.
3. Pool together the fractions containing the E1a/b heterodimer and dialyze the samples overnight against the size exclusion buffer (*see* **Note 9**).
4. Concentrate the mixture using a 50 kDa cut-off filter to a final volume of 1 mL and filtrate through 0.2 μm filter.
5. Load the sample onto a preparative Superdex 200 gel filtration column, equilibrated with size exclusion buffer. Fractions of 1 mL are collected during the chromatography.
6. Analyze by 10% SDS-PAGE gel 10 μL of the fractions corresponding to the E1a/b elution peak. Pool the fractions were SAE2/SAE1a or SAE2/SAE1b display a 1:1 stoichiometry and concentrate using 50 kDa cut-off filters to 20–50 mg/mL final concentration. Freeze small aliquots in liquid nitrogen and store at −80 °C until use.

3.3 Preparation of Recombinant SUMO Conjugation Substrate: Expression and Purification of GST-CAT3Ct

As an efficient substrate we use the catalase 3, which has a SUMOylation consensus site, Lys 423, fully exposed on the protein surface and located at the C-terminal domain (comprising amino acids 419–492). The cDNA encoding the C-terminal tail of CAT3 (419–472) was obtained from 2-week-old plants and cloned into pGEX-6p-1 to obtain an N-terminal GST (glutathione transferase)-fusion protein (Figs. 2 and 3).

1. For the protein expression and cell lysis, follow the procedure described in **steps 1–10** in Subheading 3.1. Add the appropriate antibiotics: ampicillin (pGEX-6p1) and chloramphenicol (for the bacterial strain).

2. Pass the sample through 0.2 μm filter before loading it to the affinity column (*see* **Note 4**).
3. Pre-pack a column with 3 mL of gluthatione-Sepharose (50% slurry in 20% ethanol, which corresponds to 1.5 CV). Add 7.5 mL (5 CV) of distilled water in order to remove the ethanol. Equilibrate the column with 5 CV of the equilibration buffer II.
4. Pass the protein extract (input) through the column by gravity flow and collect the FT. Perform one wash with the equilibration buffer 2 (5 CV). Elute the protein in fractions of 1 mL with elution buffer II. Quantify protein content by Bradford assay and analyze eluted fractions by Coomassie Blue staining in 12% SDS-PAGE gel.
5. Fractions containing GST-AtCAT3Ct are pooled together and desalting by passing the mixture into prepacked disposable PD-10 desalting column (GE healthcare) equilibrated with size exclusion buffer.
6. The recovered sample is concentrated using 10 kDa cut-off filters (GST-*At*CAT3Ct has a predicted MW of 34 kDa) to 5 mg/mL, flash-frozen into liquid nitrogen and stored at −80 °C in small aliquots.

3.4 In Vitro SUMOylation Assays for Analyzing Distinct SUMO Isoforms

In order to test the efficiency of each SUMO isoform for conjugation to the substrate, SUMO isoforms are used in independent in vitro assays. For simplicity, major qualitative differences in SUMO conjugation rate can be identify in single time point assays through a temperature range [3]. Once identified the SUMO isoforms displaying the highest differences in conjugation efficiency, a time-course assay is performed for quantifying conjugation kinetics. In *Arabidopsis*, *At*SUMO1, *At*SUMO3, and *At*SUMO5 are the isoforms that differ dramatically in their conjugation capacity. For performing kinetics studies, SUMO conjugation is assayed at two temperatures, 37 and 42 °C, and reaction products are analyzed at several time points: 0, 10, 20, 30, and 60 min. In order to minimize technical differences between independent reactions, a master mix is prepared by adding all the common components in the reaction mixture. The preparation of a reaction master mix for one incubation temperature is as follows (the volumes should be scaled up according to the number of temperatures to be assayed):

1. Prepare a master mix reaction by mixing the following components in the indicated order. Add H_2O, 5× Reaction Buffer, 0.5 μM *At*SAE2/*At*SAE1a, 0.5 μM *At*SCE1 and 5 μM GST-*At*CAT3Ct calculated to a final 360 μL reaction volume, although the master mix final volume is adjusted to 330 μL at this point (*see* **Note 10**).

2. Divide the preparative master mix in three PCR tubes (110 μL per tube) and add 2 μM of AtSUMO1, AtSUMO3, or AtSUMO5, calculated to a final reaction volume of 120 μL (110 μL master mix + 10 μL SUMO isoform to be tested).
3. Always include a control reaction without ATP. Transfer 20 μL of each of the three performed reactions into a new PCR tube as a negative control. A total of 6 tubes corresponding to *At*SUMO1, *At*SUMO2, *At*SUMO3, and the respective negative controls are obtained for each temperature (*see* **Note 11**).
4. Start the reaction by adding 1 μL of 100 mM ATP (1 mM final concentration) into the reaction tubes, except for the control reactions, mix gently, and collect the reaction mixture on the bottom of the tube by a short spin (*see* **Note 12**).
5. Immediately take the first time-course point (0 min), and stop the reaction. Stop reactions by removing 20 μL of the reaction mixture at a given time point, transfer them to a new centrifuge tube containing 4 μL of SDS 6× loading buffer and heat for 10 min at 70 °C.
6. Transfer the reaction tubes to a PCR machine with a gradient temperature program and incubate at 37 °C or/and 42 °C (depending on the experimental conditions being assayed).
7. Remove 20 μL of the reactions at the specified times and stop the reactions.
8. Stop negative control reactions at the last time-course point, 60 min.
9. For SUMO conjugation efficiency quantification, resolve reaction products by SDS-PAGE. For facilitating comparative quantification among SUMO isoform conjugation rate, analyze, in the same protein gel, time-course reaction aliquots incubated at the same temperature and containing either AtSUMO1 or AtSUMO3 or AtSUMO5.
10. Load 12 μL of each time point denatured sample on a Novex 4–12% Bis-Tris gradient gels and perform electrophoresis in MOPS running buffer.
11. Blot proteins into PDVF membranes using a semi-dry transfer for 30 min at 20 V at room temperature in transfer buffer.
12. Block the membrane for 1 h in blocking buffer solution at room temperature.
13. Incubate the PVDF membranes with a primary antibody solution against GST (anti-GST polyclonal antibody) in blocking buffer overnight at 4 °C (*see* **Note 13**).
14. Rinse the blots three times for 10 min with the TBST solution to remove the excess of the primary antibody unbound at the membrane.

15. Incubate the blots in the secondary antibody for 45 min at room temperature.
16. Wash the PVDF membranes three times for 10 min with TBST solution.
17. Apply the chemiluminescent substrate, ECL-prime reagent, to the blot according to the manufacturer's recommendation.
18. Capture the chemiluminescent signal with the LAS4000 imaging system.

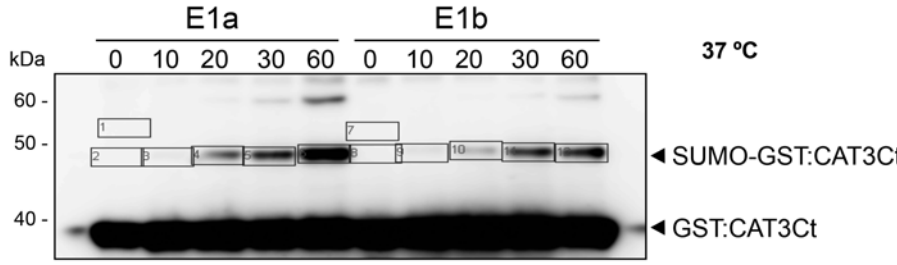
The same procedure can be applied for performing kinetics studies of other SUMO conjugation machinery components. For each specific case, the reaction master mix will be modified accordingly. In case of analyzing SUMO E1-activating enzyme kinetics, the reaction mixture will contain AtSUMO2, AtSCE1 and GST-AtCAT3Ct, and AtE1a (AtSAE2/AtSAE1a) or AtE1b (AtSAE2/AtSAE1b) will be added after distributing the reaction master mix into independent tubes.

3.5 Quantification of SUMO Conjugation Kinetics

In order to calculate the SUMOylation efficiency specific to SUMO isoform or E1 isoform present in the assay, chemiluminescent signal is quantified using the quantitative image analysis software Multigauge. In this protocol, as an example, quantification of the E1a or E1b in vitro assays is explained (Fig. 4).

1. Gel images are processed and quantified with Multi Gauge software (*see* **Note 14**). For simplification, only the GST:CAT3-monoSUMO adduct is quantified.
2. Draw a region of interest (ROI) using the drawing tools. We recommend using the rectangle shape. Draw a rectangle that encloses the largest band of interest and use the same box area for enclosing the rest, including an area of the membrane without signal, although right above or below the bands being quantified, to be used as background (Fig. 4a).
3. Export original quantification raw data to an Excel sheet (or similar).
4. Subtract background signal from all data points (AU-B) (Fig. 4b).
5. Calculate the average signal obtained from each membrane (average of all data calculated in the previous step) and use it for normalizing the obtained values ((AU-B)/signal average). This procedure facilitates the reduction of technical variability resulting from differences in western blotting and chemiluminescent capture time among experiments (Fig. 4c).
6. Plot values onto a scattered graph and determine the time-course points that fit to the linear range (Fig. 4d).

a



b

Export data to an Excel worksheet.
Normalize values to the average signal obtained in all data points.

	min	label	AU	area	AU-B	(AU-B) / signal aver.
AtSAE1a	0	2	1632065	11,61	0,5	0,01
	10	3	2399777	11,61	8,2	0,09
	20	4	8828518	11,61	72,4	0,80
	30	5	14765320	11,61	131,8	1,46
	60	6	37287113	11,61	357,0	3,95
	background	1	1584192	11,61		
AtSAE1b	0	8	2820802	11,61	8,2	0,09
	10	9	2929146	11,61	9,3	0,10
	20	10	5198183	11,61	32,0	0,35
	30	11	14865731	11,61	128,7	1,43
	60	12	17471508	11,61	154,7	1,71
	background	7	2000486	11,61		
Signal average					90,3	

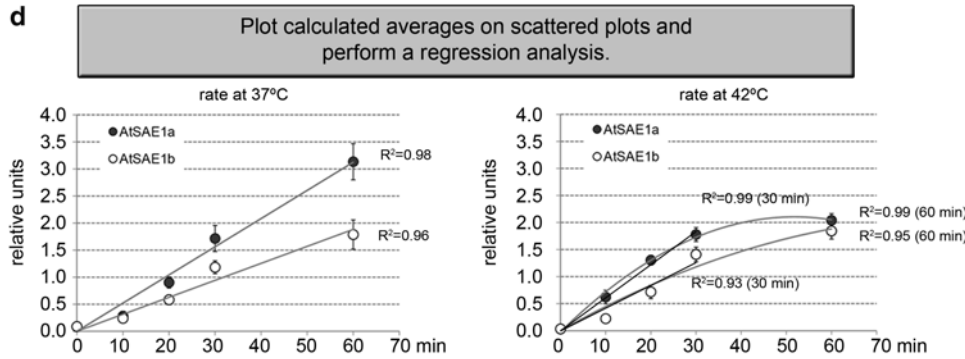
37 ºC

c

Repeat calculations for all experimental replicates and determine the average and standard error.

		(AU-B)/signal average				37 ºC			
	min	Exp. I	Exp.II	Exp. III	Exp. IV	average	Std Dev	n	Std Error
AtSAE1a	0	0,11	0,01	0,20	0,02	0,08	0,09	4	0,05
	10	0,31	0,09	0,50	0,22	0,28	0,17	4	0,09
	20	0,84	0,80	0,76	1,21	0,90	0,20	4	0,10
	30	1,60	1,46	1,38	2,43	1,72	0,48	4	0,24
	60	2,86	3,95	2,39	3,32	3,13	0,67	4	0,33
AtSAE1b	0	0,02	0,09	0,16	0,09	0,09	0,06	4	0,03
	10	0,26	0,10	0,34	0,25	0,24	0,10	4	0,05
	20	0,65	0,35	0,78	0,55	0,58	0,18	4	0,09
	30	1,21	1,43	1,24	0,86	1,18	0,24	4	0,12
	60	2,15	1,71	2,24	1,05	1,79	0,54	4	0,27

Fig. 4 Workflow of SUMO conjugation quantification. (**a**) Reaction products were resolved by SDS/PAGE and examined by immunoblot analysis with anti-GST antibodies. Luminescence signal was quantified using Multi Gauge software by using the rectangle selection tool for determining the ROIs (which all have the same area). (**b**) Data (AU) is exported to Excel, background signal is subtracted (AU-B column), and values are normalized to the average signal obtained in the particular dataset considering all data points ((AU-B)/signal aver. column). This data processing allows comparison between experimental replicates. (**c**) Average of results obtained from independent replicates is calculated. (**d**) Data obtained in (**c**) are plotted on scattered graphs and the reaction time window fitting on linear regression lines is selected for calculating the reactions slopes (e.g., for 37 °C incubation reaction linearity is maintained through 60 min, while for 42 °C incubation linearity is only maintained up to 30 min.). (**e**) Average of slopes obtained in independent replicates is calculated in order to compare multiple samples/assay conditions



e

Calculate slopes using data fitting into a linear regression model. Determine average values from slopes obtained in experimental replicates.

	Slope 37ºC							
	Exp. I	Exp.II	Exp. III	Exp. IV	average	Std Dev	n	Std Error
AtSAE1a	0,048	0,069	0,038	0,059	0,054	0,014	4	0,007
AtSAE1b	0,037	0,031	0,036	0,017	0,030	0,009	4	0,005

Fig. 4 (continued)

7. Determine slopes, relative luminescence signal versus time, at each temperature for E1a or E1b using the normalized values from each membrane and the time-course points identified in the previous step. The slope of each line represents the GST:CAT3Ct SUMOylation efficiency.
8. Repeat steps from **2** to **7** for each experimental replicate and calculate the average of obtained slopes and variability as measured by the standard error (Fig. 4e).

4 Notes

1. *E. coli* BL21 Codon Plus RIL cells carry a plasmid pLysS (chloramphenicol resistance) that codifies for T7 lysozyme that represses the expression of the other genes under the T7 promoter but does not interfere with the protein expression induced by IPTG; allowing high efficiency of the protein of interest. This strain also contains extra copies of the argU, ileY, and leuW tRNA genes in order to avoid potential translation restrictions of heterologous proteins from organisms that have AT-rich genomes.
2. Take 1 mL sample before and after induction as a noninduced and induced control. Pellet cells by centrifugation and suspend it in 100 μL of cracking buffer. Store at −20 °C until SDS-PAGE analysis.
3. Cell disruption can be followed by Bradford quantification to ensure total cell lysis by sonication.

4. Filtration was performed using a reusable vacuum filtration system (e.g., Nalgene).
5. The amount of sepharose used has to be adapted to the scale of the experiment, the capacity of the sepharose being used (refer to manufacturer instructions), and the recombinant expression levels.
6. To ensure that digestion is complete analyze the reaction by SDS-PAGE before performing the next step. If partial digestion is detected, extend digestion by adding thrombin (add units according to the efficiency of the ON digestion).
7. Filtered and degas buffer solutions and samples are used in all chromatographic steps.
8. Each aliquot should be used only 3–4 times; extensive freeze-thawing cycles may lead to lose enzymatic activity of the native protein.
9. For this sample, we skip the thrombin digestion treatment.
10. When assembled in vitro reaction thaw samples on ice, centrifuge, and quantify aliquots by Bradford. If required, dilute enzymes using the 1× reaction buffer. Aliquots of 5× reaction buffer can be stored at –20 °C for 6 months.
11. Another control reaction can be done without the substrate, GST-AtCAT3Ct. In this case, using only a negative control at the highest temperature might be enough.
12. ATP aliquots are sensitive to the freeze-thawing cycles. Avoid more than 3 cycles.
13. Primary antibody might be applied to the PVDF membrane for 90 min at room temperature.
14. Image quantification accuracy will depend on the image acquisition system used. LAS4000 reader delivers a range cope from 0 to 65,535 before the image reaches saturation, while a scanned TIFF image delivers a range scope from 0 to 255 resulting in a sensitivity reduction. One interesting feature of Multi Gauge software is that allows contrast adjustment in order to visualize better the signals without varying the raw data that will be used for quantification.

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