Sumoylation in Arabidopsis: stress response, signaling & evolution

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Chapter 7

General discussion

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Post-translation modifications (PTMs) are a major strategy used by cells to quickly modulate protein activity in response to (a)biotic stresses or during development. Sumoylation constitutes one of the most crucial PTMs, as it has been shown to be essential in model eukaryotic organisms like budding yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), roundworm (*Caenorhabditis elegans*), human and Arabidopsis (*Arabidopsis thaliana*) (Seufert et al., 1995; Epps & Tanda, 1998; Mossessova & Lima, 2000; Nacerddine et al., 2005; Nowak & Hammerschmidt, 2006; Saracco et al., 2007). Sumoylation is involved in numerous physiological processes ranging from transcriptional regulation to signaling. Understanding the complex system of sumoylation is far from being complete. This thesis has explored the involvement of sumoylation in the control of the plant immune signaling and plant development (Chapter 2), in acclimation to elevated ambient temperature (Chapter 4) and has aimed at uncovering the mechanisms that led to an expanded *SUMO* gene family in Arabidopsis (Chapter 5 and 6). In this chapter, I will discuss the main findings of this thesis on the role of *SUMO* proteins in the Arabidopsis as well as novel research questions raised from these findings.

**Are SUMO1 and SUMO2 fully redundant?**

There are eight genes that code for *SUMO* proteins in Arabidopsis. It is well established that AtSUMO1 and AtSUMO2 act as the main substrates for *SUMO* conjugation (Kurepa et al., 2003; Lois et al., 2003; Saracco et al., 2007). Using gene phylogeny and collinearity analyses, we showed that *AtSUMO1* and *AtSUMO2* represent the archetype *SUMO* gene in plants. *AtSUMO1/2* are the closest homologs of the human *HsSUMO2/3*. The sequence of the encoded SUMO1/2 proteins is extremely conserved between orthologs found in mosses and those in angiosperms (Chapter 5). In fact, *AtSUMO1* and *AtSUMO2* emerged from the same ancient *SUMO* gene by a gene duplication that occurred after the split of *Tarenaya hassleriana* (family Cleomaceae) and Brassicaceae. So far, neither biochemical nor genetic differences were observed between *AtSUMO1* and *AtSUMO2* (van den Burg et al., 2010). For example, SUMO1 and SUMO2 are used with the same efficiency for *in vitro* (de)conjugation by the different SUMO enzymes (Colby et al., 2006). Also, the single *sumo1* or *sumo2* mutants do not exhibit any aberrant phenotype. In contrast, the double mutant *sumo1 sumo2* is embryo-lethal, reinforcing the idea that these two isoforms act redundantly (Saracco et al., 2007). Our genetics studies confirmed this redundancy, as *sumo1* heterozygous *SUMO2*<sup>KD</sup> homozygous (*sumo1<sup>+/+</sup> SUMO2<sup>KD</sup>) mutants resembled wild type plants (Chapter 3 and Chapter 4). A single wild type allele for *SUMO1* is apparently enough to ensure sufficient levels of *SUMO* proteins.
However, the expression pattern of AtSUMO1 and AtSUMO2 is not identical but complementary: AtSUMO1 is apparently broadly expressed in various plant tissues (leaves, roots, but not the vasculature, the lateral root primordia, and the root apex), whereas AtSUMO2 is highly expressed in the vasculature, the lateral root primordial and the root apex, and less in the mesophyll cells (van den Burg et al., 2010). This suggests that AtSUMO1 and AtSUMO2 have most likely sub-functionalized, at least at the tissue level (van den Burg et al., 2010). A similar situation is seen in humans, as the roles of two almost identical SUMO isoforms, HsSUMO2 and HsSUMO3, largely overlap. However, they have different roles in embryonic development: HsSUMO2 is essential while HsSUMO3 is dispensable (Wang et al. 2014). This example in humans shows that experimental procedures that have been carried out so far might not be adapted to demonstrate a putative functional difference that exist between AtSUMO1 and AtSUMO2.

What happened to SUMO genes after duplication?
In addition to the two archetype genes AtSUMO1/2, the genome of Arabidopsis contains six other SUMO homologs. This contrasts with the genome of organisms like roundworm, fruit fly or budding yeast where only a single archetype SUMO gene is present and sufficient (Floto & Melchior, 2013). Explaining the origin of this high number of SUMO genes in Arabidopsis in contrast to other eukaryotes was one of the major challenges addressed in this thesis. In Chapter 5, we depicted the evolutionary mechanisms that led to the emergence of these six additional Arabidopsis SUMO copies, which show a remarkable sequence divergence from AtSUMO1/2. We revealed that alternating whole-genome and tandem-gene duplications have caused the expansion of the SUMO gene family currently found in Arabidopsis (Hammoudi et al., 2016). Importantly, overexpression of tagged variants of AtSUMO1 or AtSUMO2 leads to activation of plant immunity at the expense of plant development and fitness (van den Burg et al., 2010). Potentially, the duplication of SUMO copies results in a higher gene dosage of SUMO, which is then also harmful for the organism. Moreover, many species can survive with a single SUMO gene, and many SUMO duplicates are lost after their birth (Chapter 5). Based on plant phylogeny, we could decode the evolutionary history of the Arabidopsis SUMO gene family in order to determine how the SUMO gene family has expanded with this gene dosage constraint.

To assess whether the additional SUMO genes are possibly active in Arabidopsis or its sister species, we analyzed the following criteria: (i) the conservation of the diGly motif across species, (ii) gene expression in species based
on published data, (iii) divergence of the SUMO sequences between species and (iv) divergence of the SUMO genes within a population of 444 Arabidopsis accessions. We conclude that several Arabidopsis SUMO genes became pseudogenes. For instance, AtSUMO7 is not expressed in Arabidopsis, it has lost its diGly motif and it displays high sequence variation between Arabidopsis accessions, indicating that AtSUMO7 pseudogenized in Arabidopsis. However, SUMO7 expression was reported for C. rubella (NGS data) suggesting that this SUMO gene may encode a functional protein in sister species of Arabidopsis. Likewise, others and we concluded that AtSUMO4, AtSUMO6 and AtSUMO8 must have pseudogenized. On the other hand, SUMO3 and SUMO5, which share 50% and 35% protein sequence identity with SUMO1/2, respectively, have a conserved diGly motif and are still transcriptionally active (Kurepa et al., 2003; Chapter 5). In addition, SUMO5 showed a strong conservation within the Arabidopsis population. Thus, SUMO3 and SUMO5 appeared to be good candidates for functional proteins with a role as substrates for protein modification. Overall, we see that SUMO1 and SUMO2 remain under a strong negative selection pressure in Arabidopsis, while the other SUMO paralogs appear to be under a positive selection pressure that leads to pseudogenization, or potentially to neo-functionalization.

**Has SUMO3 neo-functionalized in Arabidopsis during evolution?**

In Chapter 6, we found that accumulation of the Arabidopsis SUMO3 paralogs can rescue the AtSUMO1 and AtSUMO2 compromised phenotype in the sumo1/2KD mutant. Moreover, high levels of Arabidopsis SUMO3 complemented a compromised SUMO1/2 function. Finally, the Arabidopsis SUMO3 gene is dispensable in wild type plants, but it is required when SUMO1/2 protein levels are low, as genetically demonstrated by the embryo-lethality of the sumo1/2KD sumo3 triple mutant (Chapter 6). These findings are remarkable, as it was previously suggested that AtSUMO3 does not act complementary, but rather antagonistically to AtSUMO1/2, at least in plant immunity and flowering (van den Burg et al., 2010). We also established that the premature form of SUMO3 could rescue the sumo1/2KD mutant. Combined this means that the endogenous SUMO3 gene is still active in Arabidopsis and likely mimics to some degree SUMO1/2 function.

Interestingly, reduced SUMO1/2 levels in sumo1/2KD result in SA accumulation, while SA in turn induces expression of AtSUMO3. AtSUMO3 expression is, therefore, likely to be up-regulated in sumo1/2KD (van den Burg et al., 2010). This places SUMO3 function downstream of SUMO1/2. Importantly, we did not see up-regulation of SUMO3 in the pad4 background in the microarray analysis performed in Chapter 4. The SA-dependent phenotype of sumo1/2KD could,
therefore, be less severe due to some accumulation of SUMO3 protein that partially complements the low SUMO1/2 levels and potentially enhances the viability of the mutant plants. However, the embryo-lethality of sumo1 sumo2 double mutant indicates that endogenous SUMO3 cannot complement the complete loss of SUMO1/2. The recovery of the sumo1 sumo2 embryo-lethality by overexpression of AtSUMO3 suggests that SUMO3 is not as good a substrate for SUMO conjugation as SUMO1/2, corroborating the findings of Castano-Miquel et al., (2011) and/or the expression of the AtSUMO3 promoter does not allow SUMO3 to accumulate to sufficient levels to take over the function of SUMO1/2, including embryo-development (van den Burg et al., 2010).

By comparing the protein sequences of SUMO1/2 and SUMO3, we found that the important SUMO interacting motif (SIM)-binding pocket in SUMO3 differs from SUMO1/2 (Chapter 6). Our data indicates that the modified SIM binding pocket of SUMO3 compromises its interaction with the SUMO E3 ligase SIZ1, suggesting that SUMO3 in Arabidopsis functionally drifts away from the SIZ1-dependent conjugation pathway. At this stage we cannot conclude whether Arabidopsis SUMO3 is on its way to pseudogenize by progressively becoming an inactive form of SUMO1/2 or whether it has already neo-functionalized and gained new properties (Fig. 1). For instance, AtSUMO3 may have neo-functionalized by encoding a protein that interacts with SIMs that are different from the SUMO1/2-interacting SIMs. This would result in paralog-specific protein interaction. For example, the SA receptor NPR1 interacts with SUMO3, but not with SUMO1/2 (Saleh et al., 2015). In addition, SUMO3 interacts with other proteins than SUMO1/2 in a yeast-two hybrid assay (Elrouby & Coupland, 2010; Elrouby et al., 2013; Mazur and van den Burg, personal communication). Also, HsSUMO2/3 (homologs of Arabidopsis SUMO1/2) and HsSUMO1 interact with different partners, confirming that neo-functionalization of SUMO paralogs via novel non-covalent interactions has occurred independently in other organisms (Zhu et al., 2008).

In humans, acetylation of HsSUMO1 and HsSUMO2 in the SIM-binding pocket controls non-covalent interaction with SIMs peptides (Ullmann et al., 2012). The acetylation of Lys37 and Lys33 in HsSUMO1 and HsSUMO2, respectively, leads to the neutralization of the positive charge in the SIM-binding pocket and prevents binding of these SUMO paralogs to SIM-containing proteins like Protein Inhibitor of Activated STAT 1 (PIAS1), a human homolog of Arabidopsis SIZ1 (Ullmann et al., 2012). Also, an acetyl-mimicking variant of HsSUMO2Ac-K37 but not HsSUMO1Ac-K37 interacts better with the bromodomain protein p300. This led to the idea that acetylation of HsSUMO2 acts as a molecular switch that shifts the SUMO-interaction
from PIAS1 to bromodomain proteins. Strikingly, the protein alignment of Arabidopsis and human SUMOs reveals that the Lys residue subject to acetylation in HsSUMO1 an HsSUMO2 (Fig. 2; blue letters) is also a Lys in AtSUMO3, whereas it is an Arg in AtSUMO1/2 (Fig. 2; red letters). Consequently, this position can no longer be acetylated in AtSUMO1/2 (Dormeyer et al., 2005; Yang & Seto, 2008). In contrast, AtSUMO3 might be acetylated and its acetylation would then likely modulate its interactome. Assuming that SUMO1/2 mainly interact with SIZ1, the acetylation of SUMO3 could (i) inhibit the SUMO3-SIZ1 interaction and (ii) promote its interaction with bromodomain-containing transcription activators (Mujtaba et al., 2007). In agreement with this hypothesis, AtSUMO3 is modified by a yet unknown PTM (van den Burg et al., 2010). As convergent evolution has happened multiple times during evolution (Owerbach et al., 2005), it would not be surprising that acetylation has independently emerged in plants and in mammals as a way to control the SIM-specificity of SUMO paralogs (Ullmann et al., 2012).

Although the function of SUMO3 apparently largely overlaps with SUMO1/2 in Arabidopsis, the question whether SUMO3 is diverging from SUMO1/2, which results in pseudogenizing or neo-functionalizing, remains open (Fig. 1). There are three arguments for pseudogenization of AtSUMO3: (i) SUMO3 is lost in several Brassicaceae species (Chapter 5). (ii) The SUMO3 protein sequence is highly variable within the Arabidopsis population (Chapter 5). Finally, (iii) a SUMO protease that can efficiently maturate the premature SUMO3 or deconjugate SUMO3 from targets remains to be identified (Chapter 5). In this light, we found that the SUMO3 precursor is apparently maturated in Arabidopsis, as it could rescue the sumo1/2KD mutant (Chapter 6).
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Figure 2: Multiple protein sequence alignment (obtained with Clustal) of the Arabidopsis isoforms AtSUMO1, AtSUMO2, AtSUMO3, and the human isoforms HsSUMO1, HsSUMO2 and HsSUMO3.

In black is shown the SIM-binding pocket. The residue numbering corresponds to the sequence of AtSUMO1. In colour are shown the corresponding residues to the acetylated Lys residue found in HsSUMO1; HsSUMO2 and HsSUMO3: the corresponding Lys are shown in blue, while the corresponding Arg are shown in red.

On the other hand, there are at least five arguments for neo-functionalization of AtSUMO3. (i) The diGly motif of AtSUMO3 is retained, suggesting that AtSUMO3 is still biochemically active and that selection pressure operates on this motif in the SUMO3 gene (Chapter 5). (ii) None of the substitutions, which were found within the Arabidopsis population, introduced an early stop codon in the open reading frame, indicating that Arabidopsis retains a functional SUMO3 gene (Chapter 5). (iii) SA strongly induces the expression of AtSUMO3 and to a lesser extent AtSUMO1/2, which suggests a specific role for SUMO3 in plant defense (van den Burg et al., 2010). (iv) The Xanthomonas campestris effector protein XopD contains a SUMO-protease that is capable of cleaving conjugated SUMO3 from an artificial substrate PCNA in vitro (Colby et al., 2006). This could indicate that SUMO3 targets as decoy XopD or that SUMO3 conjugation is suppressed by XopD to enhance bacterial disease. Finally, (v) SUMO3 non-covalently interacts with proteins that do not interact with SUMO1/2 (Elrouby & Coupland, 2010; Elrouby et al., 2013; Mazur and van den Burg, personal communication). This suggests that SUMO3 and SUMO1/2 interactoms do not entirely overlap. Taken together, neo-functionalization of AtSUMO3 appears to be the most likely scenario, thus indicating that SUMO3 is an innovation in Brassicaceae plants.

Is SUMO5 a protein modifier?

Our analysis using genome collinearity and gene phylogeny of the different SUMO genes revealed that SUMO5 ancestral gene in dicots has quickly diverged from the
archetype SUMO after its emergence near the whole genome triplication (WGT) At-γ (~135 mya). However, the SUMO5 gene lineage has retained the diGly motif up to Brassicaceae, which contrasts with the frequent loss of the diGly motif the SUMO4 orthologs (Chapter 5). SUMO4 only first emerged in a common ancestor of the Brassicaceae (~40 mya), while SUMO5 is clearly older, as it first emerged prior to the split of the Brassicaceae and Cleomaceae families (~52 mya). We also noted that the protein sequence of the SUMO5 paralog is highly conserved within the Arabidopsis population examining 444 accessions (Chapter 5, Fig. 3). Interestingly, overexpression of mature AtSUMO5 in Arabidopsis plants resulted in conjugation of SUMO5 to unknown proteins (Budhiraja et al., 2009). Combined, these data suggest that AtSUMO5 has neo-functionalized and that it is now under negative selection pressure in Arabidopsis (and perhaps more broadly in Brassicaceae). Moreover, AtSUMO5 appears to be a poor substrate for the well-characterized Arabidopsis SUMO conjugation pathway (involving SAE1/2 and SCE1) when compared to SUMO1/2 (Chosed et al., 2006; Castano-Miquel et al., 2011). In addition, none of the known SUMO proteases could effectively maturate preSUMO5 or cleave conjugated SUMO5 from a substrate (Chosed et al., 2006). If SUMO5 is actually not maturated, then the absence of maturation for this SUMO isoforms might indicate that SUMO5 acts exclusively though non-covalent interactions. However, this hypothesis can most likely be excluded considering the conjugation of the mature forms of SUMO5 in vitro and in vivo (Budhiraja et al., 2009), and the high conservation of the diGly motif in SUMO5. SUMO5 might, therefore, possess both its own E1-E2-E3 cascade and a set of proteases for maturation and deconjugation. If SUMO5 indeed has its own conjugation pathway, then we should actually consider SUMO5 as a novel class of Ubiquitin-like modifiers rather than a paralog of SUMO1/2.

**Are there unknown SUMO machinery enzymes in Arabidopsis?**

By demonstrating that SUMO3 is a functional paralog of SUMO1/2 in Arabidopsis and that overexpression of preSUMO3 can rescue the sumo1/2\(^{KD}\) (Chapter 6), we indirectly demonstrate that AtSUMO3 is maturated in planta, as suggested previously (Kurepa et al., 2003; van den Burg et al., 2010). In addition, our findings in Chapter 5 strongly suggest that SUMO5 is a functional protein modifier. Yet, Arabidopsis SUMO proteases that are biochemically active towards SUMO3/5 remain to be identified for both these isoforms. If SUMO3/5 are actually processed in planta by the known Arabidopsis SUMO protease(s), then there are at least two possibilities to explain why the SUMO proteases could not cleave these two SUMO isoforms in vitro (Colby et al., 2006; Chosed et al., 2006). (i) The proteases might need to be modified by PTM in order to recognize SUMO3/5 (Villamil et al., 2012). (ii) These proteases can only operate on SUMO3/5 as heterodimers (Liu & Chien, 2014). Alternatively,
the Arabidopsis genome may encode SUMO proteases without any homology to the known SUMO proteases. These putative SUMO3/5 specific proteases remain to be identified.

I speculate here that further unknown SUMO (de)conjugating enzymes may have emerged from ancient gene duplication and diverged enough not to share similarities with the known SUMO enzyme. For instance, many angiosperms possess multiple copies of SCE1, while only one SCE1 gene is found in the genome of Arabidopsis (Novatchkova et al., 2012). SCE1 duplicate(s), resulting from the WGT At-γ, might have immediately co-evolved with SUMOS5 ancestral gene in different dicot lineage, resulting in a SUMOS5-specific E2 enzyme in Brassicaceae. One possible strategy to verify this hypothesis is to examine by synteny and cluster analysis whether the Arabidopsis genome contains collinear genes with the ancient SCE1 prior to WGT At-γ (Chapter 6). Although the synteny is rather weak prior WGT At-γ, this approach could also reveal whether additional genes that encode SUMO E3 ligases exist.

Strikingly, there are hundreds of SUMO targets, while there are only two SUMO E3 ligases in Arabidopsis. Because SUMO E3 ligases are considered to promote sumoylation in specific circumstances, the Arabidopsis genome might, therefore, also encode additional unknown SUMO E3 ligases. The two SUMO E3 ligases characterized in Arabidopsis, SIZ1 and HPY2, share a common SP-RING domain that is typically found in SUMO E3 ligases in other eukaryotes. However, SUMO E3 ligases without a SP-RING domain have also been identified in mammals like RanBP2 and Pc2 (Wang & Dasso, 2009). These latter two SUMO E3 ligases bind SCE1 by domains other than SP-RING protein domain. Hence, additional unknown E3 ligases might be involved in the specific modification of certain substrates among the set of hundreds of SUMO substrates currently identified. However, the strong pleiotropic phenotypes of the siz1 and hpy2 single mutants, and the embryo-lethality of the siz1 hpy2 double mutant, sustain that these two E3s play a prominent role in plant development (Ishida et al., 2012).

**How does the SUMO E3 ligase SIZ1 function?**

SUMO E3 ligases are defined as proteins that promote conjugation of SUMO onto (certain) substrates in a sumoylation assay where the concentrations of the E1 and E2 enzymes are substantially lower than required for reaction with only the E1 and E2 enzymes. In contrast to the high number of ubiquitin E3 ligases that provide substrate specificity to conjugation reactions, the number of identified SUMO E3 ligases in the various model organisms is low, especially in the model plant Arabidopsis. Hence,
how, when and where SUMO E3 ligases function remain unknown. SIZ1 contains different functional protein domains (i.e. SAP, PHD, PINIT, SP-RING, SIMs and NLS) (Cheong et al., 2009; Cheong et al., 2010; Chapter 1). In contrast, the SP-RING domain is the only functional domain in HPY2 (Ishida et al., 2009). This suggests that these two SUMO E3 ligases may work through different molecular mechanisms.

There are three non-exclusive hypotheses about the different molecular mechanisms of the SUMO E3 ligases SIZ in Arabidopsis. First, SIZ1 works as a molecular scaffold that gathers the E2 enzyme SCE1, SUMO and the conjugation substrate in a single protein complex (Chapter 6). This hypothesis is supported by the phenocopy of the siz1 mutant in plants that overexpress SUMO1/2ΔGG i.e. dwarfism reverted at elevated temperature (van den Burg et al., 2010; Chapter 6). We also found that SUMO3 is drifting away from the SIZ1 conjugation pathway and that SUMO3ΔGG cannot interfere with SIZ1-mediated conjugation (Chapter 6). This corroborates the fact that overexpression of SUMO3ΔGG in a wild type background does not lead to a siz1-like phenotype (van den Burg et al., 2010). We did, however, not address whether SUMO3 still belongs to the HPY2 (or other unknown E3 ligases) pathway. If SUMO3 would only have diverged away from SIZ1, then this would constitute neo-functionalization of SUMO3 with respect to its substrate specificity via SUMO E3 ligases.

Second, SIZ1 may have additional functions in regulating gene expression. SIZ1 contains a NLS, a DNA binding domain (SAP domain) and a PHD domain that is implicated in binding histone H3K4me3 marks (Li et al., 2006; Pena et al., 2006). This means that SIZ1 possibly regulates gene expression by acting at the chromatin level. Histone H3K4me3 marks are generally associated with transcriptional activation. This epigenetic mark does not activate transcription of the gene itself, but rather promotes histone H3 acetylation at the affected loci, which in turn positively controls gene expression (Pray-Grant et al., 2005). SIZ1 might, therefore, bind the H3K4me3 marks at promoter regions and modulate the transcriptional activity of the affected genes by controlling their acetylation. In human, the silencing of the PIAS1 family (homologs of AtSIZ1) leads to a decrease in SUMO conjugation at the chromatin level, corroborating this hypothesis (Niskanen et al., 2015).

Finally, SIZ1 may promote SUMO conjugation at non-consensus sites. Since the SUMO E2 enzymes recognize and bind the SUMO acceptor peptide motif ψKxE (Bernier-Villamor et al., 2002), SIZ1 may force sumoylation of lysine residues that are not recognized by E2s. This alternative lysine sumoylation has been observed in
yeast, but remains elusive in plants, although ~20% the identified SUMO targets in Arabidopsis do not contain any sumoylation consensus sites (Pfander et al., 2005; Yunus & Lima, 2009; Miller et al., 2010). The massive SUMO conjugation caused by heat stress is largely suppressed in siz1 (Yoo et al., 2006). Beside the inactivation of SUMO proteases, SIZ1-mediated conjugation appears thus to play a crucial role in global sumoylation to a broad spectrum of proteins. This thesis provides data that supports the first hypothesis, where SIZ1 acts as a scaffold protein (Chapter 6). In particular, we showed how SUMO1 and SUMO3 compete for SIZ1 via its SIMs.

The first hypothesis could be reinforced by determining whether overexpression of SIZ1 with mutations in the SP-RING domain or in the SIM(s) results in a siz1 null mutant phenocopy. The second hypothesis could be verified by Chromatin-Immuno Precipitations (ChIP) of SIZ1 in combination with ChIP of H3K4me3. These experiment could reveal whether SIZ1 preferentially binds to H3K4me3 enriched promotors or other genomic elements. Finally, analyzing with proteomics whether there is an enrichment of sumoylation on SUMO consensus sites in SUMO modified proteins in the siz1 mutant in comparison to wild type plant should provide data to support the third hypothesis. This could be tackled by proteomics on heat stressed seedling similarly to (Miller et al., 2010; Miller et al., 2013), as this treatment triggers a massive SIZ1-dependent SUMO conjugation response (Kurepa et al., 2003; Yoo et al., 2006).

A novel SUMO1/2-dependent pathway for temperature acclimation in Arabidopsis

In Chapter 4, we showed that the SUMO-deficient Arabidopsis mutant sumo1/2\textsuperscript{KD} collapses at elevated ambient temperatures of only 28°C, a normally non-detrimental growth condition for this plant species (Quint et al., 2016). Interestingly, this phenotype was specific to growth at constantly elevated ambient temperatures, as other stresses like for instance heat shock at 37°C, high salinity and high mannitol did not cause a collapse of the sumo1/2\textsuperscript{KD} mutant. Transcriptomics and western blot analyses revealed that the constant mild increase of the ambient temperature to 28°C did not trigger a classical heat stress response that involves massive expression of Heat Shock Proteins (HSPs) and other (co-)chaperones. This indicates that the growth of Arabidopsis at 28°C does not cause accumulation of protein damage, as seen when applying a sudden heat shock of 37°C for 30 min only (Wang et al., 2004). This thermosensitive collapse of seedlings was independent of the SUMO3 E3 ligases SIZ1 and HPY2, since both the single mutants siz1 and hpy2 did not collapse at 28°C. So far, SUMO conjugation triggered by abiotic stresses was found to be mediated by one of these two SUMO E3 ligases, with a major role for SIZ1 (Saracco et al., 2007;
Kurepa et al., 2003; Yoo et al., 2006; Miura et al., 2007; Ishida et al., 2009; Miura & Hasegawa, 2009; Zhang et al., 2013). This thermosensitive phenotype of the sumo1/2KD mutant is to our knowledge the first example of a SUMO-dependent abiotic stress response where none of the SUMO E3 is involved.

Strikingly, both sumo1/2KD and eTK, a knockout mutant for three of the four Arabidopsis HsfA1s genes, collapse after an incubation of one week at a temperature of 28°C. This suggests a functional overlap in SUMO1/2 and HsfA1 function. (i) We showed that the lethality of the sumo1/2KD can be rescued by overexpressing SUMO3, confirming that the phenotype depends on loss of SUMO conjugation (Fig. 2; Chapter 6). (ii) Overexpression of HsfA1a also rescued sumo1/2KD (Chapter 4). Based on our transcriptomics data, western blot data, and the overexpression data on HsfA1 in the sumo1/2KD background, we conclude that sumoylation of SUMO1/2 acts genetically upstream of HsfA1 signaling to allow growth of Arabidopsis at elevated temperatures. However, I do not know whether sumoylation acts at the level of HsfA1 or affects proteins coded by gene-targets of HsfA1. This is a novel role of SUMO1/2 in plants, showing that SUMO1/2 is essential for growth of Arabidopsis at increased temperatures. Further analysis of the sumo1/2KD mutant should address whether the critical time spend at elevated temperatures correlates negatively with the ambient temperature. Moreover, as we suggest in Chapter 4 that mis-regulation of few specific HsfA1 targets might cause the collapse of both eTK and sumo1/2KD, it will be crucial to identify these targets in order to examine the molecular mechanism that causes plant lethality at in a few degrees warmer growth conditions.

In contrast to sumo1/2KD, the siz1 mutant does not collapse, but rather recovers at elevated growth temperatures (Chapter 2). Much of the siz1 growth defects appear to rely on increased accumulation of the defense hormone salicylic acid, which is fully suppressed by increased temperatures {Yang et al., 2004; Mang et al., 2012; Chapter 2). Moreover, sumo1/2KD plants that germinate at 22°C and then are transferred to 28°C, first show a recovery of their rosette size and leaf shape in the first days at 28°C, whereas their collapse only occurs after few weeks at 28°C (Chapter 4). Nonetheless, growth and leaf elongation are both arrested one week after the sumo1/2KD plants are placed at 28°C. This suggests that the sumo1/2KD phenotype is a blend of loss of SIZ1-dependent and -independent sumoylation. In support of this model, we noted that the transcriptional profile of siz1 and sumo1/2KD largely overlap at 22°C, but also shortly after the transfer to 28°C. The two profiles become more distinct after 4 days at 28°C (Chapter 4). Identifying genes whose expression is directly regulated by SIZ1 in normal conditions (i.e. SIZ1 marker genes), e.g. by performing a genome-wide ChIP-SEQ experiments on SIZ1, and
linking this ChIP-SEQ data to SIZ1-dependent gene transcriptional changes at 28°C, will allow us to determine to which extend the loss of SIZ1 function has still a role in the sumo1/2\textsuperscript{KD} phenotype at 28°C.

**The pivotal role SUMO in the control of SA accumulation**

Prior to this thesis, several studies linked salicylic acid (SA) with sumoylation. The dwarf phenotype of siz1 and sumo1/2\textsuperscript{KD} is SA dependent (van den Burg et al., 2010; Lee et al., 2007). Overexpression of mature SUMO1/2 and SUMO1/2\textsuperscript{ΔGG} results in enhanced SA accumulation (van den Burg et al., 2010). Also, disruption of the genes coding for the SUMO proteases OTS1 and OTS2 leads to enhanced SA accumulation (Bailey et al., 2016). A forward genetic screen for suppressors of esd4 yielded *ISOCROMATE SYNTHASE 1* (*ICS1*), also named *SALICYLIC ACID INDUCTION-DEFICIENT 2* (*SID2*) (Villajuana-Bonequi et al., 2014). Interestingly, while SUMO1/2 conjugation seems to repress the SA-related defenses, accumulation of SA causes a rapid and transient induction of SUMO3 (van den Burg et al., 2010). The different chapters of this thesis have reinforced the central role that SA plays in the phenotypes of SUMO-related mutants (Fig 3). First, we confirmed that SA accumulation is a major, but not exclusive, component of the siz1 phenotype (Chapter 2). Second, we showed that the SA accumulation is also responsible for the major part of morphological defects seen for sumo1/2\textsuperscript{KD} and siz1 at 22°C (Chapter 4). Third, our data strongly suggest that increased accumulation of SA, as a result of low levels of SUMO1/2, probably causes expression of SUMO3. Yet, SA activates its receptor NPR1, resulting in the expression of defense genes, like *PR1*. Remarkably, while SUMO3 interacts with NPR1 and promotes NPR1 interaction with the transcriptional activator TGA3 (Saleh et al., 2015), we noted that SUMO3, when its levels are up, can likely take over SUMO1/2 function, when their concentrations are low (Chapter 6). This accumulation of SUMO3 might be enough to force it to interact with SIZ1. Consequently, SIZ1 would repress the SA pathway when it uses SUMO3 for conjugation, which would down-regulate the expression of *AtSUMO3*. Hence, I propose here that *AtSUMO3* act in a feedback loop that potentially dampens the SA burst.

The collapse of sumo1/2\textsuperscript{KD} is specific for elevated ambient temperatures. Elevated temperatures also suppress constitutive accumulation of SA in mutants with auto-active alleles of resistance(-like) genes (Chapter 4; Yang & Hua, 2004; Mang et al., 2012). The findings in this thesis indicate that the collapse of this mutant is potentially caused by a combination of increased temperature and suppression of SA biosynthesis. As SA biosynthesis is suppressed in sumo1/2\textsuperscript{KD} at elevated growth temperatures, expression of SUMO3 is likely also inhibited in this mutant and the
Figure 3: Does SUMO3 buffer salicylic acid-dependent defense activation?
Schematic representation of the interplay of SUMO and salicylic acid signaling suggested by the finding of this thesis and Saleh et al., (2015). Arrow- and line-headed lines represent activation and repression, respectively. In black is shown the activated pathways, in grey the non-activated pathways.

A. In wild type plants, SUMO1/2 negatively control the SA pathway, via SIZ1.
B. When SUMO1/2 levels are low, SA accumulates. SA promotes transient expression of SUMO3 and activates NPR1, leading to the expression of defense genes, like PR1. Yet, the SUMO3 protein interacts with NPR1 and stimulates NPR1 interaction with TGA3 transcription activators (dash-lines), resulting into defense gene expression. On the other hand, SUMO3 also represses SA signaling via SIZ1. Hence, SUMO3 both activates and represses SA defense activation.

endogenous SUMO3 cannot complement the compromised SUMO1/2 conjugation. In short, SUMO1/2 but also SUMO3 is no longer available for SUMO conjugation in sumo1/2<sup>KD</sup> at 28°C (Chapter 4). This notion is supported by the fact that not only overexpression of wild type and mature SUMO3 rescued the dying phenotype of sumo1/2<sup>KD</sup> at 28°C, but also induction of the endogenous SUMO3 gene by treatment with the SA analog BTH rescued the mutant at 28°C (Chapter 6). Inhibition of AtSUMO3 expression appears, therefore, to be causal for the death of sumo1/2<sup>KD</sup> at 28°C. I propose that SUMO3 usually takes over the role of SUMO1/2 due to SA accumulation when SUMO1/2 levels are low. Yet, an increase in ambient temperature prevents SUMO3 protein accumulation. However, suppression of SA biosynthesis by introgression of the pad4 mutation or NahG transgene, results in the recovery of the morphological defects (reduced rosette size, curly leaves, short petiole) of the sumo1/2<sup>KD</sup> mutant at 22°C, but not of the growth arrest and collapse at 28°C. This most likely indicates that SA is not the only factor that promotes SUMO3 expression...
at 28°C. It would, therefore, be interesting to assess the expression levels of SUMO3 in the different mutant backgrounds (e.g. sumo1/2\textsuperscript{KD} and sumo1/2\textsuperscript{KD} pad4) at 22°C and 28°C, and to assess whether other hormones than SA control SUMO3 expression.

**The complexity of depicting the roles of SUMO**

As illustrated by Fig. 3 of Chapter 1, but also the experimental studies in Chapter 2 and 3, sumoylation is involved in various ways in plant development, plant immunity, abiotic stress responses and plant metabolism. Knocking out or knocking down genes encoding for key players of the SUMO pathways consequently affects all these processes and results in strong pleiotropic phenotypes, such as the dwarf phenotypes of siz1, hpy2, ots1 ots2, and sumo1/2\textsuperscript{KD}. The difficulty when studying sumoylation is to uncouple the different components that lead to these pleiotropic phenotypes. Uncoupling the different components results in highly complex experimental settings. For example, in Chapter 4, we uncoupled SA- and SIZ1-signaling from the thermosensitivity of sumo1/2\textsuperscript{KD} by working in the pad4 background and including the siz1 mutant, as the thermosensitivity is independent of SA and SIZ1 signaling in sumo1/2\textsuperscript{KD}. This allowed us to assess whether the transcriptional responses to elevated ambient temperatures in sumo1/2\textsuperscript{KD} are correlated with the response seen in the HsfA1 triple mutant eTK. In Chapter 2, we used genetics to uncouple the signaling induced by high ammonium/nitrate ratio, by signaling via resistance-like gene SNC1, and by SA signaling/biosynthesis. By suppressing these signaling components, we concluded that SIZ1 controls a pathway involved in plant growth, and we propose that possibly brassinosteroid (BR) synthesis is affected in the siz1 mutant.

However, the here-described reverse genetic studies did not reveal a direct interactor of SIZ1 that is causal to the activation of the SA pathway. To assess direct interactors of SIZ1 and to identify the component(s) of the SA pathway targeted by SIZ1, a tandem affinity purification proteomic approach was used (Chapter 2). However, this yielded only very few interactors of SIZ1, suggesting that the experimental set-up does not suit pull-downs with SIZ1 or that partners of SIZ1 only weakly interact with SIZ1 (under basal conditions). A better experiment could be ChIP of SIZ1 to examine whether SIZ1 binds chromatin in order to determine which genes are directly regulated by SIZ1.

An important part of the findings in this thesis relies on reverse genetics studies with the initial sumo1/2\textsuperscript{KD} line B (Chapter 4 and Chapter 6). Genotyping of this sumo1/2\textsuperscript{KD} proved cumbersome, as the sumo1/2\textsuperscript{KD} mutant is a triple locus mutant and both the amiR-SUMO2 insertions contribute to the “sumo1/2\textsuperscript{KD} phenotype”
(Chapter 3). This means that crossing of the sumol/2<sup>KD</sup> mutant with other mutants for genes-of-interest requires each time a screen for at least a quadruple mutant. An alternative experimental route would be the establishment of a pharmacological approach that can inhibit SUMO conjugation, making the inhibition of sumoylation inducible. In animals cells, SUMO conjugation inhibitors such as spectomycin B1 (Hirohama et al., 2013), ginkgolic acid or its structural analog anacardic acid (Fukuda et al., 2009) function by inhibiting the catalytic activity of E1 or E2 enzymes. One potential caveat is the limited specificity of these chemicals, as others reported that these chemicals also affect other enzymes such as lipoxygenase (Grazzini et al., 1991), histone acetylases (Balasubramanyam et al., 2003) or Aurora kinase A (Kishore et al., 2008). A pharmacological approach would not circumvent the pleotropic phenotype caused by loss of SUMO conjugation, but it would be more straightforward than genetic approaches involving extensive genotyping. Therefore, it is useful to explore a pharmacological strategy in the future by testing the efficiency of these putative SUMO conjugation inhibitors in plants and/or by identifying new specific inhibitors of SUMO conjugation for studies with Arabidopsis mutants.

**Global model**

As a summary, I integrated the findings, conclusions and ideas raised in this thesis in a general model (Fig. 4). In wild type Arabidopsis plants, SUMO1/2 are the main substrates used for conjugation (A). Via the SUMO E3 ligase SIZ1, SUMO1/2 inhibit the SA pathway and promote plant growth via brassinosteroid signalling. When Arabidopsis is grown at elevated ambient temperature, SUMO1/2 also activate temperature acclimation via the master regulator of heat stress: the HsfA1 family. In the siz1 mutant, the SA pathway is constitutively activated leading to an autoimmune

**Figure 4: General model integrating the findings and conclusion of this thesis.**

The signaling taking place in wild type plants in normal conditions is framed in blue (A), whereas the different mutants are separated by dot-lines (siz1 in B, sumol/2<sup>KD</sup> in C and D, and OX::SUMO3 in sumol/2<sup>KD</sup> in E). Horizontal boxes indicate the processes influencing the phenotype: cell death, autoimmune dwarfism, brassinosteroid-dependent growth and HsfA1-temperature acclimation. Black boxes with white text indicate activation of the process, grey boxes with black text indicate mild activation of the process, grey boxes with grey text indicate absence of activation of the process. Dashed-line boxes with black text indicate that the process (here, temperature acclimation via HsfA1) is not active at 22°C, but can be activated at 28°C. The status of these different processes are indicated by vertical colored boxes on the left, containing the considered genotypes and the conditions if necessary (green means non-stressed plants, orange means stressed plants, red means growth arrest and collapse). Activation and induction of expression is shown with arrow-headed lines, inhibition is shown with line-headed lines. Dashed-line arrows indicate indirect regulation. In black is shown the activated pathways, in grey the non-activated pathways. SIZ1-mediated conjugations are indicated with SIZ1 (in red) on the arrows. Thin line arrows indicate the subtle effect of SUMO3 cause by its mild accumulation.
General discussion
dwarf phenotype (B). Moreover, accumulation of the resistance-like protein SNC1 leads to spontaneous cell death resulting in leaf lesions. As cell death is linked to nitric oxide (NO) signaling, and results in this thesis and by others indicated that SNC1 signaling is suppressed by a high ammonium/nitrate ratio, it is likely that SNC1 functions via the NO signaling pathway. In addition, SA accumulation results in the transient expression of *SUMO3*. As *SUMO3* has potentially neo-functionalized, SUMO3 likely affects unknown processes including potentially bolting time (van den Burg *et al*., 2010). Also, it was proposed that SUMO3 specifically regulates NPR1 activity by modulating its interactions with several downstream transcription regulators (Saleh *et al*., 2015). Temperature acclimation via HsfA1 can still occur in *siz1* when grown at 28°C, meaning that SIZ1 is not essential for this process. The down-regulation of the SA pathway at 28°C is known to suppress autoimmunity and cell death in Arabidopsis, resulting in a partial recovery of the morphological defects of the *siz1* mutant at 28°C. Finally, at both 22°C and 28°C, absence of SIZ1 results in a down-regulation of brassinosteroid-mediated plant growth (Chapter 2; Catala *et al*., 2007). In *sumo1/2*KD, the SA pathway is also up-regulated at 22°C, thus explaining the reduced size of the mutant (van den Burg *et al*., 2010; C). *SUMO3* expression also occurs, potentially allowing a mild activation of the BR pathway through SIZ1, which might explain the increased size of *sumo1/2*KD compared to *siz1*. Growth of *sumo1/2*KD at 28°C prevents SA biosynthesis in a similar manner as in *siz1* (D). However, neither SUMO1/2 nor SUMO3 is present at sufficient levels in the *sumo1/2*KD to allow temperature acclimation via the HsfA1 pathway, resulting in the growth arrest and collapse at 28°C. By forcing accumulation of SUMO3 in *sumo1/2*KD, the temperature acclimation via HsfA1 can still take place, which allows *sumo1/2*KD to survive at 28°C (E). This can be achieved by introducing an overexpression construct (OX::*SUMO3*) or by inducing the expression of the endogenous *SUMO3* gene by applying the SA analog BTH. Alternatively, overexpression HsfA1 alone is already sufficient to suppress lethality of *sumo1/2*KD at 28°C. Finally, high concentrations of SUMO3 allow SIZ1 to interact with SUMO3, a situation normally prevented due to the weak interactions between SIZ1 and SUMO3. Hence, SIZ1 can suppress again the SA pathway in OX::*SUMO3 sumo1/2*KD plants, causing the plants to have wild type-like rosettes.
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