# SUMOYLATION IN PLANTS: MECHANISTIC INSIGHTS AND ITS ROLE IN DROUGHT STRESS

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Keywords: SUMO, drought, subcellular localization, conjugation machinery, diversification, abiotic stress, SUMO proteases
Running title: SUMO in drought

**Abstract**

Post-translational modification by SUMO is an essential process that has a major role in the regulation of plant development and stress responses. Such diverse biological functions are accompanied by functional diversification among the SUMO conjugation machinery components and regulatory mechanisms that has just started to be identified in plants. In this review, we focus on the current knowledge of the SUMO conjugation system in plants in terms of components, substrate specificity, cognate interactions, enzyme activity and subcellular localization. In addition, we analyze existing data on the role of SUMOylation in plant drought tolerance in model plants and crop species, we discuss the genetic approaches used in order to stimulate or inhibit endogenous SUMO conjugation. The role that potential SUMO targets identified in proteomic analyses may have in drought tolerance is also discussed. Overall, the complexity of SUMOylation and the multiple genetic and environmental factors that are integrated to confer drought tolerance highlight the need for significant efforts to understand the interplay between SUMO and drought.

## Introduction

Plants have developed sophisticated mechanisms to cope with adverse environmental conditions and, among them, protein modification by SUMO (Small Ubiquitin-like MOdifier) has emerged as a major molecular process that mediates plant tolerance to a wide range of abiotic and biotic stresses (Castro *et al.*, 2012; Lois, 2010; Verma *et al.*, 2018).

SUMO, as the other members of the Ubiquitin-like family protein modifiers (Ubl), is a small protein of approximately 100 amino acids that displays a β-grasp fold, which is characterized by a β-sheet with 5 anti-parallel β-strands and a single helical element between β-4 and β-5 strands (Fig. 1A). Although this fold is best exemplified by ubiquitin, it is also found as a domain contained in larger proteins, suggesting that it might constitute a multi-functional scaffold with different biological functions (Burroughs *et al.*, 2007). Another hallmark of the Ubl family is the conjugation/deconjugation process. Although some variations exist, previously described in detail (Vierstra, 2012), Ubl's are conjugated to protein targets through three sequential reactions catalyzed by the E1 activating enzyme, the E2 conjugating enzyme and E3 ligases. In general, Ubl's are synthesized as immature forms that become processed by a family of cysteine proteases (Vierstra, 2012), ULP (UbL-specific Proteases) (Li and Hochstrasser, 1999; Schwienhorst *et al.*, 2000). SUMO C-terminal processing exposes the Ubl C-terminal di-Gly motif required to enter the conjugation pathway (Vierstra, 2012). Protein post-translational modifications by Ubl's are reversible and ULP's also catalyze Ubl removal from the target (Fig. 1B). Components of different Ubl's conjugation machineries are conserved, but they have evolved to recognize cognate Ubl and to establish high selective interactions among them, ensuring the fidelity of the signaling cascade (Liu *et al.*, 2017a; Tokgoz *et al.*, 2012; Walden *et al.*, 2003).

In this review, we will focus on molecular aspects of the SUMO conjugation system in plants and discuss the current knowledge of the role of SUMOylation in plant responses to drought stress. Since its discovery 20 years ago, SUMO has received major attention due to its essential cellular functions and its major role in human diseases, including cancer and neurological disorders (Droescher *et al.*, 2013; Seeler and Dejean, 2017). Numerous genetic and structural studies performed in yeast and animal systems have contributed to identify the molecular mechanisms involved in SUMO conjugation/deconjugation (Cappadocia and Lima, 2018). In plants, SUMO was first identified as an interactor of the ethylene inducing xylanase (EIX) from the fungus *Trichoderma viride* (Hanania *et al.*, 1999). In the following years, the main components of the SUMOylation system in *Arabidopsis* were characterized (Chosed *et al.*, 2006; Kurepa *et al.*, 2003; Lois *et al.*, 2003; Miura *et al.*, 2005; Murtas *et al.*, 2003).

In plants, as well as in animals, SUMO conjugation is essential during embryo development (Nacerddine *et al.*, 2005; Saracco *et al.*, 2007). SUMOylation modulates plant hormone signaling (Campanaro *et al.*, 2016; Kim *et al.*, 2015; Lois *et al.*, 2003; Miura *et al.*, 2010), root stem cell maintenance (Xu *et al.*, 2013), circadian clock (Hansen *et al.*, 2017a; Hansen *et al.*, 2017b), light signaling (Lin *et al.*, 2016; Sadanandom *et al.*, 2015), plant immunity (Lee *et al.*, 2007), plant immunity and growth (Hammoudi *et al.*, 2018), defense responses to necrotrophic fungal

pathogens (Castaño-Miquel *et al.*, 2017), thermotolerance (Yoo *et al.*, 2006) and, virtually, any aspect of plant development (Ishida *et al.*, 2012; Ling *et al.*, 2012; Liu *et al.*, 2014). Considering that SUMOylation regulates physiological processes that are key determinants for agriculture productivity, uncovering the molecular insights into SUMO conjugation has a major interest for providing new markers and/or biotechnological tools to the agro-food sector.

## Components of the SUMO conjugation machinery in plants

### *The SUMO isoforms*

The existence of distinctive SUMO isoforms and their attachment to substrates as monomers, in single or multiple positions, or as polymers by building polySUMO chains, contribute to the high complexity of the molecular consequences of SUMOylation. Among others, SUMOylation regulates protein activity by inducing subcellular redistribution, modulating protein-protein interactions, competing with other post-translational modifications or promoting conformational changes. On the other hand, the most prevalent role of polySUMO chains seems to function as substrate for ubiquitination (Tatham *et al.*, 2008), so that the SUMOylated substrate is tagged for degradation by the proteasome.

*Arabidopsis* genome encodes eight SUMO isoforms, although only SUMO1, 2, 3 and 5 are expressed (Hammoudi *et al.*, 2016; Kurepa *et al.*, 2003; Novatchkova *et al.*, 2004). SUMO1 and SUMO2 are the most closely related isoforms sharing an 83% of amino acid sequence identity. SUMO3 and SUMO5 display 42% and a 30% of amino acid sequence identity with SUMO1, respectively (Castaño-Miquel *et al.*, 2011). This diversification involves residues that perform key molecular functions such as E1, E2 and SUMO Interacting Motifs (SIM) interactions (Castaño-Miquel *et al.*, 2011). SIM's are composed of hydrophobic residues flanked by acidic residues or residues that can be phosphorylated (Hecker *et al.*, 2006; Song *et al.*, 2004). SUMO1/2 isoforms establish the most favorable interactions, while SUMO5 is the less efficient isoform. Since SUMO1/2 isoforms are essential in *Arabidopsis* (Saracco *et al.*, 2007), the highest conservation of these key molecular determinants in SUMO1/2 isoforms provides a molecular mechanism to favor their attachment to substrates, which would ensure the plant viability. The biological role of SUMO3 seems to be restricted to defense responses, although the SUMO3 knockout mutant plants are not impaired in resistance to infection by *Pseudomonas syringae pv tomato DC3000* (PstDC3000) (van den Burg *et al.*, 2010). Supporting the role of SUMO3 in plant defense, the master regulator of basal and systemic acquired resistance NPR1 specifically interacts non-covalently with SUMO3 and it is modified by SUMO3 (Saleh *et al.*, 2015). In addition, SUMO3 is upregulated upon *Turnip mosaic virus* (TuMV) viral infection and modifies the viral RNA-dependent RNA polymerase of the virus, resulting in stimulation of viral infection (Cheng *et al.*, 2017). In both cases, protein conjugation by SUMO3 is dependent on the presence of a SIM in the target. Despite its functional specialization, SUMO3 is only present in some brassicas, suggesting that modulation of defense responses by SUMOylation would include evolutionary

divergent mechanisms. The biological role of SUMO5, which is evolutionary conserved in plants (Hammoudi *et al.*, 2016), remains to be elucidated.

*The E1 activating enzyme*

The E1 SUMO activating enzyme is a heterodimer formed by the SAE2 large subunit and the SAE1 small subunit. The SAE2 large subunit is organized in four functional domains: adenylation, catalytic cysteine, UFD (Ubiquitin-Fold domain) and C-terminal domains (Lois and Lima, 2005). The SAE1 small subunit also contributes to the adenylation domain, which catalyzes the formation of a high-energy SUMO acyl adenylate intermediate in the presence of ATP and magnesium. In a second reaction that requires a major rotation of the Cys domain (Olsen *et al.*, 2010), the catalytic cysteine facilitates the formation of the high-energy thioester bond between the E1 and SUMO and the release of AMP. At this stage, SUMO can be transferred to the recruited E2 through a transesterification reaction catalyzed by the E1~Ubl. The SAE2 C-terminal tail is not required for *in vitro* SUMO activation (Lois and Lima, 2005), although it contains the molecular signals that determine E1 subcellular localization (Castaño-Miquel *et al.*, 2013; Moutty *et al.*, 2011; Truong *et al.*, 2012).

The *Arabidopsis* genome encodes for one isoform of the E1 large subunit, SAE2, and two isoforms of the E1 small subunit, SAE1a and SAE1b. SAE2 knockout mutations confer lethality early during embryo development, while T-DNA mutants of the SAE1a isoform are viable, which initially suggested that SAE1 isoforms may have a redundant role *in vivo* (Saracco *et al.*, 2007). Later, kinetic analysis showed that the SAE2/SAE1a holoenzyme confers higher conjugation rates than SAE2/SAE1b in reconstituted *in vitro* SUMO conjugation assays (Castaño-Miquel *et al.*, 2013). SAE1a and SAE1b also display different subcellular distributions, and the absence of SAE1a compromises the capacity of *Arabidopsis* plants to accumulate SUMO conjugates upon stress. These results suggested an unanticipated role of the E1 as a limiting regulatory step during SUMO conjugation *in vivo* (Castaño-Miquel *et al.*, 2013). The presence of E1 variants in other plants (Castaño-Miquel *et al.*, 2013; Novatchkova *et al.*, 2012) suggests that E1 diversification could provide an additional level of regulation of SUMO conjugation *in vivo*, although additional research is needed to validate this hypothesis.

*The E2 conjugating enzyme*

The E2 conjugating enzyme can directly transfer SUMO to the substrate by means of a nucleophilic attack at the SUMO~E2 thioester by a lysine residue in the substrate (Bernier-Villamor *et al.*, 2002). In general, the acceptor lysine resides at the core consensus motif Ψ-**K**-x-E, where Ψ is an aliphatic amino acid. Recent developments in mass spectrometry methodologies have allowed the identification of variants of this consensus site that include surrounding amino acids, which contribute to strengthen binding to the E2. These variations comprise additional hydrophobic amino acids (*[VIP]*-x-Ψ-**K**-x-E), additional acidic regions (Ψ-**K**-x-E-x-*[ED]*5*),

phosphorylation dependent motifs (Ψ-**K**-x-E-x-x-*SP*), or inverted core consensus motifs ([ED]-x-**K**-Ψ)(Hendriks and Vertegaal, 2016). In addition, *bona fide* SUMOylation consensus sites also have structural requirements such as being located in extended/non-structured and exposed regions (Pichler *et al.*, 2005). In plants, advances achieved in SUMO proteomics technics have allowed the identification of 71 SUMO acceptor sites (Rytz *et al.*, 2018). The 65% of the acceptor lysines belong to a canonical SUMOylation consensus site, and the remaining 35% correspond to non-canonical sites, suggesting that additional mechanisms for substrate recognition have a relevant role in plant SUMO conjugation. Alternatively, since the proteomic studies were performed using heat shock-treated plants, it is possible that non-canonical sites are modified as consequence of SUMOylation becoming less stringent under stress (Hendriks and Vertegaal, 2016).

SUMO E2 conjugating enzymes display the so called UBC fold that includes 4 α-helices and four β-strands, and contain the HPN tripeptide motif separated by 7 amino acids from the catalytic cysteine (Michelle *et al.*, 2009). In *Arabidopsis*, T-DNA insertion mutants in *SCE1*, which codifies the only one isoform of the SUMO E2 conjugating enzyme, are embryo lethal (Saracco *et al.*, 2007). SCE1 is one of the most conserved members of the SUMO conjugation machinery sharing a 63% of sequence identity with its human ortholog. In contrast, *Arabidopsis* SAE2 and SAE1 share 36% and 30% of sequence identity with their human orthologs, respectively. SCE1 functions as a hub during the conjugation cascade by establishing multiple protein-protein interactions with the E1 activating enzyme, the substrate, E3 ligases and SUMO through dedicated surfaces, of which some overlap (Fig. 2A). This mutually excluding interactions could contribute to confer directionality in the conjugation cascade (Wang *et al.*, 2010). A hallmark in Ubl's is their capacity to establish non-covalent interactions with cognate E2 conjugating enzymes, through an E2 region structurally opposed to the catalytic cysteine containing region (Fig. 2A). In the SUMO system, SUMO-E2 interactions are required for polySUMO chain formation (Capili and Lima, 2007; Castaño-Miquel *et al.*, 2011; Knipscheer *et al.*, 2007).

*E3 ligases*

SUMO E3 ligases facilitate SUMO transfer to substrates, although some substrates do not require the presence of E3 ligases to be efficiently modified *in vitro*. Several E3 ligases have been identified in animals and all of them contain SUMO-interacting motifs (SIM) (Jentsch and Psakhye, 2013). Atypical E3 ligases such as human RanBP2 or ZNF451 base their activity only on SIM motifs (Cappadocia *et al.*, 2015; Pichler *et al.*, 2002). The most conserved SUMO ligases belong to the Siz/PIAS family, which contain a Siz/PIAS RING (SP-RING) domain essential for their activity and responsible for E2 recruitment (Garcia-Dominguez *et al.*, 2008). In addition, canonical Siz/PIAS possesses the SAP domain involved in DNA binding (Suzuki *et al.*, 2009), the PINIT (Pro-Ile-Asn-Ile-Thr) motif involved in binding to SIZ1-dependent substrates, and a SIM that also contributes to the E3 ligase activity (Streich and Lima, 2016).

The SUMO ligases identified in *Arabidopsis*, SIZ1, MMS21 and PIAL1/2, belong to the SP-RING family. *Arabidopsis* SIZ1 is the most studied ligase and contains an additional domain specific to plants, the so-called PHD (plant homeodomain) (Fig. 2B). The PHD domain is necessary for SUMO conjugate accumulation upon heat stress (Cheong *et al.*, 2009). Amino acid sequence diversification also affects the PINIT motif that is present in *Arabidopsis* SIZ1 as the variant PIIT (Pro-Ile-Ile-Thr). SIZ1 regulates phosphate deficiency (Miura *et al.*, 2005), basal thermotolerance (Yoo *et al.*, 2006), drought (Catala *et al.*, 2007), innate immunity (Lee *et al.*, 2007), freezing tolerance (Miura *et al.*, 2007), flowering (Jin *et al.*, 2008), abscisic acid signaling (Miura *et al.*, 2009), copper tolerance (Chen *et al.*, 2011), nitrogen assimilation (Park *et al.*, 2011) and sugar signaling (Castro *et al.*, 2015), among others. MMS21 ligases only possess the SP-RING domain and a putative SIM motif (Fig. 2B). In *Arabidopsis*, MMS21(HPY2) participates in cell cycle regulation (Huang *et al.*, 2009; Ishida *et al.*, 2009), stem cell maintenance (Xu *et al.*, 2013), drought (Zhang *et al.*, 2013), gametophyte development (Liu *et al.*, 2014) and flowering (Kwak *et al.*, 2016). Genetic studies showed that SIZ1 and MMS21 do not functionally complement each other, and the double knockout *siz1mms21* mutations confer lethality during embryogenesis (Ishida *et al.*, 2012). In addition, PIAL1/2 belong to another group of SP-RING containing ligases that promote SUMO chain formation (Tomanov *et al.*, 2014). PIAL1/2 ligases function redundantly to mediate transcriptional silencing (Han *et al.*, 2016), although this latter molecular role is independent of the ligase activity, supporting the notion that SUMO ligase activities can be part of multifunctional proteins. In fact, PIAL1/2 proteins present an additional domain, the IND domain, which allows dimerization of PIAL proteins and facilitates interactions with MOM1 (Han *et al.*, 2016). PIAL1/2 also mediate plant responses to abiotic stress (Tomanov *et al.*, 2014) and transcriptional silencing (Han *et al.*, 2016)

*ULPs*

ULPs are cysteine proteases responsible for SUMO maturation and release from the targets through their endopeptidase and isopeptidase activities, respectively (Colby *et al.*, 2006). They constitute the most numerous family among members of the SUMOylation machinery and display specificity for SUMO isoform and substrate (Chosed *et al.*, 2006; Colby *et al.*, 2006). ULPs are generally organized in a C-terminal domain that contains the catalytic triad Cys-His-Asp (ULP/C48 domain) and a highly divergent N-terminal domain that has a major role in the regulation of ULP activity *in vivo* (Hickey *et al.*, 2012; Mukhopadhyay and Dasso, 2007). This structural organization is observed in yeast ULP1, human SENP1, SENP2, SENP4 and SENP5. In the case of ULP2, the ULP domain is in the middle of the protein. And the most striking organization is present in vertebrate SENP6 and SENP7 that display a split ULP domain (Mukhopadhyay and Dasso, 2007).

The high amino acid sequence divergence present in ULPs has generated some controversy about the classification of ULP1-like and ULP2-like classes in plants. In *Arabidopsis*, initial analyses classified ESD4, ELS1/ULP1a, ULP1b, OST1/ULP1d and OST2/ULP1c as ULP1-like SUMO proteases, and SPF1/ASP1 and SPF2 as ULP2-like (Lois, 2010; Novatchkova *et al.*,

2004). According to sequence conservation data, ULP1d/OST1 and ULP1c/OST2 are closer to yeast ScULP2 (Castro *et al.*, 2016), but their ULP domain is located at their C-terminus as ULP1-like class (Table 1). A deep phylogenetic analysis, including *Arabidopsis*, tomato, grapevine and poplar genomes, defined the existence of four ULP groups in *Arabidopsis*, namely A, B1, B2 and C (Novatchkova *et al.*, 2012). Group A contains the At3g48480 isoform for which does not exist experimental data supporting its role as a SUMO protease. Group B1 contains *Arabidopsis* ULP1d/OST1 and ULP1c/OST2. Group B2 contains the recently characterized SPF1/ASP1 and SPF2 (Kong *et al.*, 2017; Liu *et al.*, 2017b), which contain the ULP domain in the middle of the protein as ULP2-like class. Finally, the group C contains *Arabidopsis* ESD4, ULP1a/ELS1 and ULP1b, which also displays the ULP domain at its C-terminus. Given the complexity for establishing clear phylogenetic relationships between planta and yeast sequences, we favor the option of establishing a ULP classification specific to plants that takes into consideration both sequence similarity and structural organization of the ULP domain. This classification would follow the suggested by Novatchkova and collaborators, but we propose to avoid the use of B1 and B2 classes because they represent ULP sequences with different structural organization, as described above. We propose a conservative option based on four ULP classes that take into consideration sequence and structural organization, as shown in Table 1.

Sequence diversification accompanies ULP functional specialization. *esd4* plant knockout mutants display alterations in flowering-time regulation and a dwarf phenotype (Murtas *et al.*, 2003). On the other hand, plants harboring mutations in the closest *ESD4* homolog *ULP1a/ELS1* also display defects in flowering time and plant growth, although both phenotypes are less dramatic than in *esd4* plants (Hermkes *et al.*, 2011), suggesting that both proteases have distinct biological roles. The study of ULP1d/OST1 and ULP1c/OST2 identified a link between SUMO and responses to salt and osmotic stress (Castro *et al.*, 2016; Conti *et al.*, 2009). Recently, studies of the SPF1/APS1 SUMO protease have revealed that it regulates flowering time (Kong *et al.*, 2017). In addition, *spf1/asp1spf2* double mutants exhibit severe defects in gametogenesis and embryo development (Liu *et al.*, 2017b).

## Regulatory mechanisms of SUMO conjugation

Relevant advances in understanding the biological role of SUMO have been made using plants harboring mutations in different members of the SUMO conjugation machinery. Mutations in the E3 ligases *SIZ1* (Miura *et al.*, 2010) and *MMS21* (Ishida *et al.*, 2012) and the protease *ESD4* (Murtas *et al.*, 2003) confer highly pleiotropic phenotypes that, together with the lethal phenotype displayed by the E1-activating *sae2*, the E2-conjugating enzyme *sce1* or the double *sum1sum2* mutants (Saracco *et al.*, 2007), highlight the central role of protein SUMOylation in plant physiology. Consistently with this crucial function, it is plausible that SUMOylation is a highly regulated process itself. Although the molecular mechanisms potentially involved in such regulation are largely unknown, their identification would provide valuable tools for fine-tuning SUMO conjugation *in vivo*. An example of this has been the development, based on structure-

activity relationship, of a new molecular tool for inhibiting SUMOylation *in viv*o. This tool consists in the disruption of SUMO E1-E2 interactions by means of expressing the domains UFD and Ct ($SAE2^{UFDCt}$) of the E1 large subunit SAE2 involved in E2 recruitment. The expression of the E1 $SAE2^{UFDCt}$ domains allows attenuation of *in vivo* SUMO conjugation in a dose-dependent manner (Castaño-Miquel *et al.*, 2017). In contrast to knockout mutants, this molecular tool provides the advantage that can be applied for inhibiting SUMOylation in a spatiotemporal and/or inducible manner, which will provide a more accurate information on the role of SUMO *in vivo*.

*Molecular determinants conferring specificity*

A first level of regulation consists in maintaining the high fidelity of the SUMO conjugation system. This fidelity determines which protein substrates are SUMOylated by means of interactions between conserved SUMO attachment sites present in the substrate and E2 and E3 enzymes, as discussed above. Likewise, this fidelity is very important for recognition between cognate SUMO machinery components.

A crucial selection step is the selective recognition of the Ubl by cognate E1 (Lois and Lima, 2005). In *Arabidopsis*, the E1 activating enzyme discriminates among the high diversified SUMO isoforms, displaying the highest E1 activity towards the essential SUMO1/2 isoforms, whereas it is less efficient in SUMO3 activation. SUMO5 is the most poorly activated isoform, suggesting that it has a minor or very specific biological role that remains to be identified (Castaño-Miquel *et al.*, 2011). Similar results were obtained regarding SUMO isoform specificity shown by members of the ULP protease family. However, different ULP isoforms have been characterized using different approaches, indicating that more quantitative and standard analyses are required to generate robust kinetics data. Among the tested ULPs, none displayed endopeptidase or isopeptidase activity towards SUMO5, and only ELS1/ULP1a displayed a residual endopeptidase activity towards SUMO3 (Chosed *et al.*, 2006). Interestingly, the pathogen effector XopD from *Xanthomonas campestris* is a SUMO protease that displays an efficient isopeptidase activity towards SUMO1/2 and SUMO3 (Chosed *et al.*, 2007; Colby *et al.*, 2006), supporting the biological specialization of SUMO3 in plant defense (Saleh *et al.*, 2015; van den Burg *et al.*, 2010). Alternatively, it is possible that SUMO3 processing by XopD is the consequence of a broader substrate specificity, since XopD also possesses ubiquitin endopeptidase activity (Pruneda *et al.*, 2016). Two recent reports have addressed the characterization of the ULP proteases SPF1/ASP1 (Kong *et al.*, 2017; Liu *et al.*, 2017b) and SPF2 (Liu *et al.*, 2017b). Both proteases display endopeptidase activity towards SUMO1, although less efficiently than ESD4. SUMO3 is not processed by any of them and, the most surprisingly, SUMO2 is not processed either (Liu *et al.*, 2017b). As to our knowledge, SPF1 and SPF2 are the only known SUMO conjugation machinery components that discriminate among SUMO1 and SUMO2 paralogs. Further investigation is required to understand the molecular determinants responsible for this specificity and its biological consequences. In addition, SUMO proteases with capacity to catalyze SUMO3 and SUMO5 maturation remain to be identified (Table 2).

Protein-protein interactions between cognate E1 and E2 are another key step for fidelity maintenance. In these interactions, the SAE2$^{UFDCt}$ domain has a major role and the region participating in the E1-E2 interface presents structural variations among evolutionarily distant orthologs. It has been suggested that these variations may arise from the high selective pressure to ensure Ubl specificity (Liu *et al.*, 2017a). As described above, specificity of protein-protein interactions between E3 ligases and E2-conjugating enzyme, substrates or SUMOs have also a fundamental role during conjugation.

In *Arabidopsis*, the capacity to build polySUMO chains is apparently restricted to SUMO1/2 isoforms, while SUMO3 and 5 are mainly conjugated as monomers (Chosed *et al.*, 2006; Colby *et al.*, 2006). Consistently with the role of SUMO-E2 non-covalent interactions in polySUMO chain formation, SUMO3 and 5 are not competent to interact with the E2, adding another level of specificity within the SUMO system (Castaño-Miquel *et al.*, 2011). However, increasing E1 and E2 concentrations in reconstituted conjugation reactions *in vitro* facilitates polySUMO3 chain formation, which improved in the presence of a PIAL2 ligase variant (Tomanov *et al.*, 2014). Mass spectrometry analysis of SUMO1 conjugates isolated from plants failed to identify SUMO3 or SUMO5 peptides, suggesting that these isoforms are not significantly incorporated into polySUMO1 chains *in vivo* (Rytz *et al.*, 2018). In addition to other mechanisms that may remain to be identified, the low conjugation efficiency displayed by SUMO3 and SUMO5 *in vitro* (Castaño-Miquel *et al.*, 2011), could account for these results.

Finally, as described above, variations in the consensus sequence that contains the acceptor lysine and the presence of additional E2-substrate interaction surfaces in the substrate will also translate into differences in SUMO conjugation efficiency.

*Post-translational modifications*

In animals, SUMO modification of E2 at its terminal helix has been proposed to confer substrate specificity (Knipscheer *et al.*, 2008). In *Arabidopsis*, SUMOylation of SCE1 has also been identified, although there is controversy about the acceptor lysine. Proteomic analyses of the endogenous SUMO conjugates (SUMOylome) present in plants identified SCE1 as a constitutive SUMO target, since SUMO-SCE1 conjugate levels did not change upon heat or oxidative stress (Miller *et al.*, 2010). In this report, the identified acceptor lysine is located at the C-terminal $\alpha$-helix (K154) (Miller *et al.*, 2010), which is equivalent to the SUMO acceptor lysine present in the yeast SCE1 (Ho *et al.*, 2011). On the contrary, *in vitro* SUMO conjugation assays performed in the presence of the PIAL2 ligase resulted in SUMO attachment to SCE1 at K15, equivalent to human SCE1 K14 (Knipscheer *et al.*, 2008). The *Arabidopsis* SCE1 K15R mutant variant was not impaired in SUMO conjugation to substrates, although its capacity to promote SUMO chain formation was compromised (Tomanov *et al.*, 2014). One possible explanation to these different observations may be that SUMO modification of SCE1 K14 only takes place in the presence of PIAL2 and that, *in vivo*, PIAL2 activity is not high enough to produce detectable levels of SUMO modified SCE1 K14. Another explanation is that the truncated version of PIAL2 used in

353 the *in vitro* assays (Tomanov *et al.*, 2014) may be deficient in a regulatory domain, such as the
354 IND domain, which would affect the substrate specificity. More exhaustive biochemical and
355 proteomic analyses are needed to determine the occurrence of both modifications *in vivo* and
356 their molecular implications.

357 SUMO1, SAE2, SIZ1 and ESD4 have also been identified as SUMO targets *in vivo*, although
358 validation studies have only been performed for SUMO and SIZ1. Mutagenesis analyses showed
359 that SUMO2 K10 was a major SUMO acceptor site involved in SUMO chain formation, as
360 observed in SUMOylation *in vitro* assays performed in the absence of E3 ligases (Colby *et al.*,
361 2006). However, analyses of *in vivo* SUMO conjugates identified SUMO1 K23 and K42 as SUMO
362 acceptor sites, being K23 also an acceptor for ubiquitin upon heat shock (Miller *et al.*, 2010).
363 Discrepancies about the identity of lysine acceptor sites found in SUMO between *in vitro* and *in*
364 *vivo* studies need to be addressed. It is possible that regulatory components found *in vivo*, such
365 as E3 ligases or proteases, may influence the balance between the modified lysines. Alternatively,
366 the use of SUMO variants with N-terminal tags, such as the hexahistidine tag (Miller *et al.*, 2010),
367 could introduce charge changes that may compromise the detection of the N-terminal K10 as
368 SUMO acceptor. On the other side, SIZ1 SUMOylation increases upon heat and oxidative
369 stresses, which are conditions that promote a dramatic accumulation of SUMO conjugates (Miller
370 *et al.*, 2010). SIZ1 variants harboring mutations in the SUMO acceptor sites complement *siz1-2*
371 mutant plants, suggesting that SUMO modification does not significantly alter SIZ1 ligase function
372 under the analyzed conditions (Rytz *et al.*, 2018).

373 PolySUMO chains can recruit specific ubiquitin E3 ligases (SUMO-targeted ubiquitin ligases,
374 STUbl) that promote ubiquitination and subsequent degradation of the SUMO-modified target by
375 the proteasome (Geoffroy and Hay, 2009). In *Arabidopsis*, six STUbl homologs were identified as
376 SUMO-interacting proteins and shown to functionally complement the *S. pombe* STUbL mutant
377 *rfp1/rfp2* (Elrouby *et al.*, 2013). Although further investigation is required, modification of SUMO
378 by ubiquitin upon stress could respond to an increasing demand of removal of stress-induced
379 protein damage. These damaged proteins would be degraded by the proteasome in a
380 SUMOylation-dependent manner.

381 The SUMO activating enzyme large subunit SAE2 also undergoes phosphorylation at its C-
382 terminal tail as reported by several proteomics studies. Two studies identified SAE2 T598 and
383 S603 as phosphorylated residues (Meyer *et al.*, 2012; Reiland *et al.*, 2009), but also SAE2 S618,
384 S673 and T598 have been identified as kinase substrates (Nakagami *et al.*, 2010; Reiland *et al.*,
385 2009). The fact that the mentioned studies used different plant tissues, including cell culture
386 (Nakagami *et al.*, 2010), adult plants (Reiland *et al.*, 2009) and seed development (Meyer *et al.*,
387 2012), suggests that SAE2 phosphorylation is a dynamic process that could contribute to fine-
388 tune SUMOylation to adapt it to the plant physiological requirements. Phosphorylation of
389 SUMO1/2 on Ser2 has also been identified, although its biological significance is not known either
390 (Nukarinen *et al.*, 2017).

391

*Protein levels*

Independent observations have identified increased levels of the SUMO conjugating enzyme SCE1 in SUMOylation-deficient plants (Castaño-Miquel *et al.*, 2013; Nukarinen *et al.*, 2017; Saracco *et al.*, 2007). Initially, SCE1 upregulation was observed in *siz1-3* mutant plants and it was speculated to be the result of a transcriptional compensation of suboptimal SUMO conjugation levels (Saracco *et al.*, 2007), although later studies showed that *SCE1* mRNA levels were not significantly altered in *siz1-3* (Castaño-Miquel *et al.*, 2013). The upregulation of SCE1 levels in plants overexpressing the SAE2$^{UFDCt}$ domain, which is involved in E2 binding as shown above, was more striking. In these plants, SCE1 levels were increased in direct proportion to SAE2$^{UFDCt}$ levels and at a much higher level than in *siz1-3* plants. The fact that SUMOylation defects present in *siz1-3* plants were more prominent than in SAE2$^{UFDCt}$ expressing plants, together with the higher accumulation on SCE1 in these plants versus *siz1-3* plants, does not support the existence of a compensatory mechanism contributing to SCE1 accumulation. One possibility is that the SCE1-SAE2$^{UFDCt}$ complex could mediate SCE1 stabilization. (Castaño-Miquel *et al.*, 2017). *In planta*, such mechanism could facilitate the coordination between E1 and E2 levels in order to modulate SUMO conjugation rate. Alternatively, if SCE1 SUMOylation would lead to polySUMO-SCE1 STUbl-dependent ubiquitination and subsequent degradation by the proteasome, the inhibition of SUMO conjugation by SAE2$^{UFDCt}$ expression would result in SCE1 accumulation. Although this later hypothesis does not explain the differences observed between *siz1* mutant and SAE2$^{UFDCt}$ expressing plants. Further research is required to elucidate the mechanisms involved in modulation of SCE1 levels and if some similar regulatory mechanisms affect other members of the SUMO conjugation machinery.

SUMO1/2 levels are also upregulated in *siz1* mutant plants (Nukarinen *et al.*, 2017), although it was not reported if this increase correlates with an upregulation of mRNA levels. Another explanation would be that the higher ratio of free versus conjugated SUMO found in *siz1*, in comparison to wild type plants, could have an effect on mass spectrometry quantification.

Recent studies have shown that the E3 ubiquitin ligase CONSTITUTIVE PHOTHOMORPHOGENIC 1 (COP1) regulates SIZ1 proteins levels. SIZ1 co-localizes with COP1 in nuclear bodies and mediates COP1 SUMOylation, which enhances COP1 activity. Conversely, COP1 promotes the ubiquitination and degradation of SIZ1 and this mechanism would maintain the homeostasis of COP1 activity (Lin *et al.*, 2016). Consequently, SUMO conjugates accumulate to a greater extent in *cop1* mutant plants upon drought, cold and salt treatments (Lin *et al.*, 2016). Similarly, salt triggers a downregulation of OTS1 and OTS2 protein levels, providing a possible mechanism for the accumulation of SUMO conjugates during salt stress. The same study shows that, OTS1 downregulation is the result of proteasome mediated protein degradation (Conti *et al.*, 2008).

*Subcellular distribution*

Similarly to its human and yeast orthologs, *Arabidopsis* SUMO E1 activating enzyme displays nuclear localization, which is facilitated by a nuclear localization signal, NLS, present at the SAE2 C-terminal tail (Castaño-Miquel *et al.*, 2013). In mammals, although both E1 subunits have distinct functional NLSs, *in vitro* experiments demonstrated that the E1 large subunit Uba2 NLS is the signal required for the efficient nuclear import of the E1 heterodimer (Moutty *et al.*, 2011). Also, human SAE2 is SUMOylated at its C-terminal tail and this mechanism mediates nuclear retention (Truong *et al.*, 2012). The existence of similar mechanisms that would regulate E1 nuclear localization in *Arabidopsis* remains to be elucidated.

*Arabidopsis* E2 conjugating enzyme is distributed between nucleus and cytoplasm in transient expression assays, but it mainly localizes to the nucleus, and to nuclear speckles, when co-expressed with SUMO. This SUMO-mediated SCE1 redistribution is dependent on its activity since the SCE1 C94S mutant does not display this capacity (Lois *et al.*, 2003). In similar experiments, SCE1 also displays nuclear localization when co-expressed with $SAE2^{UFDCt}$ domain (Castaño-Miquel *et al.*, 2017), suggesting that SCE1 subcellular localization greatly depends on interactions established with other members of the SUMO conjugation machinery.

SIZ1, consistently with the presence of a nuclear localization signal on its C-terminus, localizes to the nucleus and nuclear speckles (Miura *et al.*, 2005). The second known E3 ligase, MMS21, predominantly localizes to the nucleus but it is also present in the cytosol (Huang *et al.*, 2009; Ishida *et al.*, 2009). Similarly to the E2, it would be interesting to analyze if the localization of any of these ligases is modulated by interactions with other components of the SUMOylation machinery.

SUMO proteases are heterogeneous regarding their subcellular distribution. ESD4 displays nuclear localization, predominantly at the periphery of the nucleus (Murtas *et al.*, 2003). ESD4 interacts with the nuclear pore anchor protein (NUA) in yeast two-hybrid assays, although this interaction is not required for ESD4 localization at the nuclear periphery (Xu *et al.*, 2007). Surprisingly, ESD4 closest homolog ELS1 (ULP1a) is present in the cytosol (Hermkes *et al.*, 2011). In addition, OTS2 (ULP1c) and OTS1 (ULP1d) localize to the nucleus, and OST2 is also found in nuclear foci (Conti *et al.*, 2008). The recent characterization of SPF1 and SPF2 showed that they are nuclear ULP (Kong *et al.*, 2017; Liu *et al.*, 2017b).

In general, SUMO conjugation/deconjugation machinery members are enriched in the nucleus consistently with the massive accumulation of SUMO conjugates in this compartment (Saracco *et al.*, 2007). The issue about how non-nuclear SUMO conjugates are modified remains to be answered. One possibility could be that SUMO-loaded SCE1 (SUMO~SCE1) migrates to the cytosol to modify substrates. However, the fact that co-expression of SUMO and SCE1 results in SCE1 nuclear localization suggests that additional molecular mechanisms, like potential interactions with E3 ligases, are necessary to facilitate SUMO~SCE1 cytosolic targeting. If this is the case, MMS21 is the only known E3 ligase showing partial cytosolic localization and that could somehow facilitate SUMO~SCE1 cytosolic enrichment. Similarly, only one protease ELS1

(ULP1a) is candidate to be mediating cytosolic SUMO deconjugation. Interestingly, ELS1 (ULP1a) is the closest homolog to the nuclear ESD4, being both of them the ones displaying higher activity *in vitro* (Chosed *et al.*, 2006). In this scenario, and as suggested by the developmental defects conferred by mutations in *ESD4* (Murtas *et al.*, 2003) and *ELS1* (Hermkes *et al.*, 2011), it is tempting to speculate that ELS1 (ULP1a) and ESD4 would account for the main SUMO deconjugation activities of cytosolic and nuclear substrates, respectively. The other ULP isoforms would have a more specialized role, as supported by the moderate phenotypes displayed by their respective knockout mutant plants.

Regardless of the data generated in the cited studies addressing the subcellular localization of the SUMO conjugation machinery (Fig. 3), the use of fluorescence protein fusions together with overexpression strategies in heterologous systems highlights the need for complementary analyses. Future studies based on immunolocalization approaches, or similar, and using plants expressing endogenous levels of the SUMOylation machinery component analyzed will contribute to unravel subcellular SUMOylation dynamics more accurately.

**SUMOylation and abiotic stress in model species and crops**

As already mentioned, mutants in the SUMOylation machinery are often lethal or show pleiotropic phenotypes, which make it difficult to discriminate between direct and indirect regulatory roles. However, in the last decade, diverse studies revealed a clear link between SUMOylation and abiotic stress responses in plants.

In *Arabidopsis*, it is well established that the abundance of SUMO conjugates increases in response to exposure to different abiotic stresses such as high salinity (Conti *et al.*, 2008), high temperature (Kurepa *et al.*, 2003; Yoo *et al.*, 2006), freezing (Miura *et al.*, 2007), drought (Catala *et al.*, 2007), copper excess (Chen *et al.*, 2011), oxidative stress (Miller *et al.*, 2010; Miller *et al.*, 2013), ethanol treatment and proteotoxic stress caused by canavanine (Kurepa *et al.*, 2003). Despite the fact that the characterization of SUMOylation-dependent abiotic stress responses has been studied mostly in *Arabidopsis*, there is growing evidence that this link is conserved in many crop species such as rice, maize or soybean. The expression of the SUMOylation machinery components in those species is also developmentally controlled (Augustine *et al.*, 2016; Chaikam and Karlson, 2010; Li *et al.*, 2017; Reed *et al.*, 2010). Furthermore, conjugate levels increase in response to abiotic stress such as cold, high salinity or increased ABA in rice (Chaikam and Karlson, 2010), and in response to heat stress in poplar (Reed *et al.*, 2010). In maize, most of the SUMO machinery components are strongly expressed during seed development, which could have relevant implications for seed survival under normal conditions but also under stress conditions. In addition, maize SUMO conjugates also accumulate in response to heat and oxidative stress (Augustine *et al.*, 2016). Moreover, SUMO conjugates also increment in soybean plants exposed to various abiotic stresses including high salinity, heat or increased ABA (Li *et al.*, 2017). All these pieces of evidence point out to a conserved role of SUMOylation in the control of abiotic stress responses (Park and Yun, 2013).

In the framework of growing evidence for climate change-derived adverse consequences for crop productivity world-wide, it is increasingly important to study plant adaptation mechanisms to extreme and unpredictable environmental conditions, particularly increasing drought periods and high salinity in soils. Understanding those mechanisms will allow breeding or designing crop varieties tolerant to drought or salinity stress. In this review, we will focus on the role of SUMOylation on the regulation of drought stress responses and evaluate its potential biotechnological applications in agriculture.

*Impairment of SUMOylation and drought stress*

The first clear link between drought tolerance and SUMOylation came from the characterization of *siz1-3* knockout mutant, a T-DNA insertion affecting the *SIZ1* gene. The *siz1-3* mutant presents a pleiotropic phenotype being dwarf, with stunted growth and extremely early flowering (Catala *et al.*, 2007). At the molecular level, *siz1-2* and *siz1-3* present increased levels of salicylic acid (SA) (Lee *et al.*, 2007) that correlate with high expression of pathogenesis-related genes and a constitutive systemic acquired resistance (SAR) response in *siz1-2*. Moreover, *siz1* mutants are hypersensitive to the hormone abscisic acid (ABA) (Miura *et al.*, 2009), which is a key factor in the regulation of stress responses triggered by water deficit. In *Arabidopsis*, drought induces an increase in SUMO conjugates, which is partially dependent on SIZ1 and ABA (Catala *et al.*, 2007). In addition, the *siz1-3* mutant displays enhanced sensitivity to drought compared to wild type plants, suggesting a positive role of SUMOylation in the regulation of drought tolerance. However, later studies reported contradictory results regarding the tolerance of *siz1* mutants to drought stress. Miura and co-workers found *siz1-3* and *siz1-2* mutants to be drought resistant compared to wild type and proposed the regulation of stomatal aperture and the production of reactive oxygen species (ROS) as the molecular mechanisms underlying the regulation of drought tolerance through SUMOylation (Miura *et al.*, 2013). At this point, it is remarkable to note that ROS and stomata are regulated, among other factors, by the hormones ABA and SA and, as mentioned, that *siz1* plants are hypersensitive to ABA and hyperaccumulate SA. The fact that *siz1-2* drought resistance was suppressed by expression of the *nahG* gene, which converts SA to catechol, suggested that stomatal closure was regulated by SA-dependent ROS production in guard cells, and it was independent of ABA-induced ROS production (Miura *et al.*, 2013). In agreement with these results, a more recent work found the same *SIZ1* mutant alleles *siz1-3* and *siz1-2* to be drought resistant compared to wild type (Kim *et al.*, 2017). In both reports (Kim *et al.*, 2017; Miura *et al.*, 2013), the authors speculated that discrepancies with the initial observations showing *siz1* to be more sensitive to drought (Catala *et al.*, 2007) were justified as a consequence of variability of growth conditions.

On the other hand, impairment of SUMO conjugation by the expression of the $SAE2^{UFDCt}$ domain resulted in plants more sensitive to drought (Castaño-Miquel *et al.*, 2017), supporting the results obtained by Catala and co-workers. This new approach is particularly relevant since, as opposed to *siz1* mutants, $SAE2^{UFDCt}$ expressing plants show minor phenotypic defects under normal growth conditions as compared to *siz1* mutants, suggesting that the displayed drought

sensitive phenotype does not account for severe growth defects present before the stress is induced.

Despite the contradictory results regarding *siz1* drought tolerance in these studies, a clear link exists between SUMOylation and drought tolerance. Gene expression studies in the *siz1* mutant compared to wild type, under normal or drought conditions, revealed a very complex landscape where hormone crosstalk was at the center stage. SIZ1 regulates basal and stress-induced gene expression, including genes responding to ABA as well as components of the jasmonic acid (JA), brassinosteriod and auxin pathways (Catala *et al.*, 2007).

*Activation of SUMOylation and drought stress*

The relation between SUMOylation and drought resistance has been further analyzed by a series of studies where heterologous expression of SUMO conjugation machinery components from *Arabidopsis*, rice and other species conferred drought tolerance. An interesting approach has been the study of the relation between SUMOylation and drought in halophytic plants. The isolation of a SUMO conjugating enzyme (SCE) from *Spartina alterniflora* (*SaSce9*), a halophytic grass commonly used to study salt adaptation mechanisms, and whose expression is induced by salt, drought, cold and ABA, supported the idea that increased SUMOylation could render plants tolerant to several abiotic stresses (Karan and Subudhi, 2012). Heterologous expression of *SaSce9* in *Arabidopsis* conferred not only drought resistance, but also tolerance to salt stress. At the molecular level, these plants showed higher expression levels of ion transporters, genes involved in antioxidant production as well as stress-responsive genes. Proline accumulation and ROS detoxification were suggested as mechanisms conferring drought resistance.

Surprisingly, a recent work has shown that overexpression of the *SCE1* rice homologue (*OsSCE1*) confers osmotic sensitivity to transgenic rice plants in PEG6000 treatment assays, while *OsSCE1* downregulation confers osmotic resistance (Nurdiani *et al.*, 2018). Osmotic tolerance in plants with *OsSCE1* downregulation is also correlated with an increase in proline content, suggesting that indeed OsSCE1 maybe be regulating proline production under stress conditions (Nurdiani *et al.*, 2018).

In the same line of thought, overexpression of rice *SIZ1* ortholog (*OsSIZ1*) in bentgrass and cotton also enhanced drought tolerance in these species. Overexpression of *OsSIZ1* in bentgrass produced a significant increase in shoot biomass production, under normal growth conditions, and an increase in root biomass under drought conditions, with enhanced water retention capacity. As expected, SUMO conjugate accumulation was increased in *35S::OsSIZ1* bentgrass plants, supporting the positive relation between SUMOylation and drought tolerance (Li *et al.*, 2013). A complementary study has recently been published where *OsSIZ1* was overexpressed in cotton plants and the effect on growth under drought conditions was evaluated. Transgenic cotton plants constitutively expressing *OsSIZ1* showed an increased photosynthetic rate, boll and fiber production and root biomass. The improved fitness of transgenic plants compared to wild

type under stressed conditions was related to improved water use efficiency (Mishra *et al.*, 2017). The relevance of this work relies on the evaluation of the potential of SUMO-derived biotechnological tools to fight against combined stresses, as for example heat and drought. Cotton *OsSIZ1OE* transgenic plants exposed to a combined heat and drought stress, a situation that is more similar to real field conditions, still outperformed wild type plants, both in controlled environment and under field trials. Analysis of differentially expressed genes under various stress conditions (drought, heat and combined drought and heat) indicated that stress tolerance was conferred by overexpression of stress related, heat-shock and ROS production-related genes (Mishra *et al.*, 2017). Furthermore, transgenic plants showed higher photosynthetic rate than wild type plants under low irrigation conditions, leading to the proposal of a new drought-tolerance mechanism by which SUMOylation could protect the electron transport machinery. This proposal is in agreement with previous results demonstrating that *siz1* seedlings contain reduced levels of chlorophyll compared to wild type when grown in media supplemented with sucrose or glucose (but not mannitol). This observation suggests a role of SUMOylation in sugar signaling control, independent of osmotic stress (Castro *et al.*, 2015), that could account for the photosynthetic benefits of OsSIZ1 overexpression in cotton.

Another recent study describes the tomato ortholog of *SIZ1* (*SlSIZ1*) and shows that its heterologous expression in *Arabidopsis* partially complements the *siz1* mutant (Zhang *et al.*, 2017). Expression of *SlSIZ1* confers drought resistance in transgenic tobacco with increased root growth and decreased growth inhibition. In this case, transgenic plants presented elevated levels of proline, chlorophyll content and a reduction in water loss. ROS accumulation was decreased as consequence of an increased peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) activities, which are three enzymes involved in ROS scavenging. Interestingly, expression of *SlSIZ1* in *Arabidopsis* did not completely complement the *siz1* mutant phenotype under non-stressed conditions. Partial complementation was also obtained when expressing rice *OsSIZ1* and *OsSIZ2* genes in the *siz1-2* background, since expression of *OsSIZ1* and *OsSIZ2* only alleviated dwarfism and leaf development defects of the *siz1-2* mutant (Park *et al*., 2010). As described before, SUMO ligase activity in SIZ1 is confined to the SP-RING domain with the contribution of the PINIT and SIM domains. Nonetheless, additional molecular functions mapped in other regions could be more important in SUMOylation functions during development, which could account for functional diversification among orthologs.

In contrast to what has been described for several *SIZ1* orthologs, overexpression of *MMS21* in *Arabidopsis* renders the plant drought sensitive (Zhang *et al.*, 2013). Characterization of gene expression in *mms21* mutants and over-expression lines led to the proposal that *MMS21* regulates plant drought stress negatively, mainly through regulation of ABA-dependent signaling. These studies complement the work by Catala and co-workers showing the ABA-independent role of *SIZ1* in drought regulation. In this sense, MMS21-dependent conjugation could account for the small increase in SUMO conjugates observed in the *siz1* mutant in response to drought (Catala *et al.*, 2007). MMS21 appears as a key player in the regulation of proline content, linking

drought and saline stresses through the upregulation of *P5CS1* gene expression (Zhang *et al.*, 2013).

The role of SUMO proteases *ULP1C/ULP1D* (*OTS2/OTS1*) in drought stress was previously reported in *Arabidopsis*, where the double mutant *ulp1culp1d* showed enhance drought resistance compared to wild type (Castro *et al.*, 2016). Accordingly, characterization of *OTS1* RNAi plants and overexpression lines in rice correlates SUMO protease activity with a decrease in drought tolerance (increased SUMO conjugates corresponding to enhanced drought resistance) (Srivastava *et al.*, 2017).

Despite the increasing number of evidences indicating that SUMOylation indeed plays a key role in the regulation of drought responses, there are discrepancies that need to be addressed. Many of the mentioned studies do not demonstrate that the used genetic approaches translate into the desired effect at the protein level, such as increased or decreased SUMO conjugate levels. This is particular relevant considering that the SUMOylation machinery is also post-translationally regulated, as mentioned in the previous sections. For instance, the *Arabidopsis* SUMO conjugating enzyme SCE1 levels are increased in *siz1* mutants (Castaño-Miquel *et al.*, 2017; Saracco *et al.*, 2007) and attempts to overexpress *SCE1* have resulted in co-suppression (Lois *et al.*, 2003) or silencing (Tomanov *et al.*, 2013). If these SCE1 properties are conserved across evolution, drought tolerance results generated by SCE1 manipulation, as in (Nurdiani *et al.*, 2018), should be taken cautiously since the SUMO conjugation capacity of the transgenic plants was not analyzed. Only a deep molecular characterization of plants studied will provide robust conclusions about the role of SUMOylation in drought tolerance. Table 3 summarizes the above-mentioned pieces of evidence.

*SUMO targets involved in drought stress*

The modulation of SUMOylation status in plants as a tool for crop improvement and adaptation to water deficient environments is tightly dependent on the understanding of the molecular mechanisms underlying SUMO-dependent regulation of stress responses. However, despite the identification of several drought-related proteins as targets of SUMOylation, no functional confirmation has been obtained for the biological meaning of those modifications under drought conditions. In this context, the characterization of rice SUMO protease OTS1 and its direct interaction with the bZIP transcription factor OsbZIP23, points out OsbZIP23 as the first SUMOylation substrate directly involved in drought tolerance in rice (Srivastava *et al.*, 2017). A model has been proposed in which drought produces an increase in ABA levels, which in turn triggers OTS1 SUMO protease degradation. With decreased levels of OTS1 activity, SUMOylated OsbZIP23 protein increases, which results in the transcriptional activation of drought protection genes.

Recent proteomic studies have revealed that the P5CS1 protein is a substrate of SUMOylation (Miller *et al.*, 2013) (Table 4). The *P5CS1* gene codes for delta1-pyrroline-5-carboxylate synthase

1, which is the rate-limiting enzyme in the biosynthesis of proline. The level of conjugated-P5CS1 shows a discrete increase after exposure to different types of stress (heat, $H_2O_2$ or ethanol), although fold changes were not statistically significant. However, the fact that P5CS1 is identified as a SUMO substrate, and that conjugated-P5CS1 is found in both control and stress conditions, provides an interesting link between the regulation of proline accumulation, SUMOylation and response to stress that needs to be further analyzed.

All these studies present a complex landscape where SUMOylation is crucial in the control of drought stress responses. Three major signaling pathways contribute to drought tolerance: the ABA-dependent pathway, and the two pathways dependent on the transcription factors DREB2A and ERD1. SUMOylation is established now as a major hub influencing this process through ABA dependent and independent mechanisms (Fig. 4). As mentioned above, SUMOylation influences plant fitness under stress by means of:

- Controlling the hormonal signaling pathways, modulating the balance between ABA and SA-derived ROS production and regulation of stomatal aperture, as well as other hormonal signaling pathways such as GA (Conti *et al.*, 2014), JA, BR and auxin (Catala *et al.*, 2007);
- Influencing proline content, amino acid that acts as an antioxidant and provides protection against osmotic stress;
- Protecting the electron transport system, avoiding more severe effects on photosynthesis efficiency under stress;
- Possibly, although no evidence has been shown for this, influencing the DREB2A-mediated pathway by influencing the action of DREB2A-interacting proteins 1 and 2 (DRIP1 and 2) (Qin *et al.*, 2008). All three proteins, DREB2A, DRIP1 and 2 proteins have been identified as SUMO targets in three proteomic studies, together with other relevant factors related to water deficit and drought responses studies (Miller *et al.*, 2010; Miller *et al.*, 2013; Rytz *et al.*, 2018) (Table 4). DRIP proteins act as ubiquitin E3 ligases, interact with DREB2A and mediate its ubiquitination, providing a link between SUMOylation, ubiquitination and the DREB2A pathway that sets an interesting starting point for further investigation. This interplay between SUMOylation and ubiquitination in the control of transcription factors or co-activators has been revealed in different pathways such as ABA signaling (ABI5) (Miura *et al.*, 2009), defense (NPR1) (Saleh *et al.*, 2015) or gibberellin (DELLA proteins) (Conti *et al.*, 2014).

*Interplay between salt and drought stress and SUMO*

Drought and salinity stress are tightly related, both often being present simultaneously and influencing crop growth in arid regions. Together with the increase in drought periods, salinization of arable land is one of the major concerns threatening crop productivity at a global scale. Several pieces of evidence indicate that SUMOylation is also crucial in regulation of salinity tolerance. The *siz1-1* mutant was originally isolated as a suppressor of the salinity sensitive phenotype of the *sos3-1* mutant (Miura *et al.*, 2005). More recently, the *Arabidopsis ots1ots2* double mutant,

699 which has the deSUMOylation activity compromised and accumulates SUMO conjugates, has
700 extreme sensitivity to salt compared to wild type (Conti *et al.*, 2008), in contrast to their increased
701 tolerance to drought (Castro *et al.*, 2016).

702 The functional characterization of the SUMO ligases PIAL1 and PIAL2 (Tomanov *et al.*, 2014)
703 has revealed additional data regarding the consequences of increased SUMO conjugates in the
704 regulation of salinity tolerance. Surprisingly, SUMO conjugates accumulate in the *pial1pial2* under
705 salt stress, as opposed to what it was expected since PIAL1 and PIAL2 act as SUMO ligases
706 (Tomanov *et al.*, 2014). The *pial1pial2* double mutant is therefore sensitive to salt, as the double
707 mutant *ots1 ots2*.

708 In rice, *OsOTS1* overexpression lines show an increased tolerance to salt stress compared to
709 wild type, while *OsOTS1* RNAi plants were more sensitive (Srivastava *et al.*, 2016). Thus,
710 accumulation of SUMO conjugates in rice by depletion of OTS SUMO protease results in salt
711 sensitivity and drought tolerance (Srivastava *et al.*, 2017) .

712 Overall, an increase in SUMO conjugates has a negative impact on salinity tolerance while its
713 effect in drought tolerance is controversial (Table 3). Further research in this field is needed to
714 clarify how SUMOylation affects the capability of the plant to cope with salinity and, more
715 importantly, how responses to drought and salinity, including SUMOylation-dependent
716 mechanisms, are coordinated in order to optimize growth under stress conditions.

717

718 **Future challenges and perspectives.**

719 Drought tolerance is a complex agronomic trait that relies on plant genotype, environmental
720 conditions beyond water restriction, and plant culture management. The integration of these
721 factors determines drought tolerance. On the other hand, SUMO conjugation is a complex
722 regulatory mechanism that is under a tight regulation and affects multiple signaling pathways.

723 In spite of recent advances, further studies are required to determine which are the molecular
724 determinants of the SUMO machinery specificity, which will shed light on how SUMOylation
725 affects different sets of substrates in response to single or combined abiotic stresses. In this
726 sense, the major challenges in the SUMO field are the biochemical and structural analysis of
727 SUMO machinery components, in addition to the functional and mechanistic validation of the
728 hundreds of putative SUMO targets identified. Significant advances in this area will require a
729 major effort and technical improvements, mainly related to the capacity to detect *in vivo*
730 SUMOylation dynamics.

731 In summary, SUMOylation has an important role in the regulation of abiotic stress responses,
732 and particularly in drought. The immediate challenge is to identify and validate SUMO substrates
733 specifically involved in drought responses. The characterization of specific substrates, together
734 with in-depth knowledge of SUMOylation specificity and subcellular compartmentalization will
735 lead to the design of more accurate molecular tools for drought tolerance improvement in crops.

**Acknowledgments**

This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (MINECO) and the European Regional Development Fund (ERDF) (BIO2017-89874-R) to LML's laboratory and BIO2015-73491-JIN to RB's group. We also thank the Generalitat de Catalunya (Xarxa de Referència en Biotecnologia and 2017SGR 1211) for substantial support. We thank the reviewers for their insightful comments, which have contributed to increase the quality of the review. We apologize to authors that have made important contributions to the field and that have not been quoted due to space constraints.

**Conflict of Interest**

The authors declare that they have no conflict of interest

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## FIGURE LEGENDS

**Figure 1.** *SUMO conjugation system*.

A) Ribbon representation of human SUMO as determined by NMR (2KQS)

B) SUMO conjugation/deconjugation cycle. SUMO is synthesized as a precursor that is processed at its C-terminal tail by the specific ULP proteases, releasing a SUMO mature form with a Gly-Gly motif at its C-terminus. Subsequently, SUMO is activated by the heterodimeric E1 activating enzyme, SAE1/SAE2, transferred to the E2 conjugating enzyme and, finally, attached to a target lysine in the substrate. The target lysine is usually located within the consensus site ΨKxE (Ψ is a large hydrophobic amino acid, K the modified lysine, x any amino acid and E a glutamate acid residue). This final step is facilitated by E3 ligase enzymes that interact both with SUMO charged E2 and the substrate. SUMOylation is a reversible modification and the same class of cysteine proteases involved in the maturation step catalyze SUMO excision from the substrate. In *Arabidopsis*, the SUMO conjugation machinery is composed of the SUMO isoforms SUMO1 (At4g26840), SUMO2 (At5g55160), SUMO3 (At5g55170), SUMO5 (At2g32765); the E1 enzyme subunits SAE2 (At2g21470). SAE1a (At4g24940), SAE1b (At5g50580/ At5g50680); the E2 SCE1 (At3g57870); the ligases SIZ1 (At5g60410), MMS21/HPY2 (At3g15150), PIAL1 (At1g08910), PIAL2 (At5g41580); and the ULPs described in Table 1).

**Figure 2.** SUMO E2-conjugating and E3 ligase enzymes.

A) Human SCE1 structural representation, based on 2PE6 structure, showing the residues involved in non-covalent interactions established with SUMO (green) (Capili and Lima, 2007), the E1 activating enzyme (cyan) (Liu *et al.*, 2017a), the SIZ1 E3 ligase (blue) (Streich and Lima, 2016), and the overlapping residues involved in E1 and SUMO interactions (pink).

B) Schematic representation of *Arabidopsis* E3 ligases, SIZ1, MMS21 and PIAL2, showing functional domains involved in DNA binding (SAP), plant homeodomain (PHD), substrate and E2 binding (PIIT and SP-RING), and SUMO interacting motif (SIM), and IND (interacting domain).

**Figure 3.** Subcellular distribution of *Arabidopsis* SUMO machinery components.

A. Representation of the distribution reported from expression studies that analyzed SUMOylation machinery components individually.

B. Distribution as observed in co-expression experiments of E1 and E2 or co-expression experiments of SUMO and E2.

**Figure 4.** Model showing the molecular mechanisms proposed to mediate regulation of drought tolerance by SUMO conjugation.

Table 1

*Arabidopsis* ULP sequences were retrieved from Araport11 protein sequence database (https://www.*Arabidopsis*.org/tools/bulk/sequences/index.jsp) and the ULP_protease family domain (PS50600) mapped on the sequence using ScanProsite (https://prosite.expasy.org/index.html9). Protein full length sequences are represented by a rectangle and the ULP_protease family domain by a dark grey box.

| | Gene code | Name | Alternate name | Length | Protein structure |
|---|---|---|---|---|---|
| ***Class I (C)*** | At4g15880 | AtESD4 | | 489 aa |  |
| | At3g06910 | AtULP1a | ELS1 | 502 aa |  |
| | At4g00690 | AtULP1b | | 341 aa |  |
| ***Class II (B1)*** | At1g10570 | AtULP1c | OST2 | 571 aa |  |
| | At1g60220 | AtULP1d | OST1 | 584 aa |  |
| ***Class III (B2)*** | At1g09730 | SPF1 | ASP1 | 963 aa |  |
| | At4g33620 | SPF2 | | 774 aa |  |
| ***Class IV (A)*** | At3g48480 | | | 298 aa |  |

Table 2.

SUMO isoform specificity displayed by *Arabidopsis* SUMO proteases and the pathogen effector XopD. Endopeptidase and isopeptidase relative efficiency are shown in red (high), medium (blue), and low (yellow). ND, not detectable; nt, not tested. (Chosed *et al.*, 2006; Chosed *et al.*, 2007; Colby *et al.*, 2006; Hermkes *et al.*, 2011; Kong *et al.*, 2017; Liu *et al.*, 2017b)

| | | SUMO1 | SUMO2 | SUMO3 | SUMO5 |
|---|---|---|---|---|---|
| **ESD4** | endopep. | | | ND | ND |
| | isopep. | | | ND | ND |
| **ELS1** | endopep. | | | | ND |
| | isopep. | | | ND | ND |
| **ULP1b** | endopep. | n.t. | n.t. | n.t. | n.t. |
| | isopep. | n.t. | n.t. | n.t. | n.t. |
| **OST1** | endopep. | | | ND | ND |
| | isopep. | | | ND | ND |
| **OST2** | endopep. | | | ND | ND |
| | isopep. | | | ND | ND |
| **SPF1** | endopep. | | ND | ND | n.t. |
| | isopep. | n.t. | n.t. | n.t. | n.t. |
| **SPF2** | endopep. | | ND | ND | n.t. |
| | isopep. | n.t. | n.t. | n.t. | n.t. |
| **At3g48480** | endopep. | n.t. | n.t. | n.t. | n.t. |
| | isopep. | n.t. | n.t. | n.t. | n.t. |
| **XopD** | endopep. | | | ND | ND |
| | isopep. | | | | ND |

Table 3. Summary of studies analyzing mutants, constitutive expression or RNAi of different SUMO conjugation machinery components and the effects on drought and salinity tolerance. ND: not determined. Asterisks denote that expected alterations in SUMO conjugation capacity of the studied plants were confirmed at the protein level.

| | Donor Species | Gene | Receptor Species | Genotype | Expected SUMO conjug. | Phenotype in | |
|---|---|---|---|---|---|---|---|
| | | | | | | Drought/ osmotic | high salinity |
| **Mutant** | *Arabidopsis* | *SIZ1* | *Arabidopsis* | *siz1** | | | |
| | | Catala *et al*., 2007 | | | down | Sensitive | ND |
| | | Miura *et al*., 2012; | | | down | Tolerant | ND |
| | | Kim *et al* 2017 | | | down | Tolerant | ND |
| | *Arabidopsis* | *MMS21* | *Arabidopsis* | *mms21** | | | |
| | | Zhang *et al*., 2013 | | | down | Tolerant | ND |
| | *Arabidopsis* | *ULP1C/D (OTS1/OTS2)* | *Arabidopsis* | *ulp1culp1d** | | | |
| | | Castro *et al*., 2016 | | | up | Tolerant | Sensitive |
| **Constitutive expression** | *Arabidopsis* | *SAE2$^{UFDCt}$* | *Arabidopsis* | *OE** | | | |
| | | Castaño-Miquel *et al*., 2017 | | | down | Sensitive | ND |
| | *S. alterniflora* | *SaSce9* | *Arabidopsis* | *OE* | | | |
| | | Karan and Subudhi, 2012 | | | up | Tolerant | Tolerant |
| | *Oryza sativa* | *OsSIZ1* | *A. stolonifera* L. | *OE* | | | |
| | | Li *et al*., 2012 | | | up | Tolerant | ND |
| | *Oryza sativa* | *OsSIZ1* | *G. hirsutum* | *OE* | | | |
| | | Mishra *et al*., 2017 | | | up | Tolerant | ND |
| | *S. lycopersicum* | *SlSIZ1* | *N. tabacum* | *OE** | | | |
| | | Zhang *et al*., 2017 | | | up | Tolerant | ND |
| | *Arabidopsis* | *MMS21* | *Arabidopsis* | *OE* | | | |
| | | Zhang et al., 2013 | | | up | Sensitive | Sensitive |
| | *Oryza sativa* | *OsOTS1* | *Oryza sativa* | *OE** | | | |
| | | Srivastava *et al*., 2016 | | | down | ND | Tolerant |
| | | Srivastava et al., 2017 | | | down | Sensitive | ND |
| **down regulation** | *Oryza sativa* | *OsOTS1* | *Oryza sativa* | *RNAi** | | | |
| | | Srivastava *et al*., 2016 | | | up | ND | Sensitive |
| | | Srivastava *et al*., 2017 | | | up | tolerant | ND |

**Table 4.** SUMO substrates identified by proteomic studies and related to drought or water deficit stress. All identified substrates were classified according to gene ontology (GO) terms and selected those related to “abiotic stimulus” and “response to water deprivation”. The GO enrichment analysis was conducted using agriGO. V2.0 (Tian *et al.*, 2017). Asterisk (*) indicates statistical significant differences among protein-conjugate levels in control and stress conditions (Miller *et al.*, 2013) or between wild type and *siz1-2* mutant (Rytz *et al.*, 2018).

| Locus | Description | Name | | | |
|---|---|---|---|---|---|
| | | | Miller *et al*., 2010 | Miller *et al.,* 2013 | Rytz *et al.,* 2018 |
| At2g38470 | Member of the plant WRKY transcription factor family | WRKY33 | ✓ | | ✓(*) |
| At1g06770 | C3HC4 RING-domain-containing ubiquitin E3 ligase capable of interacting with DREB2A | DRIP1 | ✓ | | |
| At2g30580 | C3HC4 RING-domain-containing ubiquitin E3 ligase capable of interacting with DREB2A | DRIP2 | ✓ | ✓ (*) | ✓(*) |
| At2g22430 | Homeodomain leucine zipper class I (HD-Zip I) protein | ATHB6 | ✓ | ✓ (*) | ✓(*) |
| At2g39800 | delta1-pyrroline-5-carboxylate synthase | P5CS1 | | ✓ | |
| At1g20440 | Dehydrin protein family | COR47 | | ✓ (*) | |
| At1g54410 | KS-type dehydrin | HIRD11 | | ✓ | |
| At5g61590 | AP2/ERF transcription factor | ERF107 | | | ✓(*) |
| At5g05410 | AP2/ERF transcription factor | DREB2A | | | ✓(*) |
| At4g34000 | ABA-responsive element binding protein | ABF3 | | | ✓(*) |

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