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Distinct roles for Arabidopsis SUMO protease ESD4 and its closest homolog ELS1

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Abstract SUMO conjugation affects a broad range of
processes in *Arabidopsis thaliana*, including flower initia-
tion, pathogen defense, and responses to cold, drought and
salt stress. We investigated two sequence-related SUMO-
specific proteases that are both widely expressed and show
that they differ significantly in their properties. The closest
homolog of SUMO protease ESD4, ESD4-LIKE SUMO
PROTEASE 1 (ELS1, alternatively called AtULP1a) has
SUMO-specific proteolytic activity, but is functionally dis-
tinct from ESD4, as shown by intracellular localization,
mutant phenotype and heterologous expression in yeast
mutants. Furthermore, we show that the growth defects
caused by loss of ESD4 function are not due to increased
synthesis of the stress signal salicylic acid, as was previ-
ously shown for a SUMO ligase, indicating that impairment
of the SUMO system affects plant growth in different ways.
Our results demonstrate that two *A. thaliana* SUMO prote-
ases showing close sequence similarity have distinct in vivo
functions.

Abbreviations
SUMO   Small ubiquitin-related modifier
SAE   SUMO activating enzyme
SCE   SUMO conjugating enzyme
ESD4   EARLY IN SHORT DAYS4
ELS1   ESD4 LIKE SUMO PROTEASE

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Introduction

Conjugation of the small ubiquitin-related modifier (SUMO) to substrate proteins is essential in plants (Saracco et al. 2007; for reviews, see Novatchkova et al. 2004; Geiss-Friedlander and Melchior 2007; Miura et al. 2007a). The process impinges on the response to several forms of stress including drought, heat, cold, phosphate shortage, high salinity, and pathogens (Miura et al. 2005, 2007b; Yoo et al. 2006; Catala et al. 2007; Lee et al. 2007; Conti et al. 2008; van den Burg et al. 2010), and regulates the time of flower initiation (Murtas et al. 2003; Jin et al. 2008). SUMO conjugation utilizes a dedicated set of enzymes (Tang et al. 2008). SUMO activating enzyme SAE activates free SUMO by formation of a thioester with the SUMO carboxyl terminus. SUMO is then transferred to SUMO conjugating enzyme SCE, forming a thioester with the SCE active site Cys. SCE can directly transfer activated SUMO onto an ε-amino group of a substrate Lys residue, if this Lys residue is accessible and in a sequence environment called sumoylation consensus motif, which consists of a hydrophobic residue, followed by Lys, any residue, and Asp or Glu (ΨKxD/E; Anckar and Sistonen 2007). In addition to these SCE-binding motifs, SUMO-binding domains consisting of a short hydrophobic $β$ strand (Hecker et al. 2006) can also increase substrate affinity, or influence the conjugation reaction in other ways. Furthermore, a number of proteins have been identified as SUMO ligases, which enhance modification of substrates lacking any of the above sequence motifs (Johnson 2004; Novatchkova et al. 2004; Geiss-Friedlander and Melchior 2007).

SUMO modification can shield protein surfaces, thereby disrupting protein interactions, or generate new interaction interfaces to promote alternative protein assemblages. Sumoylation can therefore implement changes in protein interactions, and although usually only a small fraction of a protein is in the sumoylated form, the modification can be an essential trigger for proteins to cycle between alternative activity states. Furthermore, it was recently found that attachment of multiple SUMO moieties in the form of poly-SUMO chains can promote proteolytic turnover of substrate proteins (Uzunova et al. 2007; Ulrich 2008).

Most sumoylation events are believed to be reversible, because specific proteases exist that cleave conjugates at the SUMO carboxyl terminus, releasing free SUMO and substrate for further conjugation cycles. SUMO proteases have a second role in the sumoylation cycle. They are also necessary to replenish the SUMO pool with newly synthesized protein, because all SUMO genes encode precursor proteins with carboxyl-terminal extensions, which have to be cleaved for release of mature SUMO. The two roles may, however, be fulfilled preferentially by different proteases that are related in sequence. In the yeast *S. cerevisiae*,

the two SUMO proteases have distinguishable tasks, and only one enzyme is essential for maturation of newly synthesized SUMO (Li and Hochstrasser 1999, 2000; Schwienhorst et al. 2000). Functional differences in SUMO proteases of higher eukaryotes have been extensively studied in mammals, and frequently pertain to differences in subcellular localization (Hay 2007; Yeh 2009). An additional complexity is the occurrence of several different SUMO isoforms in both animals and plants (Novatchkova et al. 2004; Saracco et al. 2007; Budhiraja et al. 2009; van den Burg et al. 2010). Differences in proteolytic activity towards different isoforms have been found for plant SUMO proteases (Chosed et al. 2006; Colby et al. 2006; Budhiraja et al. 2009). In this work, we investigate the SUMO protease from *Arabidopsis thaliana* that is most similar to ESD4, a previously characterized SUMO protease with a role in plant growth and flower initiation (Reeves et al. 2002; Murtas et al. 2003). The ESD4 homolog, ESD4-LIKE SUMO PROTEASE 1 (ELS1), has a distinct role as shown by mutant phenotype, subcellular localization and complementation of yeast SUMO protease mutants. We also show that the reduced growth of *esd4* mutants is not due to increased levels of salicylic acid.

Materials and methods

Plant genotypes and growth conditions

Plant lines used in this work are: *SIZ1*, At5g60410: *siz1-2*, *siz1-3* (Miura et al. 2005); *ESD4*, At4g15880: *esd4-2* (Murtas et al. 2003); *ELS1*/AtULP1a, At3g06910: *els1-1*, 201D11 FLAGdb; *els1-2*, SAIL_318_C01. SID2, At1g74710: *sid2-1* (Wildermuth et al. 2001). *sid2-1* mutants and a *nahG* expressing transgenic line were kindly provided by M. Bartsch and Dr. J. Parker (MPIPZ Cologne, Germany). Genotyping of mutant plants was done by PCR reactions with leaf DNA, using the following primers: *siz1-2*, GTG GAG GTG GAG ATG ATA ATG CC (459A) together with TGG TTC ACG TAG TGG GCC ATC G (SALK LBa1) to detect the T-DNA insertion allele, and 459A together with CCG AGT CAA TGG AGA GGT ACA TC to detect the WT allele; *siz1-3*, GGT CTT CAG GCT CTC CCT TAA CT (439A) together with SALK LBa1 to detect the T-DNA insertion allele, and 439A together with GAC TAA TTG CTG GAG TTT AGG TTC to detect the WT allele; *esd4-2*, CTA ATG GGT GCC GTA GCG ATC ATT C (461A) together with SALK LBa1 to detect the T-DNA insertion allele, and 461A together with TAT CTG CAG AGG GCA CAG ACT AAG TT to detect the WT allele; *els1-1*, CCA ATC TAG GGT TTT GAA CTC TGA ATT (462A) together with CAG TCA TAG CCG AAT AGC CTC TCC A (Fish2) to detect the T-DNA insertion allele, and 462A together with CAC AAC GAG TGA ACT ACT

ATT AGC TT (463A) to detect the WT allele; *els1-2*, 462A together with GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C to detect the insertion allele, and 462A together with 463A for WT allele detection; *sid2-1*, PCR for 40 cycles using primers GCA GTC CGA AAG ACG ACC TCG AG and CTA TCG AAT GAT TCT AGA AGA AGC, followed by *Mun* I digestion of the ensuing fragment (the mutant allele *sid2-1* cannot be digested). The T-DNA insert position in mutant *els1-1* was determined by sequencing of the diagnostic PCR fragment indicated above to be …T-DNA–GGG ATT TTG ACC… (the first G abutting the T-DNA sequence being nucleotide 38 after the start codon). The T-DNA insert position of mutant *els1-2* was estimated from the size of the diagnostic PCR fragment, in agreement with the information provided at http://signal.salk.edu/cgi-bin/tdnaexpress. Plants used for determination of flowering time were germinated and grown on soil in 16 h light and 8 h dark cycles (long day), or 8 h light 16 h dark cycles (short day) in controlled environment at 25°C.

Vector constructs

ELS1 cDNA was cloned from mRNA by reverse transcription and PCR, and inserted into vector pDONR201 (Invitrogen, Darmstadt, Germany). From there, the ORF was recombined into vector pDEST17 (Invitrogen) for protein expression in *E. coli* (pDEST17-ELS1). A similar clone with a mutation to convert active site Cys into Ser (TGT to AGT change; pDEST17-ELS1-C461S) was made using standard methods. GFP fused to the carboxyl terminus of ELS1 (ELS1–GFP) was expressed in vector 35S-GW-GFP [a Gateway-compatible plant binary vector with 35S promoter, GFP cassette and conferring kanamycin resistance in plants; a kind gift of Dr. Franziska Turck, MPIPZ, Cologne, Germany) and in vector p3 (a plant binary vector containing a CaMV 35S promoter supplied with two additional enhancer regions (triple 35S promoter), and conferring hygromycin resistance in plants (Yin et al. 2007)]. A construct for expression of the *ELS1* promoter fused to GUS used vector pGPTV8-GW-GUS (a binary vector with a Gateway cassette in front of the GUS open reading frame; a kind gift of Dr. Hailong An, MPIPZ, Cologne, Germany). A 2.5-kb promoter fragment of *ELS1* ending at the start codon, cloned via PCR into vector pDONR207 (Invitrogen) using oligonucleotides GTC TTC ATT TCC TCC AGC and ACT CTC GGG AAG CAA GCA AG was sequenced and linked to the GUS open reading frame.

ELS1 localization

The *ELS1*p-GUS construct in vector pGPTV8 was transformed into Arabidopsis using floral dip (Clough and Bent 1998), and used for GUS staining by standard methods. ELS1–GFP in vector 35S-GW-GFP was used for floral dip of Arabidopsis, whereas ELS1–GFP in vector p3 was used for *Nicotiana benthamiana* agroinfection (Bucher et al. 2003). Tissue samples were analyzed using a Leica SP2 AOBS confocal laser scanning microscope.

Experiments in *Saccharomyces cerevisiae*

The congenic yeast strains used were KU1 (*ulp2Δ*) and YKU8 (*ulp1-2*). Both mutations cause temperature sensitivity of growth. The *ulp1-2* mutation was identified in a screen selecting for spontaneous mutations that suppressed the temperature-sensitive phenotype of the *ulp2Δ* mutation (K. Uzunova, Univ. of Cologne, Germany and R.J. Dohmen, unpublished results). For complementation studies, *ulp2Δ* and *ulp1-ts* cells were transformed with *CEN/URA3*-based plasmids expressing ESD4 or ELS1 from the galactose-inducible promoter P_{GAL1} or its weaker derivative P_{GALS}. For expression of ESD4, the cDNA was excised as an *Xba*I/*Bam*HI fragment from pBLUESCRIPT-ESD4 and cloned into p416GAL1 or p416GALS (Mumberg et al. 1994). The ELS1 cDNA was cloned into the same vector after fusing it to *Bam*HI and *Eco*RI restriction sites in a PCR reaction that used pDONR201 as a template. To score for complementation, transformants were streaked onto media containing 1% yeast extract, 2% peptone and 2% galactose, followed by incubation at 35°C for 2 days.

Protein purification and in vitro activity assays

ELS1 was purified after expression of a pDEST17 construct using a hexahistidine tag and standard procedures. Incubations for protease activity were carried out as described previously (Murtas et al. 2003).

Protein blotting and antisera

Detection of bands on Western blots was carried out with the ECL detection system (GE Healthcare, Munich, Germany), using anti SUMO1 antibody as described (Murtas et al. 2003) to detect SUMO conjugates, or anti hexahistidine antibody (Qiagen, Hilden, Germany) to detect SUMO fusion and cleavage products. Anti SUMO3 antibody was from Abcam (Cambridge, UK).

RNA gel-blot analysis and RT-PCR

RNA gel blotting was carried out using standard methods. The ELS1 probe was generated using primers ATG AAA AAC CAA TCT AGG G and CAT TAG CTG AGA ATG CAC G. Transcript level of tubulin served as a loading control (probe generated with PCR primers ACA CCA GAC ATA GTA GCA GAA ATC AAG and ACT CGT TGG

GAG GAG GAA CT). RT-PCR experiments were carried out as described before (Yin et al. 2007), using primers TTG GAG ACA AGA TGA AGA ACC A and TTG AGA TGG TAG CCC AAC CT to quantitate *ELS1* mRNA, and tubulin-specific primers as a control.

Statistical evaluation of leaf number at the time of flowering

A comparison of mean values for the two genotypes (WT vs. *els1-1*) by a *t* test gave the following results: LD total leaves, $\tau = 1.14$; LD rosette leaves, $\tau = 2.16$; LD cauline leaves, $\tau = 3.00$; SD total leaves, $\tau = 1.30$; SD rosette leaves, $\tau = 1.17$; SD cauline leaves, $\tau = 2.62$. With a *t* value of 2.08 for 20 degrees of freedom and $P = 95\%$, and 2.85 for $P = 99\%$, it follows that none of the differences was significant at the 99% level, except for cauline leaves under LD conditions. In the latter case, however, the magnitude of the difference was rather modest (0.6 leaves).

Results

Characterization of SUMO protease ELS1/AtULP1a

We have previously shown that mutations in the SUMO-specific protease *ESD4* (At4g15880) cause early flowering and reduced growth of *A. thaliana* plants (Reeves et al. 2002; Murtas et al. 2003). The closest homolog of *ESD4* in *A. thaliana* is At3g06910. This *ESD4*-like gene (called *ESD4 LIKE SUMO PROTEASE1*, *ELS1*, in the following; also termed AtULP1a; Kurepa et al. 2003; Novatchkova et al. 2004) codes for a putative SUMO protease. Figure 1a shows a domain diagram of ESD4, ELS1 and the yeast SUMO proteases Ulp1 and Ulp2. A protein sequence alignment (Fig. 1b; Suppl. Fig. S1) indicates that similarity between ESD4 and ELS1 is highest in the carboxyl-terminal protease domain (active site Cys 461 marked by a dot), but also extends towards the amino terminus of the proteins. The ELS1 coding sequence is distributed over a larger 5′ exon and eight smaller downstream exons (Fig. 1c). Figure 2a shows the pattern of GUS activity in a plant expressing an *ELS1* promoter–GUS fusion transgene, indicating that the gene is ubiquitously expressed, with high accumulation in the vasculature and in roots. That both of these tissues have a highly active SUMO conjugation system is also suggested by the recent finding that SUMO1 is highly expressed in roots, and SUMO2 in the vasculature (van den Burg et al. 2010). In order to determine expression at later stages of development, tissue of WT plants was harvested for determination of *ELS1* mRNA abundance by RT-PCR. The results shown in Fig. 2b indicate that expression of *ELS1* mRNA is high in flowers, and
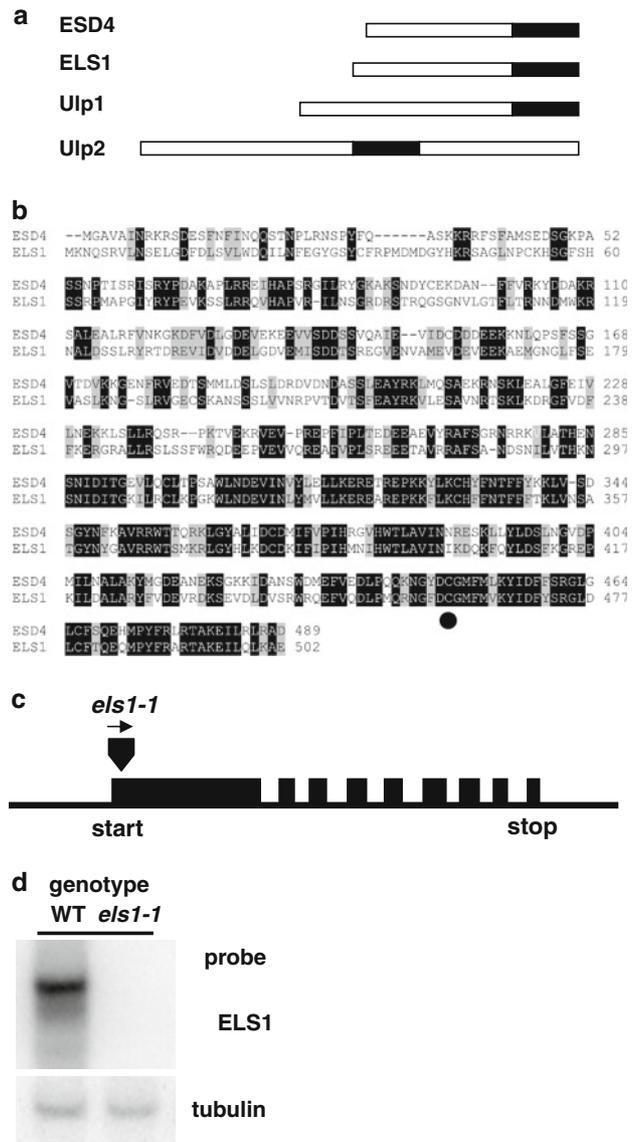

Fig. 1 ELS1 protein alignment and gene structure. **a** ESD4, ELS1 and *S. cerevisiae* Ulp1 are similar in the carboxyl-terminal protease C48 domain (*black box*), and contain an amino-terminal extension. **b** Protein sequence alignment of SUMO proteases EDS4 (*top*) and ELS1 (*bottom*). Similarity is most extensive in the carboxyl-terminal catalytic domain, but encompasses the entire length of the proteins. *Dot* indicates position of the active site Cys residue. *Black background* was used for identical residues, *grey* for conserved changes. **c** Schematic drawing of the intron–exon structure of *ELS1*. The T-DNA insertion in mutant *els1-1* lies in the first exon (*thick vertical arrow*). **d** RNA gel blot to demonstrate that T-DNA insertion mutant *els1-1* does not express full length ELS1 mRNA. WT, wild type

persists in siliques, which have an expression level comparable to leaves.

To functionally compare *ELS1* with *ESD4*, we determined the intracellular localization of ELS1. Fusion proteins of ELS1 to GFP, carrying GFP either at the carboxyl terminus, or at the amino terminus, were made and cloned
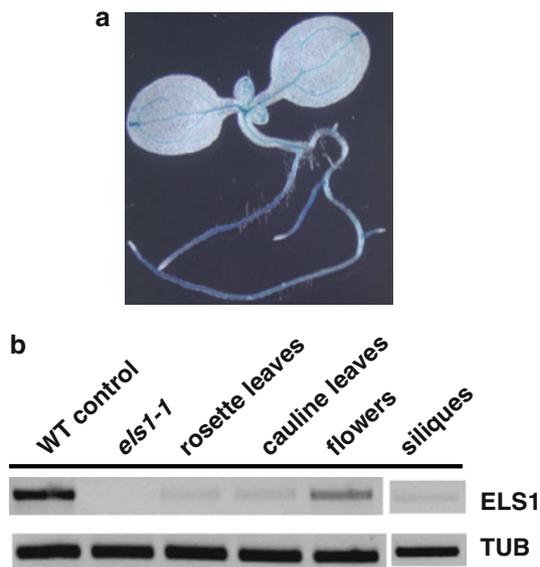
Fig. 2 Expression domains of ELS1. **a** Expression of ELS1 was assessed with a fusion transgene containing 2.5 kb of *ELS1* upstream region, fused to the GUS reporter. Staining for GUS activity in a seedling indicates ubiquitous expression, with high levels in the vasculature and in roots. **b** RNA from mature plants was used for determination of *ELS1* expression in different tissues by reverse transcription-polymerase chain reaction (RT-PCR). Expression is high in flowers, compared to leaves and siliques. *TUB* tubulin control

into binary expression vectors. Constructs were expressed in Arabidopsis by stable transformation, and in *Nicotiana benthamiana* leaves by agroinfection (Bucher et al. 2003). Transgenic Arabidopsis seedlings display ELS1–GFP fluorescence in the cytoplasm of root cells (Fig. 3a–d). Because of the generally weak expression, we also investigated distribution in *N. benthamiana*. In this heterologous host, the majority of the ELS1–GFP fusion protein is also associated with the cytoplasm and/or the endo-membrane system (Fig. 3e–g), and is absent from the nucleus (stained by propidium iodide, red color). In some cases, a faint ELS1–GFP signal was found in the nucleolus (data not shown). While sumoylation is essential for ribosome biogenesis in yeast (Panse et al. 2006), and human SUMO proteases SENP3 and SENP5 are nucleolar proteins (for reviews, see Hay 2007; Yeh 2009), we cannot rule out that the faint nucleolar fluorescence is a side effect of overexpression, or due to the use of a heterologous host. Similarly, a GFP–ELS1 construct had a generally non-nuclear localization, being detectable at membranes and in the cytoplasm (data not shown). In contrast, previous experiments had demonstrated that ESD4 localizes to the nuclear envelope or lamina (Murtas et al. 2003; Xu et al. 2007). We therefore conclude that ELS1 and its closest homolog ESD4 have distinct cellular localizations.

We next tested whether ELS1 is a SUMO-specific protease. The ORF was expressed in *E. coli* with a hexahistidine tag, purified and used in an in vitro proteolysis assay as previously described (Murtas et al. 2003). Figure 4 shows that ELS1/AtULP1a can cleave sequences from the carboxyl terminus of SUMO1 (At4g26840). The protein is sensitive to *N*-ethyl maleimide (NEM), a broad specificity inhibitor of cysteine proteases. Furthermore, substitution of the predicted catalytic center Cys at position 461 by Ser resulted in an inactive protein (Fig. 4, third lane). These results demonstrate that ELS1/AtULP1a acts as a SUMO protease in vitro and confirm the experiments of Chosed et al. (2006).

An insertion mutant in *ELS1* was obtained from the FLAGdb collection (line 201D11, Ws genetic background; Samson et al. 2002). Sequencing of a PCR-generated genomic fragment revealed the position of the T-DNA in the first exon, which interrupts the Gly codon of position 13 (Fig. 1b, c; "Materials and methods"). The mutant was called *els1-1*. The putative loss of gene function in the *els1-1* mutant was indicated by the absence of mRNA in an RNA gel blot experiment (Fig. 1d). The appearance of *els1-1* mutants is generally similar to WT plants, except for slightly reduced growth (Fig. 5) and thinner stems. Staining of tissue sections suggested that the vasculature of the *els1-1* mutant is less developed (data not shown). However, the relationship of this trait to *ELS1* remains to be determined, because mutant allele *els1-2*, which carries a T-DNA insertion in *ELS1* in the Col-0 background, does not lead to thin stems. Interestingly, it was recently reported that the phenotype of a mutation in a ubiquitin-specific protease depended strongly on the genetic background (Schmitz et al. 2009). Protein gel blot experiments indicated that, unlike *esd4* mutants, the T-DNA insertion in *els1-1* did not cause a gross change in intracellular abundance of SUMO conjugates (Fig. 6, lanes 1–3), suggesting a more limited role in SUMO regulation.

We determined the time of flowering of WT and *els1-1* plants by measuring leaf numbers (Table 1). Statistical analysis (cf. "Materials and methods") indicated no significant difference in the number of rosette leaves, or in total leaf number. The number of cauline leaves formed before flowering under long-day conditions differs, but the magnitude of the difference is small (0.6 leaves; Table 1; "Materials and methods"). The same results were obtained by comparing mutants carrying another allele, *els1-2*, with its WT accession Col-0 (data not shown). In contrast, a strong, statistically significant difference in flowering time between WT and *esd4* plants was previously reported (Reeves et al. 2002). We conclude that, unlike *ESD4*, *ELS1* is not a determinant of flowering time.

Complementation of yeast (*S. cerevisiae*) mutants by expression of Arabidopsis SUMO proteases

Saccharomyces cerevisiae has two SUMO-specific proteases, Ulp1 and Ulp2 (Fig. 1a). They have the same type of

Fig. 3 Subcellular localization of ELS1 protein. **a–d** ELS1–GFP fusion protein was expressed in Arabidopsis and assessed by microscopy. Images show the hypocotyl–root transition of a young seedling. **a** *Red channel* detects chloroplasts of the hypocotyl. **b** *Green channel* detects ELS1–GFP throughout the cytoplasm. **c** Phase contrast image. **d** Overlay image. **e–g** Section of an *N. benthamiana* leaf cell after transient expression of ELS1–GFP. ELS1–GFP localizes to endomembranes and to the cytoplasm, but not to the nucleus (panel **e**). Consequently, staining of nuclear DNA by propidium iodide (**f**) does not overlap with ELS1–GFP expression domains (**e**). Note that there is a gap between the membrane decorated by ELS1-GFP (*green*), and the stained DNA (*red*), indicating that the nuclear periphery does not contain ELS1–GFP (overlay image **g**)
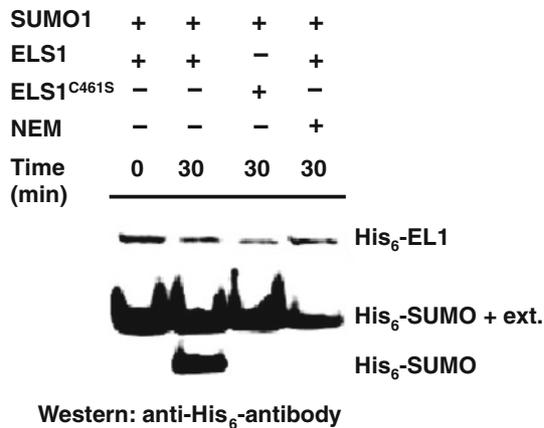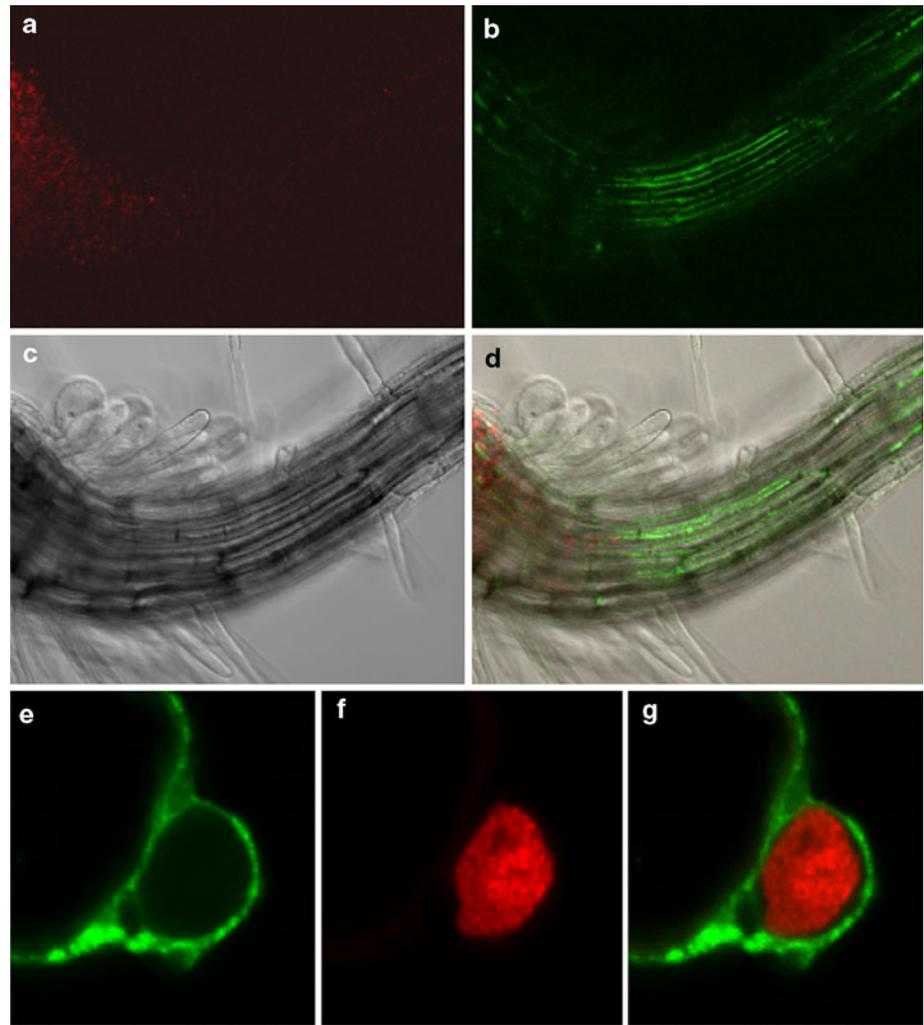

Fig. 4 Desumoylation activity of ELS1. ELS1, or its C461S derivative, in which the predicted active site cysteine was mutated, and SUMO1 with carboxyl-terminal extension were purified from *E. coli* via amino-terminal His tag and combined for incubation. Aliquots from these reactions were subjected to Western blotting with anti hexahistidine antibody. ELS1, but not ELS1[C461S], was capable of cleaving the extension (ext.) off the carboxyl terminus of SUMO1

protease domain, but different amino-terminal domains. Ulp2 has, in addition, a carboxyl-terminal extension following the catalytic domain. Both ELS1 and ESD4 have an amino-terminal (but no carboxyl-terminal) extension and based on their sequence may therefore be grouped with yeast protease Ulp1, rather than with Ulp2 (Novatchkova et al. 2004). We cloned the ELS1 and ESD4 ORFs into plasmids for expression in yeast, using two types of vectors. One vector, carrying the galactose-inducible GAL1 promoter, allows a relatively high expression level. The other vector expresses the Arabidopsis SUMO proteases under control of a truncated variant of the GAL1 promoter, which results in attenuated galactose-inducibility, while maintaining tight repression by glucose (Mumberg et al. 1994; GALS in Fig. 7). Complementation of mutations in either *ULP1* or *ULP2* was tested using both vectors. *ULP1* is essential, and the complementation assay used the temperature sensitivity of growth of an *ulp1-ts* mutant with similar phenotypes as reported previously for another *ulp1* allele (Li and Hochstrasser 1999). In contrast, mutants with
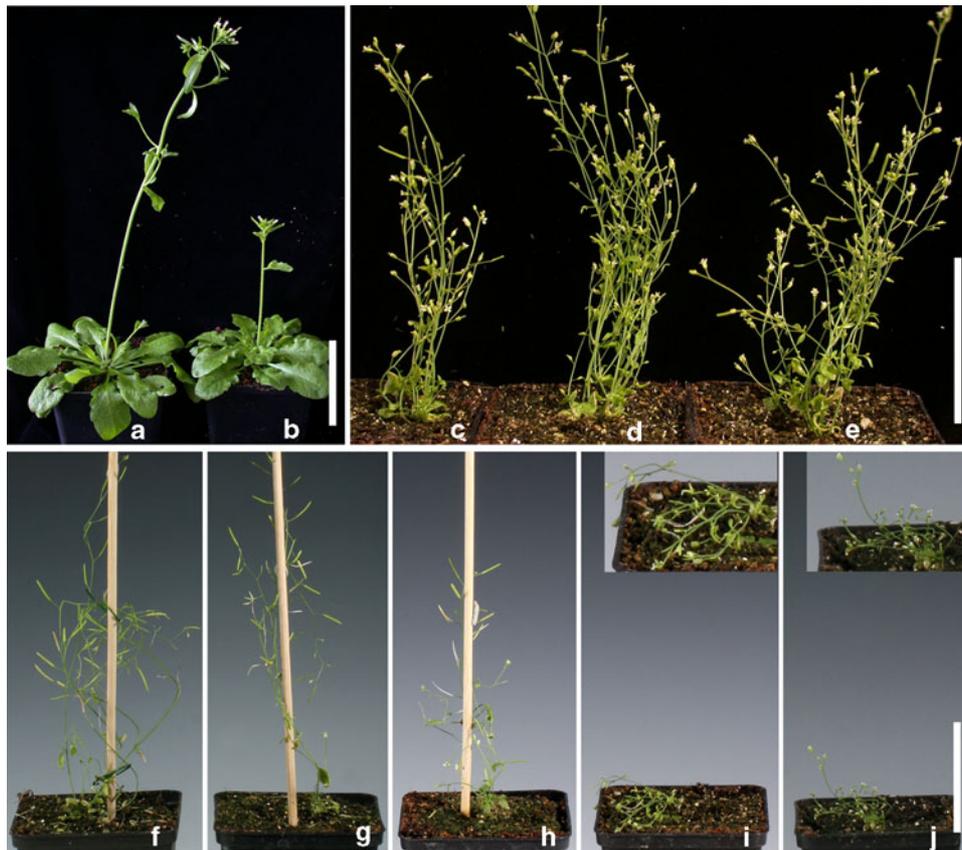
Fig. 5 Growth habit of mutants impaired in activity of components of SUMO conjugation and de-conjugation. **a**, **b** WT plant (**a** accession Ws) next to *els1-1* plant (**b**) after growth in short day conditions. **c**–**e** A mutation in the *ELS1* gene does not change the growth characteristics of *esd4-2* mutants. Two *esd4-2/esd4-2 ELS1/els1-1* plants (**c**, **e**), and one *esd4-2 els1-1* double mutant (**d**) are siblings of the same *esd4-2/esd4-2 ELS1/els1-1* mother plant. Differences in growth between plants homozygous or heterozygous for the *els1-1* mutation are small, compared to effects of genetic background or environment (plant age ca. 16 weeks). **f**–**j** A block in salicylic acid biosynthesis by mutation *sid2-1* does not alleviate dwarfism of *esd4* mutants. **f** WT plant, **g** *sid2-1* mutant, **h** plant homozygous for *sid2-1*, and heterozygous for the *esd4-2* mutation; **i** *sid2-1 esd4-2* double mutant, **j** *esd4-2* single mutant. Plants were grown in parallel under the same conditions for 5 weeks, *insets* in panels **i** and **j** are close-ups. *Scale bars* represent 5 cm

a deletion of the *ULP2* gene are viable, but sensitive to a variety of stresses including elevated temperature (Li and Hochstrasser [2000](#); Schwienhorst et al. [2000](#)). The temperature-sensitive phenotype of a ULP2 deletion allele (*ulp2Δ*) was used to score complementation by an Arabidopsis cDNA. Figure [7](#) shows that *ELS1* can complement the *ulp1-ts* mutant, whereas the *ESD4* cDNA cannot. In striking contrast, the *ulp2Δ* temperature sensitivity is not complemented by *ELS1*, but by *ESD4* cDNA, when expressed from a weak promoter. We therefore conclude that ELS1 and ESD4 differ in their ability to complement yeast mutants. While it is the essential function of ULP1 to generate mature SUMO, ULP2 predominately acts on polymeric SUMO chains (Li and Hochstrasser [1999](#); Schwienhorst et al. [2000](#); Bylebyl et al. [2003](#)). These results suggest that in vivo ELS1 has a similar role in the SUMO cycle to ULP1 while ESD4 has that of ULP2.

Double mutant analysis of Arabidopsis SUMO proteases ESD4 and ELS1

Lack of co-localization and distinct mutant phenotypes of *els1* and *esd4* mutants makes direct functional redundancy or overlap in function unlikely, but does not exclude all forms of mutual influence. In order to reveal interactions between *ELS1* and *ESD4*, we constructed the double mutant *esd4-2 els1-1*. *esd4-2* is a loss of function mutation in the Col-0 background (Murtas et al. [2003](#)) and apparently affects growth and fertility of plants more severely than the *esd4-1* allele, which is in the Ler background. *esd4-2* plants are almost completely sterile under our standard growth conditions. A double heterozygous *esd4-2 els1-1* plant was backcrossed to Col-0, and F2 plants were genotyped. Both *esd4-2 els1-1* double homozygous, and *esd4-2/esd4-2ELS1/els1-1* plants could be identified. *esd4-2/esd4-2 ELS1/els1-1*
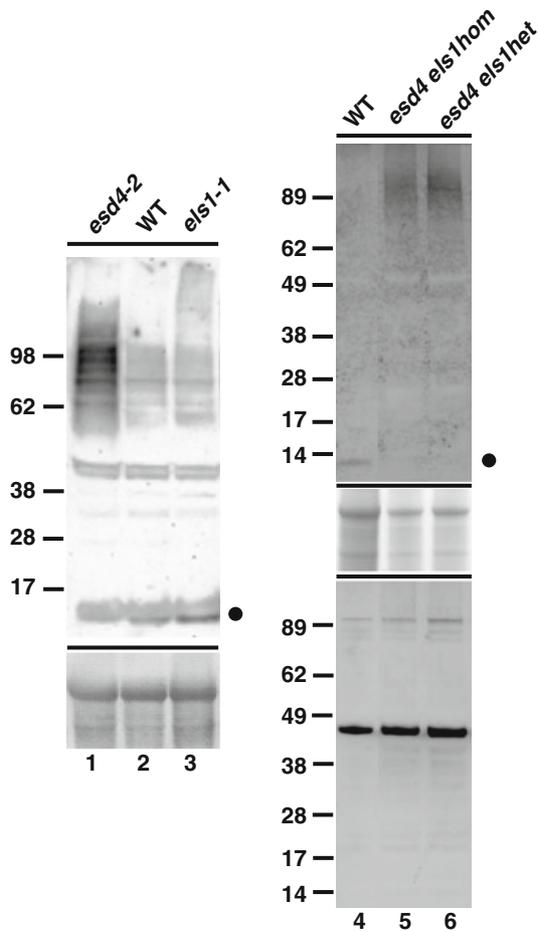
Fig. 6 Determination of in vivo SUMO conjugate levels. Lanes *1–3* extracts of WT (accession Ws), of *esd4*, and of *els1-1* mutant leaves were used for a protein gel blot probed with anti SUMO1 antibody. Whereas SUMO conjugate levels are significantly elevated in *esd4* cells, no significant change compared to the WT was detected in *els1-1*. A section containing the Rubisco large subunit band of a filter stained with Ponceau S is shown below the gel as a loading control. Lanes *4–6* extracts from WT (accession Col-0), from *esd4-2 els1-1* double mutants, and from plants homozygous for *esd4-2* and heterozygous for the *els1-1* mutation were compared by blotting and hybridization with anti SUMO1 antibody (*top panel*), and anti SUMO3 antibody (*bottom panel*). The *middle panel* shows a control gel stained with Coomassie blue to document protein concentration in the extracts. Molecular weight marker positions are shown to the *left of the blots*. A *dot* indicates position of free SUMO1

plants carried more seeds than *esd4-2* single mutants. Progeny plants of *esd4-2/esd4-2 ELS1/els1-1* parents were grown and genotyped. Interestingly, 36 of 38 plants were again *ELS1/els1-1*, indicating that the *els1-1* mutation, if present in one of the two gametes (we suspect the male gametes), acts as a fertility restorer. However, apart from fertility, there is little difference in greenhouse growth habit between *esd4-2*, *esd4-2 els1-1* and *esd4-2/esd4-2 ELS1/els1-1* plants. Figure 5c–e shows a comparison of two *esd4-2/esd4-2 ELS1/els1-1* plants (plants c, e), and one *esd4-2 els1-1* double homozygote (plant d). Growth differences due to the mixed (Ws/Col-0) genetic background and/or to environmental influences exceed those caused by loss of *ELS1* function.

We also determined the level of SUMO conjugates in *esd4-2 els1-1* double mutants and in sibling plants with genotype *esd4-2/esd4-2 ELS1/els1-1* (Fig. 6, lanes 5, 6). Consistent with the results shown in Fig. 6, lanes 1–3, which indicate only a minor influence of the *els1-1* mutation on SUMO conjugate levels, the double mutant does not have more SUMO conjugates than the single *esd4* mutant.

Growth defect of *esd4* mutant plants is SA-independent

In contrast to ELS1, SUMO protease ESD4 is a determinant of flowering time (Reeves et al. 2002). As recently reported, mutants in SUMO ligase SIZ1 (At5g60410) are also early flowering (Jin et al. 2008). Whereas *siz1* mutants are impaired in SUMO conjugation, *esd4* mutants accumulate SUMO conjugates, presumably as a consequence of decreased SUMO removal from substrates. Correct flower initiation may therefore require both efficient sumoylation and efficient de-sumoylation. Yet another similarity between *siz1* and *esd4* mutants is that both have a small stature, indicating significant growth retardation. It was recently shown that *siz1* plants have elevated levels of salicylic acid, and that their stunted growth is largely abolished if the *nahG* reading frame, encoding a salicylate hydroxylase from *Pseudomonas* bacteria, is expressed in these mutants (Lee et al. 2007; Jin et al. 2008). We were therefore interested in whether the reduced growth of *esd4* mutants is also

Table 1 Flowering time of *els1-1* mutants

Genotype	Condition	Number of rosette leaves	Number of cauline leaves	N
WT Ws	LD	9.6 ± 0.5	3.3 ± 0.2	10
els1-1	LD	10.8 ± 0.2	2.7 ± 0.1	12
WT Ws	SD	63.0 ± 10	7.6 ± 0.2	11
els1-1	SD	49.0 ± 7	5.8 ± 0.5	11

Flowering time was determined as the number of leaves present at the time of opening of the first flower. Values are mean ± standard error of the mean. Further statistical analysis of these data is shown in "Materials and methods"

N number of plants assayed, *Ws* accession Wassilewskija, *LD* refers to long-day conditions with 16 h light, 8 h darkness; SD refers to short day conditions with 8 h light and 16 h darkness

suppressed by a decrease in intracellular SA levels. To that end, the *esd4-2* mutation was combined with a *nahG* transgene, and, in separate crosses, with the *sid2-1* mutation, which affects SA biosynthesis by abolishing isochorismate synthase 1 activity (Wildermuth et al. 2001; Strawn et al. 2007). Analysis of the contribution of SA to the growth habit of *esd4* mutants indicates an independence of SA, as judged by both crosses (Fig. 5; data not shown). We therefore concluded that *esd4* and *siz1*, two mutations in nuclear-localized components of the sumoylation machinery, influence plant growth by distinct mechanisms, because growth regulation by ESD4 is SA-independent.

Discussion

In this work, we investigated a protease from the Arabidopsis sumoylation cascade. Arabidopsis encodes more than 65 reading frames with the C48 cysteine protease fold, which encompasses the SUMO-specific proteases (Novatchkova et al. 2004; van der Hoorn 2008). Only a limited number of them have been tested for activity towards SUMO (Murtas et al. 2003; Chosed et al. 2006; Colby et al. 2006). ESD4, the first characterized SUMO protease, has an important role in flower initiation and plant growth (Reeves et al. 2002; Murtas et al. 2003).

We investigated the closest relative of SUMO protease ESD4, ELS1 (termed AtULP1a by Kurepa et al. 2003), and show that it is an active SUMO protease (Fig. 4), confirming previous results by Chosed et al. (2006). Alignment of the ESD4 and ELS1 protein sequences indicates that similarity extends beyond the carboxyl-terminal catalytic domain towards the amino terminus (Fig. 1, Suppl. Fig. S1). ELS1 is ubiquitously expressed (Fig. 2). Its localization was determined by expression of ELS1–GFP and GFP–ELS1 fusion proteins in Arabidopsis and *N. benthamiana* (Fig. 3). Despite the overall similarity between ELS1 and ESD4, ELS1 localization is distinct from that of ESD4, which localizes to the nuclear periphery (Murtas et al. 2003; Xu et al. 2007). In contrast, ELS1 is mostly cytoplasmic, possibly associated with endo-membranes. While there was occasionally a weak signal detectable in the nucleolus, the protein was absent from all other parts of the nucleus, as opposed to its abundant presence in the cytoplasm. Expression in the homologous host, and the transient heterologous expression data are therefore strong indicators that ELS1 and ESD4 operate in different cellular compartments.

Another indicator of the functional difference between ELS1 and ESD4 is that *ELS1* loss of function has only mild phenotypic consequences, whereas *esd4* mutant plants are severely dwarfed (Fig. 5). ESD4 and ELS1 open reading frames were also expressed in *S. cerevisiae* cells with mutation in either of the two yeast SUMO proteases, ULP1 or ULP2. Whereas both ESD4 and ELS1 belong to the same structural group as yeast Ulp1 (Fig. 1a), only ELS1 can complement the essential function of Ulp1 in growth promotion (Fig. 7). It may seem surprising that ESD4 cannot complement, because both ESD4 in the plant, and ULP1 in yeast, localize to the nuclear periphery, whereas ELS1 is non-nuclear in the plant expression systems, and is therefore not likely to localize to the nuclear periphery upon expression in yeast. Interestingly, the requirement for Ulp1 can be bypassed by expression of mature SUMO in yeast, suggesting that an essential function of Ulp1 is its participation in SUMO maturation (Li and Hochstrasser 1999; Schwienhorst et al. 2000). If ELS1 can, in yeast, assist in generation of mature SUMO by proteolytic removal of the carboxyl-terminal extension of the primary translation product, it may complement the *ulp1-ts* mutant independent of its localization. If this explanation holds true, it follows that ESD4 is less able to assist in yeast SUMO maturation, although it can carry out this reaction in vitro (Murtas et al. 2003). Conversely, loss of ULP2, the yeast SUMO protease residing in the nucleoplasm, can be complemented by expression of ESD4 at low levels (Fig. 7). Neither ESD4 at high expression levels, nor ELS1 can complement the *ulp2Δ* mutation. The lack of complementation at high ESD4 expression levels may be attributed to a toxicity
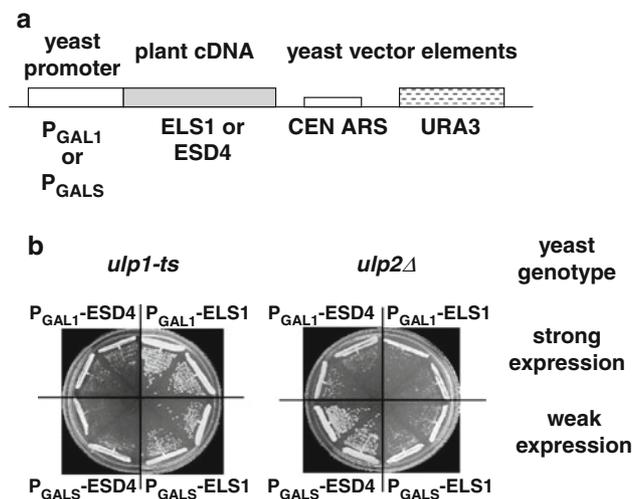

Fig. 7 Complementation of yeast mutants in SUMO proteases by Arabidopsis proteins. **a** Schematic drawing of the constructs used. An inducible yeast promoter (either strong or weak) is followed by Arabidopsis SUMO protease cDNA of either ESD4, or ELS1. The vectors also contain sequences for selection (URA3) and single copy propagation (CEN ARS) in yeast. **b** Growth of yeast mutants carrying either a ts allele of *ULP1* or lacking the *ULP2* gene is normally impaired at elevated temperature. In contrast, ELS1 (but not ESD4) supports growth of *ulp1-ts* mutant cells, and low levels of ESD4 (but not ELS1) support growth of *ulp2Δ* mutant cells at elevated temperature. Strains were grown for 2 days at the semi-permissive temperature of 35°C

effect, similar to the finding that high level expression of full length or truncated versions of yeast Ulp1 is toxic to yeast cells (Li and Hochstrasser 2003; Panse et al. 2003). These results might indicate that in *A. thaliana* the major role of ESD4 is SUMO recycling from substrates while that of ELS1 is SUMO maturation.

The double mutant *els1 esd4* was also constructed. As shown in Fig. 5, the double mutant looks similar to the *esd4* single mutant. This result is consistent with the other observations, pointing to distinct roles. The purpose of this experiment was to reveal more subtle interactions between the two SUMO proteases. For instance, the two SUMO-specific proteases of baker's yeast, Ulp1 and Ulp2, do not co-localize, yet a double mutant in both genes does not show the sum of both single mutant phenotypes, but is actually less severely affected than one of the single mutants (Li and Hochstrasser 2000). As ELS1 and ESD4 showed functional complementarity in the yeast assay, a similar role might be observed in the Arabidopsis double mutant. We found that the growth habit of *esd4* plants was unaffected by the simultaneous presence of the *els1-1* mutation, but that one of the germ lines (presumably the male germ line) displayed higher viability as *esd4 els1* double mutant, than as *esd4* single mutant, leading to a segregation distortion of the *els1* marker in the *esd4* homozygous background. The pattern of SUMO1 conjugates was also similar between the *esd4-2* single, and the *esd4-2 els1-1* double mutant. Neither mutation influences the pattern of SUMO3 conjugates (Fig. 6).

We also investigated whether the dwarf phenotype of *esd4* mutants is due to increased levels of salicylic acid, because decreased growth of Arabidopsis mutants can be due to induction of (biotic or abiotic) stress responses, and sumoylation has already been linked to stress response regulation. It was also recently found that decreased growth of mutants in another gene of the sumoylation cascade, the *SIZ1* SUMO ligase, can be suppressed by decreasing endogenous SA levels. The SA levels in *esd4* mutants therefore impinge on the question whether the increase in SUMO conjugates in the *esd4* mutant affects the same processes as the decrease caused by a *siz1* mutation. Preliminary characterization of *esd4 siz1* double mutant plants indicated that this double mutant has the growth characteristics of *esd4* single mutants, suggesting that the two mutations do not mitigate each others' effects (R. Hermkes and A. Bachmair, unpublished results).

A possible role of altered SA levels in the *esd4* mutant was assessed by expression of a *nahG* gene, which degrades SA to catechol, thereby decreasing the SA concentration. However, it was suggested that independent confirmation of results from *nahG* experiments is necessary, because catechol has biological activity and may therefore influence the outcome (van Wees and Glazebrook 2003). Therefore,

we investigated SA dependence of growth by generation of both *esd4-2 nahG* plants, and *esd4-2 sid2-1* plants. The *SID2* gene is essential for SA biosynthesis (Wildermuth et al. 2001; Strawn et al. 2007), and its absence drastically reduces SA levels (without production of catechol). The finding that *esd4-2* growth retardation is not suppressed by decreasing SA levels (Fig. 5, panels f–j) implies that excess SA is not the cause of the growth defects of *esd4* mutants. Therefore, although both *esd4* and *siz1* mutants affect the same pathway and show reduced growth, the molecular mechanisms underlying this phenotype are likely to be distinct.

In summary, mutations in sumoylation components ESD4 and ELS1, as well as in SIZ1, have unique consequences for plants, even though these genes operate in the same pathway. ELS1 may be redundant with other de-sumoylating proteases because of the relatively weak and accession-dependent phenotype of the *els1* mutation, but the closest homolog, ESD4, has little or no functional overlap with ELS1.

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