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SUMO chain formation
by Alexandrina Katardzhieva

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Abstract

The small ubiquitin-related modifier (SUMO) is conserved throughout the eukaryotic kingdom. This globular protein takes part in an important post-translational modification cycle, similar to, but quite distinct from ubiquitylation. SUMO is conjugated to a substrate protein via an isopeptide bond, connecting the C-terminal glycine of SUMO to a lysine in the substrate. This process is accomplished by the coordinated action of three enzymes: an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). SUMOylation can have opposing consequences, as some SUMOylated proteins are targeted for degradation, while others are protected from it. SUMO can be both activation and repression mark for transcription processes. Conjugation of SUMO can create or hide interaction surfaces for its substrate, or it can change the subcellular localisation of the substrate.

Several features make the study of SUMOylation in plants an exciting endeavour. Plants have one activating and one conjugating enzyme for SUMO. There are eight different SUMO isoforms in *Arabidopsis thaliana*, only half of which have been studied. As SUMOylation is essential, knocking out either the E1, or the E2, or SUMO1/2 is lethal. On one hand, only two plant SUMO ligases are currently known. This is a striking contrast to the ubiquitin E3 ligases, which number more than a thousand. On the other hand, a myriad of SUMOylation substrates have been identified, with functions ranging from stress signal transduction, through flowering time control, to the maintenance of key cellular features such as amino acid biosynthesis and DNA damage repair.

Despite the fact that many proteins can be SUMOylated, only a subset of them is modified at any given moment. Some substrates are SUMOylated only transiently, in a response to a certain stimulus, after which the modification is reversed by the action of specific proteases. How specificity to the large amount of identified substrates is achieved was the driving question behind this study. A bioinformatic search looking for novel possible SUMO ligases discovered two genes with unknown structure and function. The studies to characterise these two proteins led to the following results:

A. Describing the biochemical mechanism of the thioester transfer from Arabidopsis E1 to E2 with the help of an E2 active site point mutant, which additionally displayed stunted growth.
B. The analysis of SUMO ligase knock-out mutants demonstrated that they have a cumulative effect on the global SUMOylation in plant osmotic and salt stress.
C. Identifying a possible role for the SUMO ligase SIZ1 in post-germination abscisic acid signal transduction.
D. Identification of a SUMO E4 ligase, PIAL2, which converts monomeric SUMO and short SUMO chains into longer ones *in vitro.*
E. The biochemical characterisation of this E4 ligase showed that its chain extension activity is regulated by SUMO *in vitro*.
F. The SUMOylation of Lys15 in E2 is necessary for SUMO chain formation, especially in the absence of a ligase.

Zusammenfassung

Das Protein SUMO (small ubiquitin-related modifier) ist im ganzen eukaryotischen Reich konserviert. Dieses globuläre Protein ermöglicht eine post-translationale Modifikation, die der Ubiquitylierung ähnelt. SUMO wird durch eine Isopeptidbindung an Substratproteine konjugiert, die das C-terminale Glycin von SUMO mit einem Lysin im Substrat verbindet. Drei Enzyme koordinieren diesen Vorgang: ein aktivierendes Enzym (E1), ein konjugierendes Enzym (E2), und eine Ligase (E3). SUMOylieriung kann je nach Substrat untershiedliche Konsequenzen haben. Manche SUMOylierte Proteine werden abgebaut, während andere durch die Modifikation vor Abbau geschützt werden. SUMO kann Transkription sowohl aktivieren, als auch reprimieren. Durch die SUMO-Konjugation können neue Oberflächen zum Wechselwirkung mit anderen Proteinen entstehen, oder bereits vorhandene Interaktionsoberflächen maskiert werden, um Protein-Protein-Interaktionen zu unterbinden. Ein SUMOyliertes Protein kann auch seine subzelluläre Lokalisation ändern.

Einige Besonderheiten machen die Forschung von SUMO in Pflanzen zu einer lohnenswerten Aufgabe. Pflanzen ein aktivierendes und ein konjugierendes Enzym für SUMO. Es gibt acht verschiedene SUMO-Isoformen in *Arabidopsis thaliana,* aber nur die Hälfte davon ist bisher charakterisiert. SUMOylierung ist essenziell, Funktionsverlust von E1 oder von E2 oder von SUMO1/2 ist letal. Einerseits wurden bisher sind nur zwei SUMO-Ligasen beschrieben. Das ist ein auffallend großer Unterschied zu den Ubiquitin-E3-Ligasen, von welchen mehr als Tausend bekannt sind. Andererseits wurden viele SUMOylierungssubstrate gefunden, mit Funktionen in der Stresssignaltransduktion, der Kontrolle der Blütezeit, sowie der Regulation von essenziellen biochemischen Prozessen wie etwa der Synthese von Aminosäuren oder der Reparatur von DNA-Schäden.

Obwohl viele Proteine SUMOyliert werden können, ist nur ein Teil davon zu einem gewissen Zeitpunkt modifiziert. Manche Substrate sind nur für kurze Zeit SUMOyliert, als Reaktion auf einen bestimmten Reiz, und danach wird die Modifikation durch eine spezifische Protease rückgängig gemacht. Die zentrale Frage der Arbeit ist: „Was führt zur Spezifität in der SUMOylierung trotz der Vielzahl an Substraten?" Eine bioinformatische Suche nach weiteren möglichen SUMO-Ligasen führte zur Identifikation zweier Gene mit bisher unbekannter Struktur und Funktion. Eine nähere Untersuchung dieser beiden Proteine führte zu folgenden Ergebnissen:

A. Die Beschreibung des Reaktionsmechanismus des SUMO Transfers von E1 zum E2 mit Hilfe von Mutanten. welche zusätzlich reduziertes Wachstum aufwiesen.
B. Durch die Analyse von Mutanten mit Funktionsverlust in mehreren SUMO-Ligasen- wurde der Einfluss von SUMOylierung auf osmotischen und Salzstress beschrieben.
C. Identifizierung einer möglichen Rolle für die SUMO-Ligase SIZ1 in der Abszisinsäuresignaltransduktion nach der Keimung.
D. Identifizierung einer SUMO-E4-Ligase, PIAL2, die *in vitro* monomeres SUMO oder kurze SUMO-Ketten zu langen Ketten verlängert.
E. Die Biochemische Charakterisierung dieser E4-Ligase zeigte, dass ihre Eigenschaft, SUMO-Ketten zu verlängern, *in vitro* von SUMO beeinflusst wird.
F. Das SUMOylierte Lys15 von E2 ist notwendig für SUMO-Ketten-Bildung, insbesondere in der Abwesenheit einer Ligase.

Chapter 1: Introduction

The Oxford English Dictionary gives two definitions of the word "stress". A stress can be "pressure or tension exerted on a material object", or "a state of mental or emotional strain or tension resulting from adverse or demanding circumstance". While people still debate whether non-human organisms possess mentality or emotions, the identification of stress with the actual adverse or demanding circumstances is an accurate description in the purely biological sense. Stress factors can be divided in two categories. The biotic stress is caused by living organisms such as bacteria, viruses, fungi, parasites, predators/grazers, competitors etc. Non-living conditions, on the other hand, can cause abiotic stress. These are naturally occurring factors which can harm the living organisms exposed to them. Extreme temperatures, floods and droughts, intense sunlight and differences in the nutrient and water contents count among these.

Living organisms can employ different strategies to cope with abiotic stress. Some try to adapt their internal conditions to the ambient ones and are known as conformers. Others strive to maintain a parameter at a constant value and are regulators. The same organism can be regulator for one factor and conformer for another. A third option is to try to evade the stress condition altogether. However, it is not available to plants. Being (in most cases) firmly rooted to the ground, plants have to adapt to whatever combination of biotic and abiotic factors are present around them.

On the molecular level, plants respond to abiotic stress by opening or closing stomata on the leaves to manage temperature and water loss (Raschke, 1970a; 1970b), changing the plasma membrane composition to survive cold (winter wheat, (Pomeroy et al., 1983), storing dangerous compounds in the vacuole or exporting them using various transporters (Emmerlich et al., 2003; Li et al., 2006), secreting a protective wax layer on the leaf surface to minimise water loss (Eglinton and Hamilton, 1967), to mention a few possibilities. However, for all these events to happen, an extreme condition has to be correctly recognised as such, and then the signal has to be conveyed to the nucleus, where the appropriate transcription factors can trigger the corresponding adaptation processes. The post-translational modifications of key proteins are a quick and effective means of signal transduction. They include the covalent attachment or removal of phosphate, methyl and acetyl groups, redox modifications by reactive oxygen (ROS) and nitrogen species (RNS), and even the attachment of whole proteins, as exemplified by the ubiquitin and ubiquitin-like proteins (Dahan et al., 2011).

1.1 Ubiquitin and ubiquitin-like proteins

The main focus of this work is on SUMO (small ubiquitin-related modifier[1]) in plant stress. However, one cannot talk about ubiquitin-like proteins without introducing the founding member of the group. Ubiquitin is a small protein, 8 kDa in size and 76 amino acids in length, which is extremely well conserved throughout the eukaryotic kingdom. It has a characteristic fold (the ubiquitin roll, CATH classification 3.10.20), composed of a beta sheet flanked by two short alpha helices. One of the hallmark features of ubiquitin is its C-terminal diglycine motif, which is the binding point to the substrate protein (Schlesinger et al., 1975).

Ubiquitin is synthesised as a multimeric protein chain (Ozkaynak et al., 1984), which is then cleaved by a protease to expose the C-terminal glycine. The single monomer is added to a substrate using a three-step enzymatic cascade. First, an activating enzyme (E1) hydrolyses a molecule of ATP to AMP and uses the stored energy to create a thioester bond between a cysteine residue in its active site and the C-terminal glycine of ubiquitin (Hershko et al., 1980). The thioester is then transferred to an active site cysteine in a ubiquitin conjugating enzyme (E2). This conjugating enzyme is brought in close proximity to the substrate, which is bound by the ubiquitin ligase (E3) (Hershko et al., 1983).

[1] For a list of all abbreviations used in this study, see Appendix A.

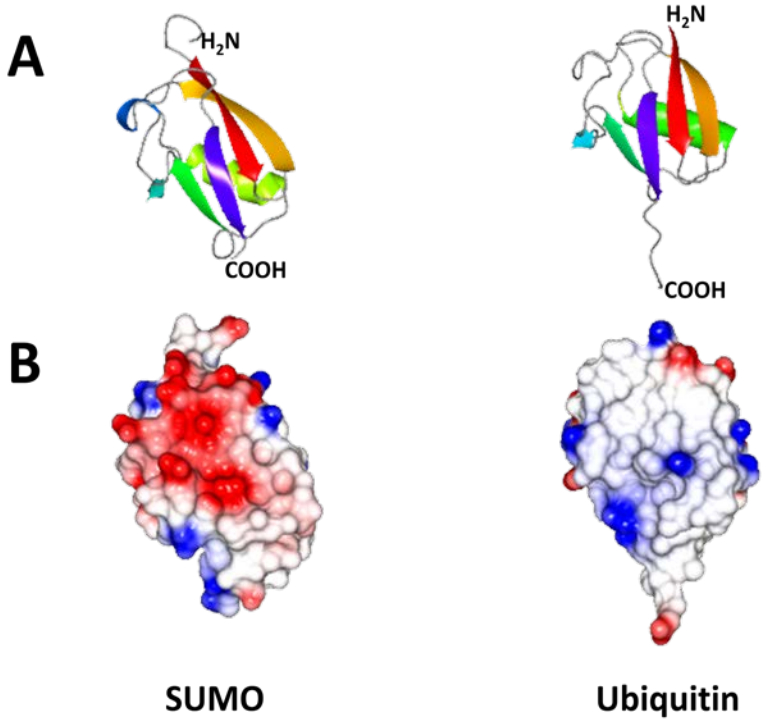


Figure 1. Comparison between the three-dimensional structure of SUMO (1WM2) and ubiquitin (1UBQ). A: A ribbon diagram of the ubiquitin-like fold, showing the beta-strands and the alpha-helix behind them. The model of SUMO is missing 12 disordered residues on the N-terminus. B: Space-filling model with residue electrostatic potential of the same orientation of the two molecules as in A. Clearly visible is a negative (red) patch in SUMO.

Depending on the exact mechanism of action of the ligase, the ubiquitin is either bound directly to the ε-amino group of a lysine residue in the target protein (RING ligases (Lorick et al., 1999)), or is first transferred to a cysteine on the E3 ligase and then to the substrate (HECT ligases and RBR ligases (Morett and Bork, 1999; Scheffner et al., 1995)). As ubiquitin itself has seven lysine residues, it can be further modified with another ubiquitin molecule to create a chain. An additional class of enzymes, termed E4 ligases, can extend these chains, rather than binding a specific substrate (Koegl et al., 1999). The chains created using the different lysines differ in their topology and function. The best known types are the Lys48 chains (Chau et al., 1989), which target the substrates for proteasomal degradation (Hochstrasser, 1996), and Lys63 chains, which have more regulatory properties (Hofmann and Pickart, 1999).

1.2 SUMO and the SUMOylation cascade

Besides ubiquitin, there are a number of ubiquitin-like proteins which regulate various processes in the cell. APG12, AUT7, FAT10, HUB1, ISG15, MNSF, NEDD8, SUMO, Ufm1, and URM1: they all have their specific targets and are involved in specific processes (Komatsu et al., 2004; Schwartz and Hochstrasser, 2003). They also have their own distinct sets of activating, conjugating and ligating enzymes. SUMO is slightly larger than ubiquitin, but possesses the same ubiquitin fold (Figure 1A). The difference in size is due mainly to the longer N-terminal part of SUMO. The surface of SUMO generally contains more charged amino acids than ubiquitin (Figure 1B). Just like ubiquitin, SUMO starts its life as a precursor, albeit a monomeric one. It has to be processed by a specific protease which exposes its C-terminus (Gong et al., 2000; Li and Hochstrasser, 1999). Similarly to ubiquitin, SUMO is added to its substrate via a three-step enzymatic cascade, which covalently binds the C-

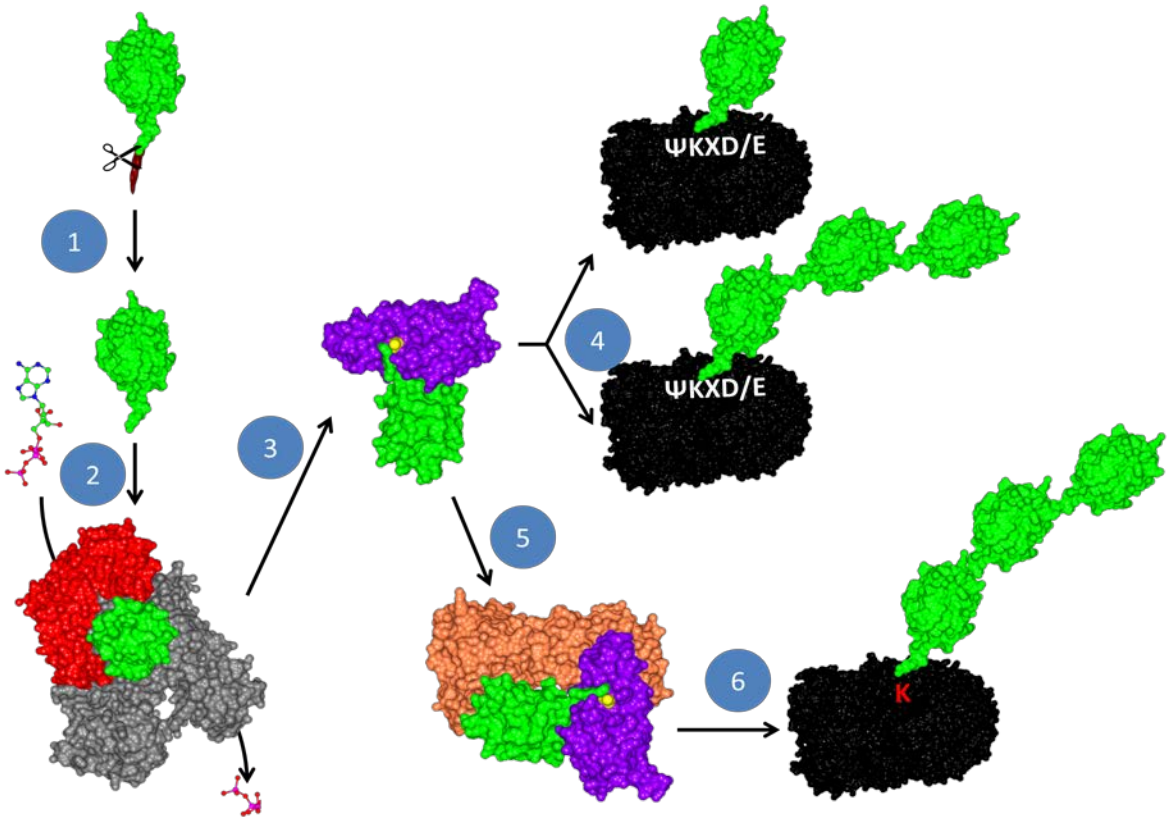


Figure 2. The SUMOylation cascade. SUMO (green) is synthesized as a precursor (brown tail), which is cleaved by a SUMO-specific protease (1) to expose the C-terminal –GG. SUMO is bound by the large subunit of the SUMO activating enzyme (2), which first addenylates its C-terminus and then forms a thioester to an active cystein. The ATP molecule is bound in the interface between SAE1 (red) and SAE2 (grey). The thioester is then transferred to an active cystein (yellow) in the SUMO conjugating enzyme (purple) (3). From there on, SCE1 can attach the SUMO to a substrate (black) directly, provided the acceptor protein contains a consensus SUMOylation motif (4). Otherwise, a SUMO ligase (orange) binds the E2 (5) and orchestrates the transfer of SUMO to a non-consensus lysine on the substrate (6). Protein structure IDs: HsSUMO2 1WM2; complex between E1 and SUMO 1Y8R; complex between E2 and SUMO 1Z5S; ScSIZ1 3I2D.

terminal glycine of SUMO to a lysine side chain (Figure 2). In fact, some substrate proteins can be ubiquitylated and SUMOylated at the same residue (Leach and Michael, 2005; Miller et al., 2010). Since SUMO also contains lysine residues, it is able to build chains. So far, no linkage specificity has been reported, i.e. the relative orientation of the donor and the acceptor SUMO should not have an effect.

The SUMO activating enzyme (E1: SAE) is a heterodimer, composed of the smaller SAE1 (Aos1, Sua1) subunit and the larger SAE2 (Uba2) subunit (Desterro et al., 1999; Dohmen et al., 1995; Johnson et al., 1997). The SAE2 contains the active cysteine which forms the thioester to SUMO, and the ATP is bound in the interface between the two subunits. The adenylation of SUMO and the subsequent thioester formation demand two different conformations of SAE, and the transfer of the bond to the E2 requires yet another position (Olsen et al., 2010). There is a single SUMO conjugating enzyme (E2: SCE, Ubc9) which, despite being rather small, around 17 kDa, can form interfaces with SUMO, with a SUMO ligase, and with a set of substrates (Seufert et al., 1995; Watanabe et al., 1996). The E2 can modify some substrates with a SUMO moiety even without the presence of a ligase. The lysine residues preferred by SCE1 sit in the so called consensus SUMOylation motif, a ΨKXD/E, where Ψ is a bulky hydrophobic amino acid, and X is any amino acid (Melchior, 2000). Other SUMOylation sites sit in inverse consensus motifs (D/EXKΨ) (Matic et al., 2010). Still others can be flanked by additional residues, such as a nearby phosphorylatable residue, making a phosphorylation-dependent

SUMOylation motif (PDSM)(Hietakangas et al., 2006), or by closely positioned negatively charged amino acids (negatively charged amino acid-dependent SUMOylation motif, NDSM) (Yang et al., 2006), or even by a sequence of hydrophobic residues (hydrophobic cluster SUMOylation motif, or HCSM) (Matic et al., 2010).

Nevertheless, SUMOylation sites exist which do not fall into any of these categories. Additional factors are needed for the modification of these, namely the SUMO E3 ligases (Johnson and Gupta, 2001; Takahashi et al., 2001). There are two types of ligases. The more abundant one, called SP-RING ligases, are found in all eukaryotes. SP-RING stands for Siz/PIAS-RING and is the domain that contacts the E2 (Hochstrasser, 2001; Takahashi and Kikuchi, 2005). It contains three cysteines and one histidine which coordinate a single zinc ion. This is similar to a corresponding domain in ubiquitin RING (really interesting new gene) E3 ligases, which coordinates two Zn^{2+} (Lorick et al., 1999). While the SUMO ligases in individual species may be very diverse, the SP-RING remains a highly conserved feature. The other type of SUMO ligases is termed the IR1-M-IR2 class, where IR stands for internal repeat and M is an intermediate sequence. The internal repeats do not bear similarity to either HECT or RING ubiquitin ligases and are thus unique for the SUMO pathway. The only identified IR1-M-IR2 SUMO ligase identified to date is the RanBP2/Nup358, a large nucleoporin which controls the GTPase Ran by SUMOylating its GAP (GTPase activating protein) (Pichler et al., 2002).

1.3 What happens to a SUMO-modified protein?

When a protein has been SUMOylated, it can undergo a variety of processes (Figure 3). First of all, the mere presence of a SUMO conjugate inhibits other modifications on the same lysine. Methyl and acetyl groups cannot be attached to it, and neither can ubiquitin (Buschmann et al., 2000; Leach and Michael, 2005). Thus, a SUMOylated protein might be protected from degradation. The SUMO itself may create new interaction surfaces for other proteins to dock with the substrate, or it may hide existing ones. SUMO interacting motifs (SIM) have been described and they are as numerous as the SUMO attachment sites. Generally, a SIM has a consensus sequence of four consecutive hydrophobic residues, of which the third one is the most variable, and followed by a serine/acidic rich region. SIMs of this type are called SIMa. A reverse SIM, or SIMr, is a mirror image of a SIMa – it is preceded by an acidic residue-rich region, and the second residue in the motif, mirroring the third in SIMa, is the most variable one. Another SIM type is the SIMb, which is composed of the Val-Ile-Asp-Leu-Thr stretch. The aspartic acid residue is well conserved, as opposed to the hydrophobic amino acid in a SIMa, and the motif is functional even without a neighbouring acidic cluster. The SIMs interact with SUMO in a non-covalent manner, by aligning to and extending its beta sheet (Uzunova et al., 2007).

Another possible fate of a SUMOylated protein is a change in its subcellular or subcompartmental localisation. Examples of this include RanGAP, the first identified SUMOylation substrate (Matunis et al., 1996), which transfers from the cytoplasm to the nuclear pore complex when SUMOylated. SUMOylation of NEMO causes its import into the nucleus (Huang et al., 2003), while the modification of the tumour suppressor PML is important for its integration into PML nuclear bodies (Zhong et al., 2000).

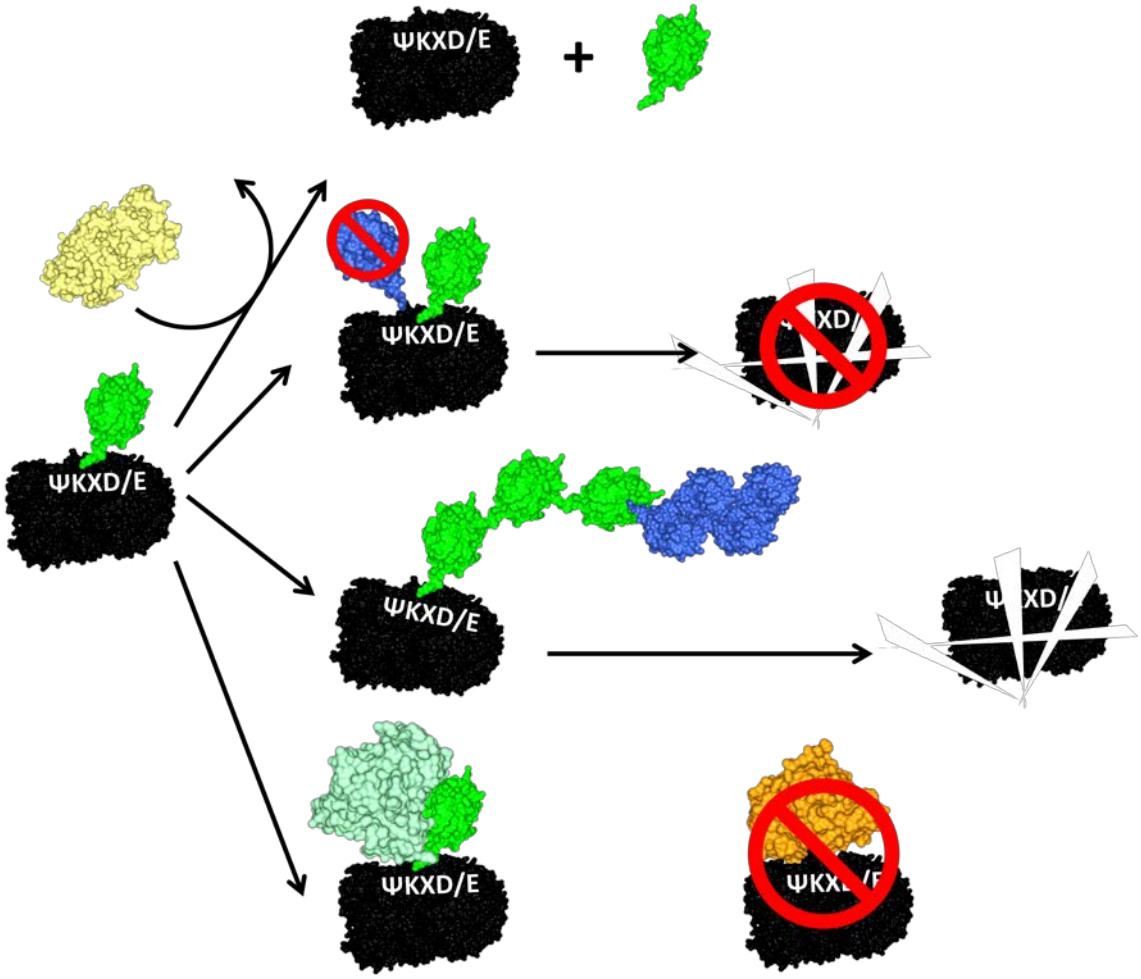


Figure 3. Possible fates of a SUMOylated substrate. Top: a SUMO protease (yellow) can hydrolyse the isopeptide bond between SUMO (green) and its substrate (black), leaving the proteins in their initial states. Upper middle: The presence of SUMO can block the attachment of ubiquitin (blue) and protect the substrate protein from degradation. Lower middle: In other cases, the presence of a SUMO chains can function as a signal for polyubiquitylation and proteasomal degradation. Bottom: The presence of SUMO can allow the binding of other proteins (cyan) or inhibit the formation of a previously existing complex. Protein structure IDs: HsSUMO2 1WM2; SUMO protease HsSENP2 1TGZ; ubiquitin 1UBQ.

As mentioned above, SUMO is able to build chains. PolySUMO chains are often a hallmark of stress (Feligioni and Nisticò, 2013; Miller and Vierstra, 2011) and have also shown to be assist in the higher order organisation of chromatin (Srikumar et al., 2013). One of the best characterised fates of a SUMO chain is secondary degradation targeting. A class of enzymes called SUMO targeted ubiquitin ligases (STUbL) have an array of SUMO interacting motifs which recognise and bind to SUMO chains (Prudden et al., 2007; Uzunova et al., 2007). The SUMO chain is then ubiquitylated, creating a heterologous chain, and the original substrate protein is degraded via the 26S proteasome system. Prior to degradation, ubiquitin is removed from the substrate and recycled. Although SUMO has a similar turnover rate to ubiquitin (Hanna et al., 2003; Rosas-Acosta et al., 2005), a deSUMOylating role of the proteasome has not yet been demonstrated.

SUMOylation is reversible. SUMO specific proteases are involved not only in exposing the C-terminal glycine of the SUMO precursor, but also for the removal of a SUMO conjugate from a substrate (Li and Hochstrasser, 1999). They are all cysteine proteases and regulate a variety of cellular processes

(Colby et al., 2006). One possible exception is the yeast Wss1 metalloprotease, which might be capable of removing mixed ubiquitin-SUMO chains from a substrate protein (Mullen et al., 2010).

1.4 Some SUMOylation substrates

SUMO is also involved in the organisation of chromosomes by regulating the DNA methylation and demethylation (Cubeñas-Potts and Matunis, 2013). It maintains constitutive heterochromatin (Shin et al., 2005), especially at telomeres (Lescasse et al., 2013), and is associated with mitotic chromosomes (Azuma et al., 2005). SUMOylation has a dual role in the regulation in gene expression. It is mostly a repressive mark, being targeted to specific promoters and recruiting other factors (Chupreta et al., 2005; Shiio and Eisenman, 2003), or directly altering the functions of transcription factors (Gill, 2005), but in several cases it can act as an activator (Arco et al., 2005). The creation of novel interaction surfaces allows SUMO to function as a binding partner recruiter or as a scaffolding protein. SUMOylation of histones, for example, recruits other proteins to the chromatin, promoting transcriptional repression. Also in PML bodies, associated with transcriptionally active regions (Xie et al., 1993), the contacts between PML and partner proteins are mediated by SUMO. In the nucleolus, SUMO regulates the processing of rRNA and the initial assembly of ribosome components.

Outside of the nucleus, SUMO can be involved in signal transduction pathways. For example, SUMOylation of IκB kinase protects it from degradation, because it hides the lysine that ubiquitin would be attached to (Huang et al., 2003). It is also involved in the regulation of the cystic fibrosis transmembrane conductance regulator (CFTR) (Ahner et al., 2013), in the hypoxia signalling cascade (Chien et al., 2013), in synapse formation (Gwizdek et al., 2013), and more. In plants, the nitrate reductase is a prominent substrate for SUMOylation, and the modification is required for its function in converting nitrate into nitrite, an important step in the biogenesis of ammonia and amino acids (Park et al., 2011).

1.5 Interactions with other post-translational modifications

As mentioned above, some substrates bear SUMOylation sites which are phosphorylation dependent. Recent studies indicate a cross-talk between phosphorylation and SUMOylation, integrating SUMO in the network of intracellular signalling. An example of such an event is the synapse formation, mentioned in the previous paragraph. When a calcium dependent serine protein kinase (CASK) becomes SUMOylated, its interactions with other proteins are weakened, which disrupts the formation of dendritic spines (Craig and Henley, 2012).

A well studied example of SUMOylation cross-talk is the modification of the DNA clamp, the PCNA (proliferating cell nuclear antigen). Besides being important for the processivity of DNA replication, this protein is also involved in DNA damage repair. When the DNA polymerase encounters an obstacle, the PCNA can be either mono- or polyubiquitylated. These modifications trigger error-prone and error-free break repair, respectively. The same lysine of PCNA, K164, can also be SUMOylated, which suppresses the template switch and this has a role in the normal S-phase (Leach and Michael, 2005).

1.6 SUMO in plants

Interestingly, although SUMO itself is conserved in eukaryotic organisms, it can have a different number of isoforms in different species. The baker's yeast, *Saccharomyces cerevisiae,* has one SUMO protein, Smt3. Humans have four, three of which have detectable transcripts, and *Arabidopsis thaliana*, the model organism used in this study, has eight (Table 1). Four of these are expressed, and functions have been assigned to three. AtSUMO1 and AtSUMO2 are evolutionarily very conserved and account for the vast majority of SUMOylation events. They can build chains, despite lacking consensus SUMOylation sites, and roughly correspond to HsSUMO2 and HsSUMO3. AtSUMO3 is similar to HsSUMO1, in the sense that it cannot build chains (Castaño-Miquel et al., 2011). It has been implicated in plant immunity and defence (van den Burg et al., 2010). AtSUMO3 and AtSUMO5 cannot compensate for the loss of AtSUMO1 and AtSUMO2, at least when expressed under the control of their own promoters (Miller and Vierstra, 2011). They do, however, accumulate in certain tissues and might have a more specific role there. Henceforth, AtSUMO shall be referred to as SUMO for the sake of simplicity. Unless mentioned specifically, work in this study was done using and detecting SUMO1.

Arabidopsis has two variants of the gene that codes for the small subunit of the SUMO activation enzyme, named SAE1a and SAE1b. Each of them can form a dimer with SAE2 and participate in the SUMOylation cascade. The SUMO conjugation enzyme is expressed from a single gene, the SCE1[2], which is in contrast with the more than 40 existing ubiquitin E2s. This difference is even more striking in the case of E3 ligases, which in plants number more than 1400 for ubiquitin, but only two have been described for SUMO: SIZ1 and MMS21/HPY2, which have specific functions in development and regulation processes (Ishida et al., 2012a). SIZ1 is involved in flowering time control, abscisic acid and salicylic acid signalling, cold and heat stress, drought and phosphate starvation (Cheong et al., 2009; Ling et al., 2012; Saracco et al., 2007). HPY2, on the other hand, is regulating meristem maintenance and cell proliferation (Ishida et al., 2009). Furthermore, two additional candidate ligases, PIAL1 and PIAL2, are the subject of the present study. All these ligases are SP-RING class ligases. An IR1-M-IR2 class SUMO ligase has not been discovered in plants, and the Arabidopsis RanGAP1 is SUMOylated by SIZ1 (Garcia-Dominguez et al., 2008). A SUMO E4 has not been described in any organism to date.

The Arabidopsis genome encodes at least eight SUMO proteases, which have been grouped in four functional classes (Novatchkova et al., 2012). The C-class protease ESD4 (early in short days) is a nuclear protein and, among other things, functions in controlling flowering time by regulating the flowering repressor FLC (Flowering Locus C). Another protease of the same class, ELS1, is extranuclear and has only been shown to deSUMOylate substrates *in vitro* (Hermkes et al., 2011). When overexpressed, the B1-class proteases OTS1 and OTS2 (overly tolerant to salt) cause increased survival of the plants in salt stress (Conti et al., 2009). Interestingly, *Xanthomonas campestris* pv. vesicatoria, a pathogen on paprika and tomato, uses a SUMO protease in its Type III secretion system to invade the host (Kim et al., 2013). Even though Arabidopsis is not a host to *Xanthomonas* pathovars, this protein, called XopD, can specifically remove SUMO moieties from all plants. However, it cannot cleave animal SUMO. Another example of host-pathogen interaction involving SUMO is the turnip mosaic virus (TuMV) RNA-dependent RNA polymerase. This viral protein has to interact with SCE1, for an infection to be successful (Xiong and Wang, 2013).

As in other organisms, SUMOylation in plants is essential. Knock-outs in the SAE2 or in the SCE1 single genes are embryonic lethal (Saracco et al., 2007). So are double knock-outs of SUMO1 and SUMO2, as well as double knock-outs of the two hitherto known SUMO ligases, SIZ1 and HPY2 (Ishida et al., 2012a). Knocking out the ESD4 SUMO protease causes stunted growth and early flowering,

[2] There is an enzyme called UBC9 in Arabidopsis, but it is a ubiquitin E2.

Table 1. List of the known SUMO and SUMO-processing proteins in *Arabidopsis thaliana*

Enzyme category	Arabidopsis enzyme	Chromosome locus	*S. cerevisiae* homologue
SUMO activating enzyme	SAE1a SAE1b SAE2	At4g24940 At5g50580 At2g21470	Aos1 Uba2
SUMO conjugating enzyme	SCE1	At3g57870	Ubc9
SUMO ligase, SP-RING class	SIZ1 MMS21/HPY2 PIAL1 PIAL2	At5g60410 At3g15150 At1g08910 At5g41580	Siz1, Siz2/Nfi1 Mms21 Pli1[1]
SUMO ligase, IR1-M-IR2 class[2]	None known		None known
SUMO protease, class A	(No name yet)	At3g48480	
SUMO protease, class B1	OTS1/ULP1d OTS2/ULP1c	At1g60220 At1g10570	
SUMO protease, class B2	(No name yet) (No name yet)	At1g09730 At4g33620	Ulp2
SUMO protease, class C	ESD4 ELS1/ULP1a ULP1b	At4g15880 At3g06910 At4g00690	Ulp1
SUMO-targeted ubiquitin ligase	AtSTUbL1 AtSTUbL2 AtSTUbL3 AtSTUbL4 AtSTUbL5 AtSTUbL6	AT5G48655 AT1G67180 AT3G07200 AT1G66650 AT5G04460 AT2G44410	Slx5, Slx8, Ris1, Rad18
SUMO	SUMO1 SUMO2 SUMO3 SUMO4 SUMO5 SUMO6 SUMO7 SUMO8	At4g26840 At5g55160 At5g55170 At5g48710 At2g32765 At5g48700 At5g55855[3] (At5g55855-At5g55860)[4]	Smt3

[1] As PIAS-like proteins are not conserved across kingdoms, Pli1 is mentioned solely as an example of another SP-RING class SUMO ligase
[2] The only known example of an IR1-M-IR2 class SUMO ligase is the mammal RanBP2
[3] The precise location of SUMO7 overlaps, but is not identical with At5g55855
[4] The precise location of SUMO8 is between At5g55855 and At5g55860

similar to a SIZ1 single knock-out, and mutants in OTS1 and OTS2 are extremely sensitive to salt stress (Conti et al., 2009). As there is no complete data on all SUMO proteases, a prediction about their combined impact on plant survival is difficult to make. In humans, however, knock-out of either SENP1 or SENP2 is embryonic lethal (Bawa-Khalfe et al., 2010). Several hundred proteins have been identified as SUMOylation substrates in Arabidopsis, with functions ranging from chromatin modification to nuclear membrane transport, including more than 100 with unknown functions (Miller et al., 2010).

Chapter 2: Material and methods

2.1 Material

2.1.1 Bacterial strains

General cloning
Escherichia coli XL1-blue (Stratagene):
endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)

Escherichia coli OmniMax (Invitrogen):
F' {proAB+ lacIQ lacZΔM15 Tn10(TetR) Δ(ccdAB)} mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80(lacZ)ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD

Heterologous expression
Escherichia coli Rosetta (DE3) pLysS:
F$^-$ ompT hsdS_B(r_B^- m_B^-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

Transformation in Arabidopsis
Agrobacterium tumefaciens C58C1 pCV2260

2.1.2 Plant material

Arabidopsis thaliana: wild type, ecotype Columbia (Col-0) (Huala and Dickerman, 2001)
T-DNA insertion lines in the Col-0 background:

siz1a: SALK_065397 (identical to *siz1-2* (Miura et al., 2007) in the gene At4g2490 (*SIZ1*)
pial1a: SALK_083748 in the gene At1g08910
pial1c: SAIL_738_B09 in the gene At1g08910
pial2a: SALK_043892 in the gene At5g41580
pial2b: GK712B09 in the gene At5g41580
35S::SCE1 OX: a construct overexpressing the gene At3g57870 (SCE1) behind the constitutive CaMV promoter, provided by Christian Hardtke
35S::SCE1C94S OX: a construct overxpressing the dominant negative variant of the gene At3g57870 (SCE1 C94S) behind the constitutive CaMV promoter, provided by Christian Hardtke
35S::PEX11E-YFP: a construct overexpressing the gene At3g61070 (Peroxin 11E) behind the constitutive CaMV promoter, provided by Friedrich Kragler.

2.1.3 Vectors and constructs

Vectors
pCR® 2.1 TA vector (Life Technologies) – this vector contains 3'-T overhangs, which enables the direct ligation of Taq-amplified PCR fragments. It can be selected with both kanamycin and ampicillin. The vector was used as a part of the The Original TA Cloning Kit (Life Technologies).

pMAL-c2 (NEB) – this vector contains the coding sequence of the maltose binding protein (MBP), positioned as an N-terminal fusion partner and optimized for cloning of cytosolic proteins. It confers ampicillin resistance.

pET9d – this vector was used for the expression of SAE, SCE1 and all SUMO isoforms (Budhiraja, 2005), conferring kanamycin resistance.

pET42c – this vector was used for the cloning and expression of NAF. The original N-terminal GST was replaced by the NAF-FLAG-6xHis construct (Budhiraja, 2005). It confers kanamycin resistance.

pLysSRARE (Novagen): This plasmid contains the genes for the T7 lysozyme, as well as for tRNAs for eukaryotic codons. It confers chloramphenicol resistance.

pTCSH1 – this binary vector, based on pGPTV-BAR (as described in (Tomanov et al., 2013), was used to express SCE1 wild-type and the dominant negative mutant SCE1 C94S *in vivo*. It confers kanamycin resistance to bacteria and Basta resistance to plants (See 2.1.2 Plant material, 35S::SCE1).

pBIN-UBI – this binary vector was used to clone candidate proteins for *in vivo* tracking and subsequent isolation. It contains YFP as a C-terminal fusion partner, and the expression in plants is driven by a ubiquitin promoter. The vector confers kanamycin resistance in bacteria and Basta resistance in plants.

Constructs

The constructs used or created in this work are described in Appendix B.

2.1.4 Cloning and detection tools

Enzymes

Restriction endonucleases were provided by Thermo Fisher (formerly Fermentas), New England Biolabs, or Roche. The suppliers of other enzymes (polymerases, ligases etc) are mentioned in the respective method descriptions.

Size standards

The size standards for both DNA and proteins were purchased from Thermo Fisher: GeneRuler 1 kb Plus DNA Ladder, GeneRuler 100 bp Plus DNA Ladder, PageRuler Plus Prestained Protein Ladder.

Oligonucleotides

Primers for PCR, sequencing and site directed mutagenesis were purchased from Microsynth AG and are listed in Appendix C.

Antibodies

Strep-Tactin alkaline phosphatase conjugate (IBA): used to identify Strep-tagged recombinant proteins in Western blot

anti-MBP antibody (NEB): used to identify MBP-tagged recombinant proteins in Western blot

anti-FLAG-M2 antibody (Sigma): used to identify FLAG-tagged recombinant proteins in Western blot

anti-SCE1 antibody: a polyclonal antibody against SUMO conjugating enzyme, produced in rabbits by Ruchika Budhiraja during her doctoral studies, used to detect recombinant SCE in Western blot

anti-SUMO1 antibody (Agrisera): a polyclonal antibody against *Arabidopsis* SUMO1, used to detect native SUMO in Western blot

anti-Phospho-Threonine antibody/P-Thr antibody (Cell Signaling): a polyclonal antibody against phosphorylated threonine, allowing detection of phosphorylated proteins

anti-mouse IgG alkaline phosphatase conjugate (Sigma): an antibody produced in goat, coupled to alkaline phosphatase and used as a secondary antibody against antibodies derived from mice, such as the anti-MBP and the anti-FLAG antibodies

anti-rabbit IgG alkaline phosphatase conjugate (Sigma): an antibody produced in goat, coupled to alkaline phosphatase and used as a secondary antibody against antibodies generated in rabbits, such as the anti-SCE antibody

anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare): an antibody produced in goat, coupled to HRP and used as a secondary antibody against antibodies derived in rabbits, such as the anti-SUMO1 or the P-Thr antibody in Western blots of plant extracts

2.1.5 Databases and software

NCBI: The National Center for Biotechnology Information, http://www.ncbi.nih.nlm.gov

TAIR: The Arabidopsis Information Resource (Huala and Dickerman, 2001), http://www.arabidopsis.org

Arabidopsis eFP Browser, a database for expression profiles of plant genes in development and stress, http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi, (Winter et al., 2007)

T-DNA express, an *Arabidopsis* gene mapping tool: http://signal.salk.edu/cgi-bin/tdnaexpress (Alonso et al., 2003)

ESyPred3D: An engine for predicting the tertiary structure of a peptide (Lambert et al., 2002)

Serial Cloner 2.6.1: a freeware DNA sequence viewer, including a restriction end nuclease library and sequence alignment tool

Benedict: a chromatogram viewer, allowing the simultaneous viewing and editing of multiple sequences

CCP4MG: a molecular graphics software for 3D imaging of protein structure (.pub) files (McNicholas et al., 2011)

SwissPDB viewer: another molecular graphics software, allowing editing of the .pdb files (Guex and Peitsch, 1997)

SUMOsp 2.0: a dedicated software for prediction of SUMOylation sites (Xue et al., 2006)

PerlPrimer: an oligonucleotide analyser for detecting primer dimers, calculating melting temperatures etc. (Marshall, 2004)

2.1.6 Growth media

E. coli were grown in LB (Luria-Bertani) medium: 10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.2, with 1.5 % w/v agar in case of plates.

A. tumefaciens were grown in YEB (Yeast extract broth) medium: 5 g/L tryptone, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose, 2 mM $MgSO_4$, with 1.5 % w/v agar in case of plates.

A. thaliana seedlings were selected on plates containing MS (Murashige-Skoog) medium: 4.3 g/L MS salts, 0.5 g/L MES, 10 g/L sucrose, 8.0 g/L agar (or 4 g/L GelRite), pH 5.7, MS vitamin mix (Sigma) was added after autoclaving.

2.2 Methods

2.2.1 Handling bacteria

Handling *E. coli*

Storing bacteria
A single colony was picked and inoculated into 3 ml LB with appropriate antibiotics. After an overnight incubation at 37 °C with shaking, 500 µl were withdrawn from the culture and mixed with 500 µl 75 % glycerol. The suspension was incubated for 30 minutes at room temperature and stored at -80 °C. When needed, a small amount of the frozen slurry could be taken with a sterile toothpick and streaked out on an LB plate.

Preparing competent cells
A single colony of the selected strain was picked and inoculated into 3 ml LB without antibiotics (25 mg/L chloramphenicol in case of Rosetta). The culture was grown overnight at 37 °C with shaking and diluted 1/100 in fresh LB. The culture was kept at 37 °C until OD_{600} = 0.4-0.5, and centrifuged for 10 minutes at 2500 xg and 4 °C. The pellet was resuspended in 1/10 volume ice cold TSS and kept on ice for 5-15 minutes. Aliquots of 200 µl were withdrawn and snap-frozen in liquid nitrogen. The cells were kept at -80 °C.

Table 2. TSS

10 % w/v PEG 4000
20 mM $MgCl_2$
5 % v/v DMSO
ad LB
pH 6.5-6.8

Heat-shock transformation
A 200 µl aliquot of competent cells was thawed on ice. The plasmid (1 µl from ca. 100 µg/ml miniprep or 20 µl overnight ligation) was added and gently pipetted up and down to mix it with the cells. The cells were incubated on ice for 30 minutes, then heat-shocked at 37 °C for 3 minutes and put back on ice for at least 30 seconds. Nine hundred microliters LB were added and the cells were incubated for 1 hour at 37 °C with gentle shaking. Afterwards, the cells were centrifuged for 3 minutes at 3000 xg. One millilitre from the supernatant was discarded, and the cells were resuspended in the remaining 100 µl. Then they were spread on an LB plate with the corresponding antibiotics. The plate was incubated overnight at 37 °C.

Handling A. tumefaciens

Preparing competent cells (Mattanovich et al., 1989)
A single colony of the C58C1 strain was picked and inoculated into 20 ml YEB containing 25 mg/ml rifampicin. The culture was incubated overnight at 30 °C and diluted 1/100 into 300 ml YEB with rifampicin. After reaching $OD_{600} \approx 0.5$, the culture was centrifuged for 20 minutes at 3000 xg. The pellet was washed three times in 10 ml 1 mM Hepes pH 7.0 and once in 10 % glycerol. It was finally resuspended in 3 ml 10 % glycerol. Aliquots of 100 µl were withdrawn and snap-frozen in liquid nitrogen. The competent cells were kept at -80 °C.

Transformation by electroporation (Mattanovich et al., 1989)
A 100 µl aliquot of competent *Agrobacterium* cells was thawed on ice and 200 ng plasmid DNA was added. Forty microliters of this suspension were placed in an electroporation cuvette with an electrode distance of 0.2 cm (BioRad). A single electric pulse of 2.5 kV voltage using a 25 µF capacitor (BioRad) was applied. Immediately after the pulse, the cells were transferred into 800 µl YEB with appropriate antibiotics. They were incubated for 1 hour at 30 °C with gentle shaking. Afterwards, the cells were centrifuged for 3 minutes at 3000 xg. One millilitre from the supernatant was discarded, and the cells were resuspended in the remaining 100 µl. Then they were spread on a YEB plate with the corresponding antibiotics. The plate was incubated for 48 hours at 30 °C.

2.2.2 Handling Arabidopsis

Growing plants
Wild type (Col-0) plants intended for transformation were grown on soil on short days (8 hours light, 16 hours dark) for 6-15 weeks, then transferred to long days (16 hours light, 8 hours dark). The first shoots were cut off and when the next shoots gave flowers, the plants were transformed using floral dip transformation (Clough and Bent, 1998). Stable lines intended for seed scale-up were grown directly on long days.

In some cases, plants with knocked-out genes in the SUMOylation pathway (mostly *siz1a* containing lines), were watered using tap water supplemented with 5 mM $(NH_4)_2SO_4$.

Floral dip transformation
The floral dip transformations were essentially done as described by Clough and Bent, 1998. An *A. tumefaciens* colony, strain C58C1 Rif^R, containing the plasmid of interest, was inoculated into 20 ml YEB medium with appropriate antibiotic concentration and grown for 48 hours at 30 °C. Two millilitres of this culture were transferred to fresh 100 ml YEB medium with antibiotics and grown overnight at 30 °C. The overnight culture was centrifuged for 30 minutes at 3500 xg and the pellet was resuspended in 200 ml 5 % w/v sucrose, containing 0.05 % v/v Silwet-L77, a mild surfactant which enhances the transformation efficiency. The plants (usually three plants per construct) were dipped into this suspension for 30 seconds, so that the flowers would be completely submerged. The plants were covered with a transparent plastic dome in order to maintain high humidity and grown on long days. The dipping was repeated 48 hours later, and the dome was removed 48-72 hours after the second dip.

Quick isolation of genomic DNA from *Arabidopsis*
About 100 mg leaves were put into a 1.5 ml Eppendorf tube and frozen in liquid nitrogen. One micro spoon of sand and 200 µl Extraction buffer were added and the mixture was immediately ground

using an IKA Labortechnik mixer with a glass pestle until the plant tissue was homogenized. The suspension was centrifuged for 5 minutes at 15 000 xg in a tabletop centrifuge, and the supernatant was transferred to a fresh 1.5 ml tube. The DNA was precipitated with 200 µl isopropanol, the solution was mixed for 5 minutes at room temperature and centrifuged again for 5 minutes at 15 000 xg, room temperature. The supernatant was discarded and the pellet was washed with 500 µl 70 % ethanol. After another centrifugation for 5 minutes at 15 000 xg, the supernatant was discarded and the pellet was air dried. Then it was resuspended in 50 µl TE buffer and heated for 10 minutes at 65 °C. The suspension was centrifuged for 2 minutes at 15 000 xg, the supernatant was transferred to a fresh Eppendorf tube and stored at -20 °C.

Table 3. Plant genomic DNA extraction buffer

20 mM Tris
250 mM NaCl
25 mM EDTA
0.5 % w/v SDS
pH 7.5

Large scale isolation of genomic DNA from *Arabidopsis*

The quick isolation requires the samples to be homogenized one at a time and can be time consuming when there are many samples to process. For the simultaneous work with many samples, about 100 mg leaves were put into a 2 ml Eppendorf tube and frozen in liquid nitrogen. One to three pre-chilled metal beads (⌀ = 3 mm) were added to each sample and the tissue was ground in a Qiagen Tissue Lyser II for 3x 1 minute at 30 shakes/sec. The tissue lyser plates were routinely kept at -20 °C to prevent sample thawing. After the frozen leaves were pulverized, 1 ml extraction buffer was added and the sample was kept for 10 minutes at 65 °C. Afterwards, 300 µl "5M" potassium acetate were added and the mix was kept for at least 10 minutes on ice. Then it was centrifuged for 10 minutes at 15 000 xg. The supernatant was transferred to a new 2 ml tube and the DNA was precipitated with 500 µl 2-propanol. The suspension was centrifuged for 5 minutes at 15 000 xg, the supernatant was discarded and the pellet was washed with 500 µl 70 % ethanol. After centrifuging for 5 minutes at 15 000 xg, the pellet was air dried and resuspended in 50 µl TE buffer. The isolated genomic DNA was stored at -20 °C.

Table 4. Plant genomic DNA extraction buffer

100 mM Tris
500 mM NaCl
50 mM EDTA
1.5 % w/v SDS
10 mM β-mercaptoethanol
pH 8

Table 5. "5M" Potassium acetate

60 ml 5M CH_3CO_2K
11.5 ml CH_3COOH
28.5 H_2O

Total RNA isolation from *Arabidopsis*

About 100 mg leaves were put into a 2 ml Eppendorf tube and frozen in liquid nitrogen. One to three pre-chilled metal beads (⌀ = 3 mm) were added to each sample and the tissue was ground in a Qiagen Tissue Lyser II for 3x 1 minute at 30 shakes/sec. The tissue lyser plates were routinely kept at -20 °C to prevent sample thawing. After the frozen leaves were pulverized, RNA was extracted according to the Qiagen RNeasy Plant kit. Isolated RNA was kept at -20 °C.

Extracting plant proteins for Western blot analysis

A 1.5 ml Eppendorf tube was loosely filled with *Arabidopsis* leaves and frozen in liquid nitrogen. One to three pre-chilled metal beads (⌀ = 3 mm) were added to each sample and the tissue was ground in a Qiagen Tissue Lyser II for 3x 1 minute at 30 shakes/sec. The tissue lyser plates were routinely kept at -20 °C to prevent sample thawing. After the frozen leaves were pulverized, 200 µl Buffer B+ were added, and the samples were heated for 6 minutes at 95 °C. Then they were centrifuged for 5 minutes at 15 000 xg and the supernatant was transferred to a new 1.5 ml Eppendorf tube. The protein concentration was measured using a NanoDrop and confirmed with SDS-PAGE and Coomassie staining.

Table 6. Plant protein extraction buffer

90 mM Hepes
30 mM DTT
2 % w/v SDS
20 µg/ml pepstatin
1 tab protease inhibitor cocktail/7ml
pH 7.5

Sterilization of *Arabidopsis* seeds

Small scale

A small amount of seeds (not more than 100 µl) were put in a 1.5 ml Eppendorf tube. Five hundred microliters 70 % ethanol were added, the seeds were shaken quickly and immediately centrifuged for 1 minute at 15 000 xg. The supernatant was removed and 500 µl of sterilizing solution were added. The tubes were shaken for 15 minutes, so that all the seeds would be washed with the solution. Afterwards, the seeds were quickly pelleted (30 seconds at 300 xg) and the supernatant was removed under the sterile hood. The seeds were washed two times with 500 µl distilled sterile water. Seeds meant to be sown on soil were air dried overnight. Seeds meant to be sown on plates were suspended in distilled sterile water and deposited on the plates with a cut-off pipette tip.

Table 7. Sterilizing solution

5 % $Ca(OCl)_2$ [1]
0.02 % Triton X-100

[1] Supernatant from an oversaturated solution

Large scale

The seeds (0.5 – 2 ml) were put in a 50 ml Falcon tube. Five hundred milligrams trichlor-isothiocyanate (Chloriklar, Bayrol GmbH) were dissolved in 5 ml dH_2O and mixed 45 ml absolute ethanol. The mixture was added to the seeds and the tubes were shaken for 10 minutes. The sterilization solution was decanted under a sterile hood and the seeds were washed three times with absolute ethanol. Finally, the seeds were air dried overnight and sown on soil.

2.2.3 Handling DNA

E. coli plasmid DNA isolation

A single colony was picked and inoculated into 3 ml LB + 100 mg/L ampicillin or 25 mg/L kanamycin. The culture was incubated overnight at 37 °C on a shaking platform. Bacterial plasmid DNA was isolated from the overnight culture using the Promega Wizard Plus SV Miniprep DNA purification kit.

In cases where a higher concentration of DNA was desired, the colony was inoculated into 50 ml LB. On the next day, plasmid DNA was isolated using the Promega PureYield Plasmid Midiprep System purification kit.

PCR

For colony PCR and analysis of DNA fragments shorter than 2000 base pairs, the Promega GoTaq DNA polymerase was used. All primer stocks had a concentration of 100 µM.

Table 8. GoTaq PCR

Component	Final concentration	Volume used	Step		Time
Buffer	1x	10 µl	95 °C		5 min
dNTPs	200 µM	4 µl	95 °C		30 sec
Forward primer	0.5 µM	0.25 µl	55 °C	30 cycles[1]	30 sec
Reverse primer	0.5 µM	0.25 µl	72 °C		1 min/kb
GoTaq	1.25 U	0.25 µl	72 °C		7 min
Template		1 µl / 1 colony	4 °C		∞
H_2O	ad 1 ml	34.25 µl			

[1] 25 cycles for colony PCR

For PCR-based cloning and analysis of DNA fragments longer than 2000 base pairs, the TaKaRa LA Taq was used.

Table 9. LA Taq PCR

Component	Final concentration	Volume used	Step		Time
Buffer	1x	5 µl	95 °C		5 min
dNTPs	400 µM	8 µl	95 °C		30 sec
Forward primer	0.2 µM	0.1 µl	55 °C	30 cycles	30 sec
Reverse primer	0.2 µM	0.1 µl	68 °C		1 min/kb
LA Taq	2.5 U	0.5µl	72 °C		7 min
Template		1 µl[1]	4 °C		∞
H_2O	ad 1 ml	35.3 µl			

[1] 2-5 µl for plant genomic DNA

cDNA synthesis

cDNA was made using the Life Technologies SuperScript III Reverse Transcriptase, according to the manufacturer protocol.

Site directed mutagenesis

Point mutations in SCE and PIAL2 were introduced via the Agilent QuikChange site directed mutagenesis kit. The mutagenesis primers (See Oligonucleotides) were designed to be complementary to each other. They spanned at least 5 codons on either side of the one being changed. In case a SIM was changed (See Domain structure of PIAL2), the primers changed up to 12 consecutive nucleotides and spanned at least 7 codons on either side of them.

DNA electrophoresis

DNA fragments were separated on agarose gels in TAE buffer. The gels were made with 0.001% ethidium bromide (Sigma), in later experiments replaced by 0.001% Midori Green Advanced (Nippon Genetics). The bands were visualized in a BioRad GelDoc trans-illumination system.

Table 10. TAE buffer

Component	Final concentration
Tris	40 mM
CH_3COOH	20 mM
EDTA	1 mM

Table 11. Agarose gel density

Fragment size	Gel density
< 1000 bp	2 %
1000 – 2000 bp	1 %
> 2000 bp	0.8 %

Purification of DNA fragments

Gel bands identified via trans-illumination were excised with a scalpel and DNA was purified from them using the Promega Wizard SV Gel and PCR Clean-up System. The DNA concentration was measured with a NanoDrop 2000c photometer (PeqLab) at 280 nm.

DNA ligation

A ligation reaction was set up based on the concentrations of the DNA fragments. Generally, 50 ng of vector were used, while the insert had a 3 times higher molar concentration. The molar concentrations were calculated according to the following formula:

$$Molar\ concentration\ (nM) = \frac{Measured\ concentration\ \left(\frac{ng}{\mu l}\right)}{Molecular\ weight\ (kDa)} * 1000,$$

where

$$Molecular\ weight\ (kDa) = \frac{Fragment\ length\ (bp) * 650}{1000}.$$

The ligations were set up in 20 µl, using a T4 DNA ligase (Thermo Scientific) and run overnight at 14 °C. A negative control contained only the vector. On the next day the reactions were transformed into competent *E. coli* cells.

2.2.4 Handling proteins

SDS-PAGE

Proteins were separated in an electric field according to their size. In order to compensate for the differences in charged amino acids, the proteins were denatured by heating for 5 minutes at 95 °C in SDS containing Laemmli sample buffer. The proteins were then separated in 10 % to 15 % polyacrylamide gels using the BioRad Mini Protean Tetra system. The gels were run at 120 V in 1x Tris-glycine electrophoresis buffer.

Table 12. SDS-PAGE stacking gel

30 % acrylamide mix, 29:1 (SERVA)	330 µl
1.5 M Tris-HCl pH 6.8	250 µl
10 % SDS	20 µl
10 % ammonium persulfate	20 µl
0.004 % bromophenol blue	20 µl
TEMED	2 µl
H_2O	ad 2 ml

Table 13. SDS-PAGE separating gel

	10 %	12 %	15 %
30 % acrylamide mix, 29:1 (SERVA)	1.7 ml	2 ml	2.5 ml
1.5 M Tris-HCl pH 8.8	1.3 ml	1.3 ml	1.3 ml
10 % SDS	50 µl	50 µl	50 µl
10 % ammonium persulfate	50 µl	50 µl	50 µl
TEMED	4 µl	4 µl	4 µl
H_2O	ad 5 ml	ad 5 ml	ad 5 ml

Table 14. Laemmli sample buffer

SDS	4 % w/v
Glycerol	20 %
βME	10 %
bromophenol blue	0.005 %
Tris-HCl pH 6.8	125 mM

Table 15. 5x Tris-gly electrophoresis buffer

Tris	125 mM
glycine	1 M
SDS	0.5 % w/v

Bis-Tris protein gels

For better resolution, precast 4-12% NuPAGE Novex Bis-Tris gels (Life Technologies) were used with a MOPS buffer optimised for separation of heavy proteins (Lars Hellman, personal communication).

Table 16. 5x Bis-Tris electrophoresis buffer

MOPS	250 mM
Tris	250 mM
EDTA	5 mM
SDS	0.5 % w/v

Coomassie gel staining

The gels were covered with a staining solution, heated in a microwave for 30 seconds and incubated for 30 minutes with shaking. Afterwards, the staining solution was discarded and the gel was covered with destaining solution, using the same procedure. After 30 minutes, new destaining solution was applied and the gel was incubated overnight with shaking.

Staining solution: 10 % 2-propanol, 10 % acetic acid, a spoonful of Coomassie Brilliant Blue G 250 (Serva)
Destaining solution: 10 % 2-propanol, 10 % acetic acid

Western blot

In order to detect proteins by antibodies, Western blots were performed. First, the proteins were separated using SDS-PAGE, and then transferred to a PVDF membrane (Immobilon-P, pore size 0.45 µm, Millipore) by wet blotting. To accomplish this, the membrane was rinsed in methanol and then soaked in transfer buffer. The gel was also soaked in transfer buffer for at least 15 minutes. A BioRad blotting cassette was assembled as follows: black part of the blotting cassette, sponge, filter paper, gel, membrane, filter paper, sponge, clear part of the blotting cassette. The cassette was covered with transfer buffer during the whole assembly process, and then put into a buffer-filled BioRad Mini Protean 3 blotting cell. The protein transfer was performed for 1 hour (1.5 hours in case of very heavy proteins) at 50 V and 4 °C. Afterwards, the membrane was blocked for 2 hours in 20 % new-born calf serum in TBS at room temperature, and then washed once with TBS. The primary antibody was diluted in TBS and applied on the membrane overnight at 4°C. On the next day, the membrane was washed twice with TBS + 0.05 % Tween-20 for 5 minutes and once with TBS only. For conjugated antibodies, the membrane was developed immediately. For non-conjugated ones, the secondary antibody was diluted in TBS and applied on the membrane for two hours at 4 °C. After the secondary antibody incubation, the membrane was again washed twice with TBS + 0.05 % Tween-20 for 5 minutes and once with TBS only.

In later experiments, the membrane was blocked with 5 % skimmed milk powder (Gerbu) in TBS + 0.05 % Tween-20.

For alkaline phosphatase (AP) conjugated antibodies: 66 µl 5 % NBT (Sigma) in 70 % DMF and 33 µl 5 % BCIP (Gerbu) in 100 % DMF were added to 10 ml TE buffer and applied on the membrane in the dark. The NBT serves as an oxidant, while BCIP is a substrate for AP, yielding a distinct purple colour when processed by the phosphatase. The membrane was incubated in the dark for 10 minutes to 4 hours, depending on the intensity of the staining. The reaction was stopped by rinsing the membrane in water and letting it dry. The detection mix was always prepared fresh.

For horseradish peroxidase (HRP) conjugated antibodies: Amersham ECL Western blotting detection reagents (GE Healthcare) or WesternBright Sirius detection reagents (Pierce) were mixed in 1:1 ratio and put on a glass plate. The membrane was laid on the plate with the protein side down and incubated in the dark for 5 minutes. Excess detection mix was removed by dragging filter paper on the edge of the membrane. The membrane was wrapped in plastic foil and placed in a film development cassette. A Fuji medical X-ray film (Fujifilm) was placed in the cassette for 30 seconds to 10 minutes and then developed in a Curix 60 developing machine (AGFA).

Table 17. Western blotting buffers

Transfer buffer	TBS	TE
190 mM glycine	50 mM Tris	10 mM Tris
25 mM Tris base	150 mM NaCl	1 mM EDTA
20 % v/v methanol	pH 7.5	pH 8.0
0.05 % w/v SDS		

Antibody dilutions
Strep-tactin II AP conjugate (IBA), 1:4000 dilution
anti-MBP (NEB), 1:10 000 dilution
anti-SUMO1 (Agrisera), 1:1000 dilution
anti-SCE1 (home-made), 1:50-1:100 dilution
anti-mouse IgG-AP conjugate (Sigma, A3562), 1:25 000 dilution
anti-rabbit IgG-AP conjugate (Sigma, A3812), 1:25 000 dilution
anti-mouse IgG-HRP conjugate (GE Healthcare, part of RPN2108 kit), 1:10 000 dilution

anti-rabbit IgG-HRP conjugate (GE Healthcare, part of RPN2108 kit), 1:10 000 dilution
anti-phospho-threonine (Cell Signaling), 1:1000 dilution

2.2.5 Recombinant protein expression in *E. coli*

A single colony was picked and inoculated into 3 ml LB medium containing the appropriate antibiotics (100 mg/L ampicillin and 25 mg/L chloramphenicol, or 25 mg/L kanamycin and 25 mg/L chloramphenicol). The culture was incubated overnight at 37 °C on a shaking platform. On the next day, the culture was diluted 1:100 in fresh LB with antibiotics and grown until OD_{600} 0.6-0.8. Protein expression was induced by adding 1 mM IPTG, after which the cultures were grown for three hours. The cells were harvested by centrifuging the cultures for 20 minutes at 4500 xg, 4°C. The pellet was washed with PBS pH 7.4 and centrifuged again for 20 minutes at 4300 xg, 4°C. The supernatant was decanted and the pellet was frozen at -20 °C.

2.2.6 Protein purification

Purification of His-tagged proteins

All SUMO isoforms, SAE and NAF carried a hexahistidine tag. His-tagged proteins were purified using IMAC with Ni^{2+}-NTA Sepharose. The frozen pellet was thawed on ice and resuspended in 5 ml Binding buffer, with 1 µg/ml aprotinin and 1 µg/ml leupeptine. After 15 minutes on ice, the bacteria were lysed using a Bandelin Sonoplus HD70 sonicator with an MS 73 tapered probe. The ultrasound bursts had 60 % intensity, 50 % on-time and were applied for 3 x 30 seconds. The suspension was centrifuged for 20 minutes at 4300 xg, 4 °C. During the centrifugation, a BioRad PolyPrep Chromatography column (2 ml bed volume) was prepared by adding 200 µl 50 % Ni^{2+}-NTA Sepharose or Talon Co^{2+} Sepharose (GE Healthcare). The resin was washed with 3 cv water and equilibrated with 6 cv Binding buffer. The lower end of the column was capped and the centrifugation supernatant was added, along with 10 µg DNase I. The column was sealed and incubated for 30-60 minutes on a rotating wheel at 4 °C. The flow-through was collected and the column was washed with 20 cv Wash buffer. The protein was eluted with 3x 1 cv Elution buffer and glycerol was added to a final concentration of 20 % v/v. The purity and concentration of the eluted protein was checked with SDS-PAGE, and the fractions were snap-frozen in liquid nitrogen and kept at -80 °C.

Purifying SAE

The plant SUMO activating enzyme is a heterodimer. The two subunits were expressed from a dicistronic construct and only the SAE2 subunit was His-tagged. The addition of 5 mM ATP to the buffers ensured that the complex was formed and purified in stoichiometric amounts.

Table 18. His-tag purification buffers

Binding buffer	Wash buffer	Elution buffer
50 mM NaH_2PO_4	50 mM NaH_2PO_4	50 mM NaH_2PO_4
300 mM NaCl	300 mM NaCl	300 mM NaCl
10 mM imidazole	20 mM imidazole	250 mM imidazole
10 % glycerol	pH 8.0	pH 8.0
0.5 % Triton X-100		
pH 8.0		

Purification of MBP-tagged proteins

Every construct containing a PIAL ligase was made with maltose binding proteins as a fusion partner. This ensured both solubility and detectability in Western blot assays. The frozen pellet of expressing

bacteria was thawed on ice and resuspended in 5 ml MBP Column buffer, with 1 µg/µl aprotinin and 1 µg/µl leupeptine. After 15 minutes on ice, the bacteria were lysed using a Bandelin Sonoplus HD70 sonicator with an MS 73 tapered probe. The ultrasound bursts had 60 % intensity, 50 % on-time and were applied for 3 x 30 seconds. The suspension was centrifuged for 20 minutes at 4300 xg, 4 °C. During the centrifugation, a BioRad PolyPrep Chromatography column (2 ml bed volume) was prepared by adding 200 µl amylose resin (NEB). The resin was washed with 3 cv water and equilibrated with 6 cv Column buffer. The lower end of the column was capped and the centrifugation supernatant was added, along with 10 µg DNase I. The column was sealed and incubated for 30-60 minutes on a rotating wheel at 4 °C. The flow-through was collected and the column was washed with 24 cv Column buffer. The protein was eluted with 3x 1 cv Elution buffer and glycerol was added to a final concentration of 20 % v/v. The purity and concentration of the eluted protein was checked with SDS-PAGE, and the fractions were snap-frozen in liquid nitrogen and kept at -80 °C.

Table 19. MBP-tag purification buffers

Column buffer	Elution buffer
20 mM Tris	20 mM Tris
200 mM NaCl	200 mM NaCl
1 mM EDTA	1 mM EDTA
pH 7.4	10 mM maltose
	pH 7.4

Purifying SCE

The *Arabidopsis* SUMO conjugating enzyme loses its activity if tagged on either terminus. This necessitated the use of an untagged protein in a crude lysate. After harvesting the bacteria, the pellet was resuspended in 6 ml phosphate buffer and frozen at -80 °C. On the next day, the pellet was thawed on ice and 1 µg/ml aprotinin and 1 µg leupeptin were added, as well as 10 mM DTT in order to protect the active site cysteine. The suspensions were centrifuged for one hour at 100 000 xg, 4 °C in a Beckman Optima ultracentrifuge. The supernatant was collected and loaded on a Sartorius VivaSpin 500 column, in order to remove the DTT which would interfere with the thioester reaction. The column was centrifuged 4x 15 minutes at 15 000 xg in a tabletop centrifuge. After every centrifugation step, the top compartment was filled with SUMO buffer. Finally, glycerol was added to 20 % v/v, the SCE was snap-frozen in liquid nitrogen and stored at -80 °C.

Table 20. SCE purification buffers

Phosphate buffer	SUMO buffer
68.3 ml 50 mM NaH_2PO_4	20 mM Tris
31.5 ml 50 mM Na_2HPO_4	5 mM $MgCl_2$
pH 6.5	pH 7.4

2.2.7 *In vitro* SUMOylation

Thioester reaction

In order to monitor the transfer of SUMO from the E1 to the E2, a thioester reaction was designed:

Table 21. SUMO thioester reaction

2 µl 10x reaction buffer
2 µg SUM1-Strep
10 ng SAE
2 µg SCE1
1 µl ATP solution
ad H_2O to 20 µl

The reactions were set up in duplicates and incubated for 30 minutes at 30 °C. The process was terminated either by adding 20 µl non-reducing sample buffer and incubating for additional 15 minutes at 30 °C, or by adding 20 µl Laemmli sample buffer and incubating for 10 minutes at 95 °C. The reaction mixtures were separated via SDS-PAGE at 4 °C at a running current of 20-24 mA.

Table 22. Thioester buffers

Reaction buffer	Non-reducing SB	ATP solution
20 mM Tris-HCl	50 mM Tris-HCl	20 mM Hepes
50 mM NaCl	2 % w/v SDS	100 mM ATP
10 mM	10 % v/v glycerol	100 mM $Mg(OAc)_2$
pH 7.6	4M urea	pH 7.4
	pH 6.8	

Substrate SUMOylation, SUMO chain formation

The dynamics of the covalent attachment of SUMO to substrates, whether to SUMO itself or to other proteins, were studied using a SUMOylation reaction:

Table 23. SUMOylation reaction

2 µl 10x reaction buffer
100 µg SUM1-Strep
4 µg SAE
1 µg SCE1
5 µg PIAL1 or PIAL2
10 µg substrate
1 µl ATP solution (See above)
ad H_2O to 20 µl

Table 24. 10x reaction buffer

200 mM Tris
50 mM $MgCl_2$
pH 7.5

In order to distinguish between the intrinsic properties of the E2 to catalyse SUMOylation and the action of the E3, a negative control was prepared without the ligase. In the cases where the SUMO chain formation was studied, no substrate was added. Unless otherwise specified, SUMO1 was used in all standard *in vitro* reactions.

Chapter 3: Results

Despite the vast amounts of confirmed SUMO substrates in plants (Miller and Vierstra, 2011; Miller et al., 2010; Saracco et al., 2007), only two SUMO ligases have been characterized, the SIZ1 (At5g60410) and the HPY2/MMS21 (At3g15150) (Ishida et al., 2009; Miura et al., 2007). This is in striking contrast with the ubiquitin E3 ligases, which number more than 1500 (Mazzucotelli et al., 2006) and often have unique specificities. Both SIZ1 and HPY2 contain an SP-RING (zf-MIZ) (Hochstrasser, 2001), a fold that binds a single Zn^{2+} ion and serves as an E2 docking site during the transfer of SUMO from the E2 to the substrate, thus facilitating the reaction. This mechanism of action is also common for the RING type of ubiquitin ligases (Lorick et al., 1999). Using the SP-RING as a search query, two other genes have been identified in *Arabidopsis* (Novatchkova et al., 2004), At1g08910 and At5g41580. They were named PIAL1 (Protein Inhibitor of Activated Stat-like) and PIAL2, respectively.

3.1 Phenotypic analysis of *pial1* and *pial2* mutants

An initial analysis of the growth phenotype and heat-shock response of *pial1*, *pial2* and *siz1* mutants was performed by Rebecca Hermkes during her doctoral studies (Hermkes, 2008). For more detailed

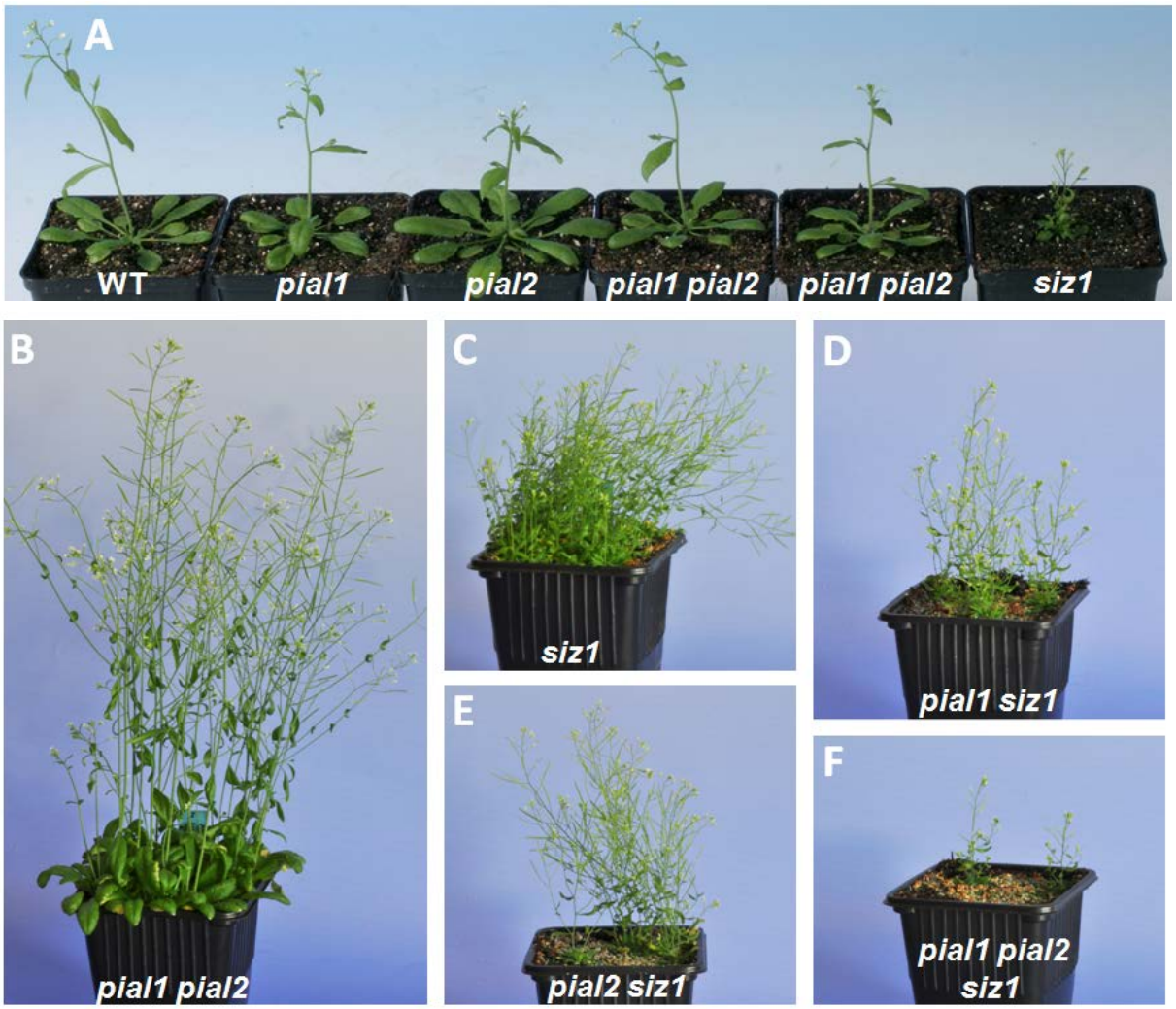


Figure 4. Growth patterns of *pial1, pial2* and *siz1* mutant plants. A: Plants were grown for three weeks in long day conditions with normal watering. The *pial1, pial2* and *pial1pial2* mutants had no growth phenotype, while plants with a *siz1* knock-out were small and flowered earlier. Homozygous *siz1* mutants could not produce fertile seeds. B: Plants were grown for six weeks in long day conditions, and the water was supplemented with 5 mM $(NH_4)_2SO_4$. The extra ammonium allowed *siz1* plants to produce fertile seeds. Combining the *pial* mutants with *siz1* had an even stronger phenotype, and the triple mutant was most severely affected.

analysis, *pial1pial2* double knock-out plants were crossed with *siz1* and the resulting progeny was genotyped, selecting for various combinations. Two allelic variants from each *pial1* and *pial2* mutant were used, creating a total library of 13 mutant lines.

3.1.1 Growth of SUMO ligase mutants under normal conditions

Arabidopsis mutant lines were grown for 3-6 weeks under long-day conditions. There were no apparent phenotypic differences between *pial1, pial2* or *pial1pial2* plants and the Col-0 wild-type plants (Figure 4A). On the other hand, the *siz1* plants were short, bushy and flowered very early. When the *pial1* or *pial2* mutations were combined with a *siz1* mutation, a cumulative effect could be seen, where the double mutants were even smaller than the single *siz1* mutant plants. This cumulative effect was even more pronounced in the *pial1 pial2 siz1* triple mutant (Figure 4B).

3.1.2 Growth of SUMO ligase mutants under stress conditions

SUMO is known to be involved in the response to biotic and abiotic stress. To test the importance of PIAL1 and PIAL2 in stress, sterilised *Arabidopsis* seeds were germinated on GelRite plates containing MS medium complemented with 150 mM NaCl for salt stress, 300 mM mannitol for osmotic stress or 2.5 µM ABA (abscisic acid) as a germination inhibiting hormone. The plates were vernalised for 96 hours at 4 °C in order to synchronise germination, and then transferred to long-day conditions. The seedlings were grown for two weeks and their fresh weight was measured on an analytical scale. Between 50 and 100 seedlings were weighed for each of the 14 genotypes (including a Col-0 wild-type control), and for each of the four conditions (three stresses and a non-stressed control), or around 5500 plants in total. See Appendix D for the raw data.

Due to the large variation in fresh weight within each individual group, the plants were divided in four subgroups, ranging from the lightest to the heaviest plant for a particular stress. The numbers of plants in each range were scored and the scores were plotted. Generally, wild-type plants weighed between 0.1 and 40 mg in the cases of no stress and salt stress, and between 0.1 and 10 mg in the cases of osmotic stress and ABA stress. The effects of each stress condition are summarised in Figure 5. As the allelic variants of the *pial1* and the *pial2* knock-outs were showing comparable phenotypic effects, they were quantified collectively.

All genotypic combinations showed little or no variation in their fresh weight distribution in the absence of stress (Figure 5A, C). Single mutants in either SUMO ligase, however, were growing better on 150 mM NaCl (Figure 5B, D), which would imply a role of SUMOylation in the regulation of growth during salt stress. This was also the case for the *pial1 pial2* double mutants, but neither for the other double mutants, nor for the *pial1 pial2 siz1* triple mutants. When germinated on 300 mM mannitol, all genotypes experienced a severe difficulty, which is reflected in their weight. No plant was heavier than 10 mg (Figure 5E, G). Knocking-out the SUMO ligases had a cumulative effect, with the single mutants being smaller than the Col-0 wild-type plants, the double mutants being even smaller, and the triple ones having more than 80 % of the plants lighter than 2.5 mg. When the plants were grown on 2.5 µM abscisic acid, *siz1* mutant plants started dying shortly after they germinated (Figure 5F, H). This was not the case in *pial1* or *pial2* single mutants, or in the *pial1pial2* double mutants. The presence of at least one functional ligase was able to keep some of the plants alive, as evidenced by the *pial1 siz1* and the *pial2 siz1* double mutants. The triple mutants, however, showed only one plant surviving, i.e. having any green tissue, from the more than 200 that germinated.

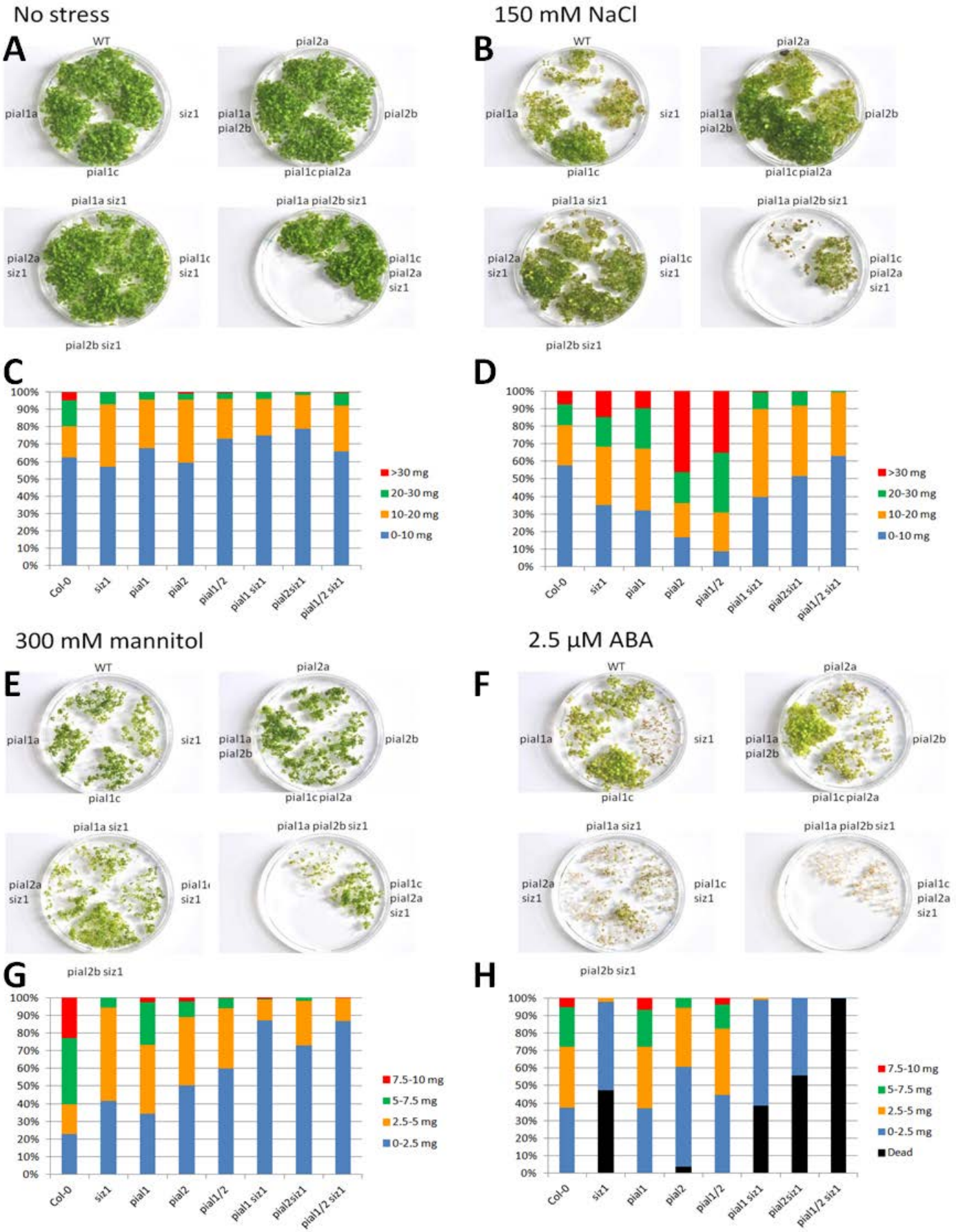


Figure 5. The effect of various stresses on pial1, pial2 and siz1 mutant plants. Between 50 and 100 seeds of each genotype were spread on plates containing either MS salts only, 150 mM NaCl, 300 mM mannitol or 2.5 µM ABA. The fresh weight of each seedling was measured two weeks after germination. A: No-stress control, including all genotypic combinations. C: Quantification of the measured non-stressed fresh weights, combining the pial1 and pial2 allelic variant pairs. B, E, F: Plants grown on 150 mM NaCl, 300 mM mannitol or 2.5 µm ABA, respectively. D, G, H: Quantification of the plant fresh weight distribution in the corresponding stress conditions.

3.1.3 Global SUMO levels in chronically mannitol stressed plants

As a follow-up to the germination and fresh weight assay, the global SUMO levels were measured in plants grown on constant osmotic stress. For simplicity, only a subset of the available genotypes was tested, including Col-0, *siz1, pial1a, pial2b, pial1a pial2b, pial1a siz1, pial2b siz1,* and the triple mutant *pial1a pial2b siz1.* The eight genotypic combinations were once again germinated on MS plates containing either no additives or 300 mM mannitol, and grown under long-day conditions. The plants were harvested two weeks after germination and total soluble proteins were extracted. The total protein concentration was measured using a NanoDrop photometer and the amounts loaded on gels were adjusted. The protein extracts were separated with SDS-PAGE on gradient gels, transferred to a PVDF membrane and blotted with an anti-SUMO1 antibody. Another gel with the same samples was stained with Coomassie Blue. The lane intensities on both the Western blots and the Coomassie stained gels were measured with ImageJ and normalised to one another. The total SUMO levels of the analysed plants, in the presence or absence of osmotic stress, are reflected in Figure 6.

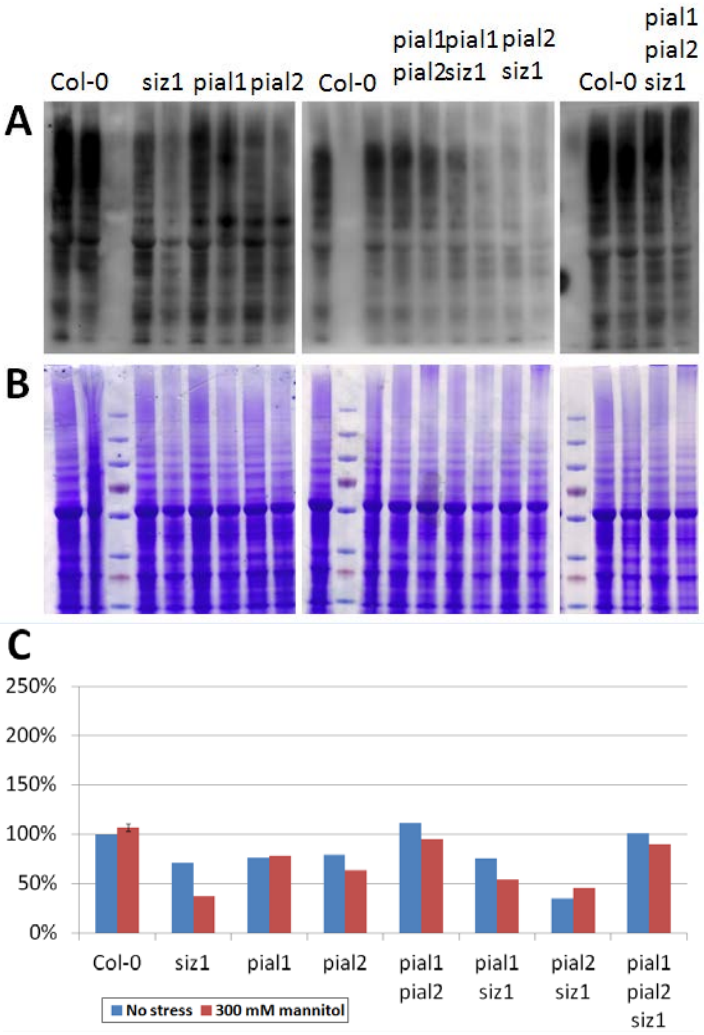


Figure 6. Global SUMO1 levels in two week old seedlings. Plants bearing the corresponding SUMO ligase knock-outs were germinated on plates containing either MS salts or MS salts with 300 mM mannitol. A: A total protein extract from leaf tissue was blotted with anti-SUMO1 antibody. The lanes always show the non-stressed plants first, followed by the stressed ones. B: Coomassie stained gels with the same amounts of protein as in A. C: Quantification of the lane intensities with double normalization. The colour intensity of each lane was scored using ImageJ. First the intensity of the SUMO stain from panel A was normalised to the total protein amount from panel B. Then the stressed samples were compared to the corresponding non-stressed Col-0 WT standard. Each gel contained an internal Col-0 standard, in order to minimise the batch differences. (The scale was set to 250% so that the results of the chronic stress can be easily compared to the shock stress ones, see Figure 7.)

While the total SUMO conjugates in stressed single ligase mutant plants were reduced when compared to the Col-0 wild-type, this effect was rescued in the *pial1 pial2* double mutant, as well as in the *pial1 pial2 siz1* triple mutant. In contrast, the osmotic stressed *pial1 siz1* mutant had an intermediate level of global conjugation, when compared with the *siz1* and the *pial1* single mutants. Quite surprisingly, the *pial2 siz1* mutants had low SUMO levels even in normal conditions, which would imply a role of PIAL2 in maintaining the SUMO homeostasis. A notable observation is that neither the wild-type plants, nor the mutants did exhibit elevated SUMO levels in these chronic stress conditions.

3.1.4 Global SUMO levels in heat-shocked and osmotic-shocked plants

Plants can adapt to long-lasting stress (Bohnert and Sheveleva, 1998)(Cushman and Bohnert, 2000). As such, their global SUMO levels may be elevated during an initial shock, but are then reduced again when the adaptation programs are triggered. To eliminate adaptation and observe immediate stress induced SUMOylation differences, a heat shock and an osmotic shock assay were developed. The same eight genotypic combinations as for the chronic stress were tested. Seedlings were grown on MS salt plates without any stress for two weeks under long-day conditions. Then they were transferred to 24 well plates, one plant per well, six plants per genotype, including the Col-0 wild-type control. Each well contained 1 ml MS medium. The plates were set up in triplicates, sealed and left under long-day conditions for 24 hours. One plate was left as a non-stressed control. To the second one, 300 mM mannitol were added to each well, while the third plate was put in a 37 °C water bath. After one hour, all plants were harvested and soluble proteins were extracted. The total protein concentration was measured using a NanoDrop photometer and equal amounts of proteins were loaded on SDS-PAGE gradient gels. The gels were run in duplicates. One was stained with Coomassie (Figure 7B), while the other one was transferred to a PVDF membrane and blotted with anti-SUMO1 antibody (Figure 7A).

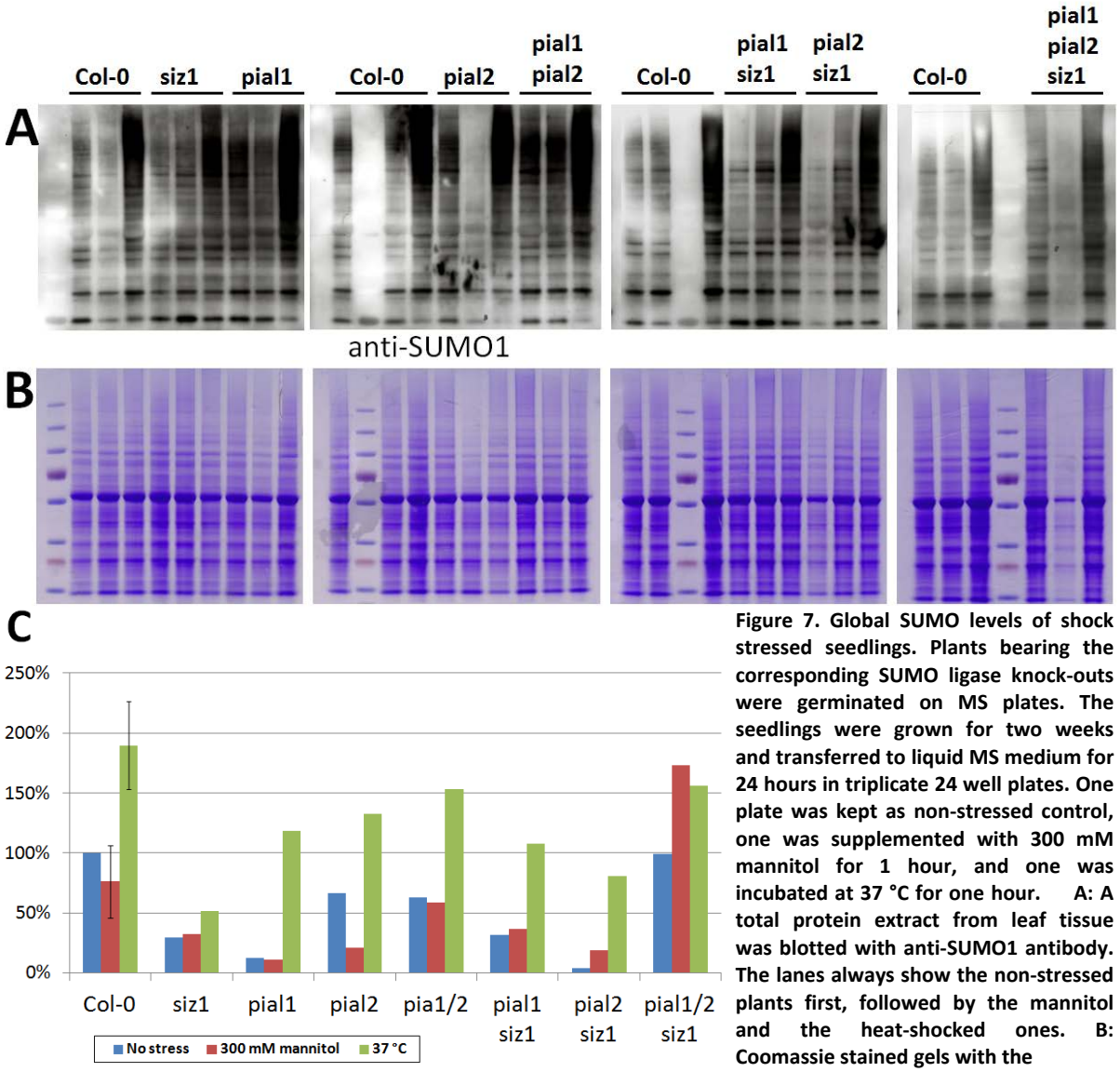
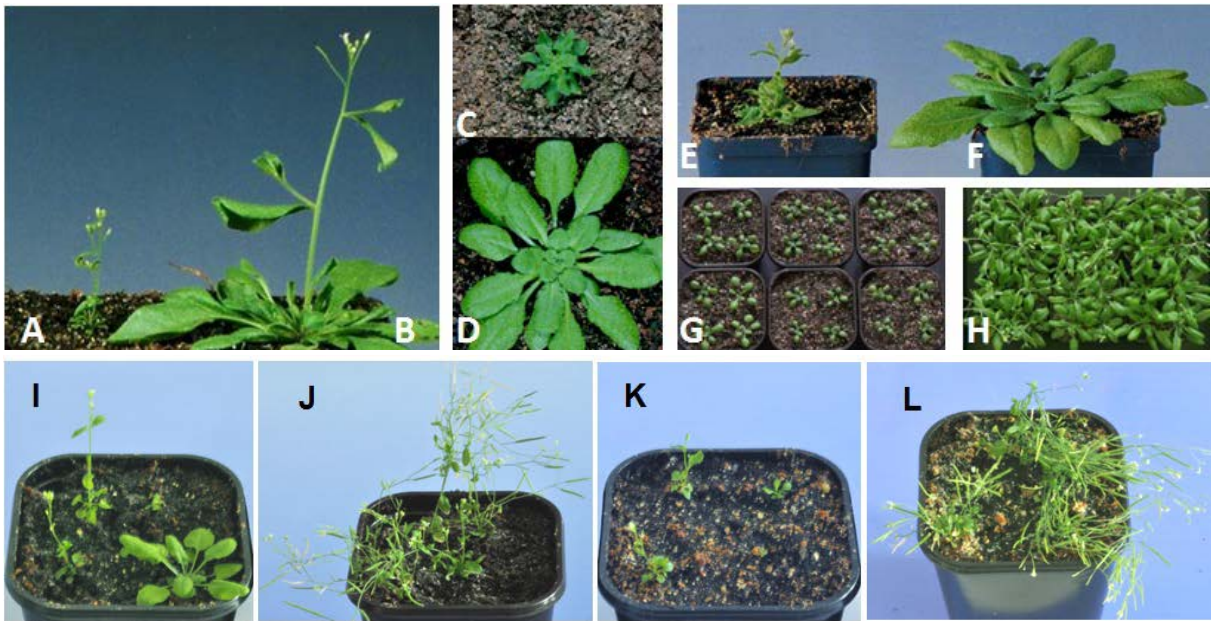


Figure 7. Global SUMO levels of shock stressed seedlings. Plants bearing the corresponding SUMO ligase knock-outs were germinated on MS plates. The seedlings were grown for two weeks and transferred to liquid MS medium for 24 hours in triplicate 24 well plates. One plate was kept as non-stressed control, one was supplemented with 300 mM mannitol for 1 hour, and one was incubated at 37 °C for one hour. A: A total protein extract from leaf tissue was blotted with anti-SUMO1 antibody. The lanes always show the non-stressed plants first, followed by the mannitol and the heat-shocked ones. B: Coomassie stained gels with the same amounts of protein as in A. C: Quantification of the lane intensities with double normalization. The colour intensity of each lane was scored using ImageJ. First the intensity of the SUMO stain from panel A was normalised to the total protein amount from panel B. Then the stressed samples were compared to the corresponding non-stressed Col-0 WT standard. Each gel contained an internal wild type standard, in order to minimise the batch differences.

Similarly to the chronic stress test (Figure 6), a shock mannitol treatment did not elicit a notable SUMOylation response, with the surprising exception of the *pial1 pial2 siz1* triple mutant (Figure 7C). Heat shock, on the other hand, caused a strong increase in SUMO conjugates of the wild type plants relative to the resting conditions. The *siz1* knock-out plants failed to respond to heat shock, and all other single and double knock-outs had lower global SUMO levels even in non-stressed conditions. Nevertheless, heat-shocking these mutants caused an elevation in the global SUMO levels, albeit not as strong as in the wild type plants. The SUMO levels of the *pial1 pial2 siz1* triple mutant were comparable to the wild type plants in normal conditions, and heat-shock caused elevation was again observed.

3.2 Mutation in the active site cysteine of SCE1 has a dominant negative effect on SUMOylation

Changing the SCE1 active site cysteine to a serine (C94S) had caused a dwarf, early flowering phenotype in the plants (Figure 8A-F). Overexpressing the wild-type gene *in vivo* had no effects on the plant growth (Figure 8G, H), and the mutant phenotype could be partially rescued by watering the plants with $(NH_4)_2SO_4$ (Figure 8I-M).



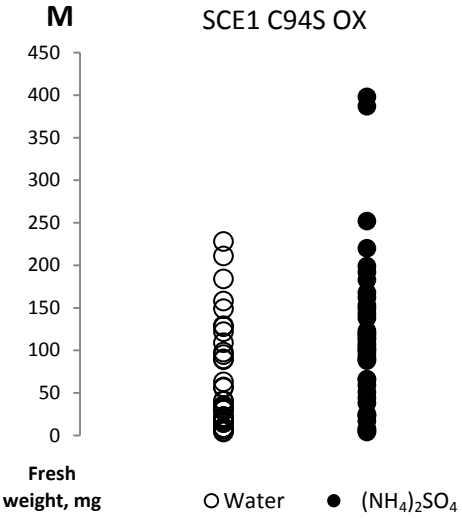


Figure 8. Growth phenotype of the plants with a mutation in the SUMO conjugating enzyme. Plants bearing the SCE1 C94S (A) construct are smaller than the ones containing a transgene with the wild-type copy (B). In short day conditions, they are both smaller (C vs. D) and flower earlier than wild-type expressing plants (E vs. F). G: Three week old plants, the left and middle pots contain two different SCE1 overexpressing lines, while the right pots have Col-0 wild-type plants. Overexpression of the wild-type SCE1 does not cause any difference in the growth habits of the plants. H: Same plants as in (G), but five weeks old. I, K: Seedlings expressing two different lines of SCE1 C94S. The size of the plants depends on the levels of the transgene expression. Pot in (I) has a Col-0 wild-type plant for comparison, which was removed later .J: Plants from (I), watered with tap water, one month later. L: Plants from (K), watered with 5 mM $(NH_4)_2SO_4$, one month later. M: Quantification of the fresh weights of the water-grown and ammonium-grown SCE1 C94S expressing plants, showing a 75% increase in the average weight of plants watered with ammonium.

In order to test the effects of the mutant SCE1 C94S on global SUMOylation *in vivo*, it was transiently expressed in *A. thaliana* plants bearing an inducible AvrPto gene (Tsuda et al., 2012). The leaves were infected with *Agrobacterium* strains, and the global SUMO levels in the infected leaves were tested with a Western blot (Figure 9A). Whereas infection with a wild-type SCE1 overexpressing construct had the same effects as infection with a mock solution, the SCE1 C94S construct caused a decrease in the overall SUMO levels.

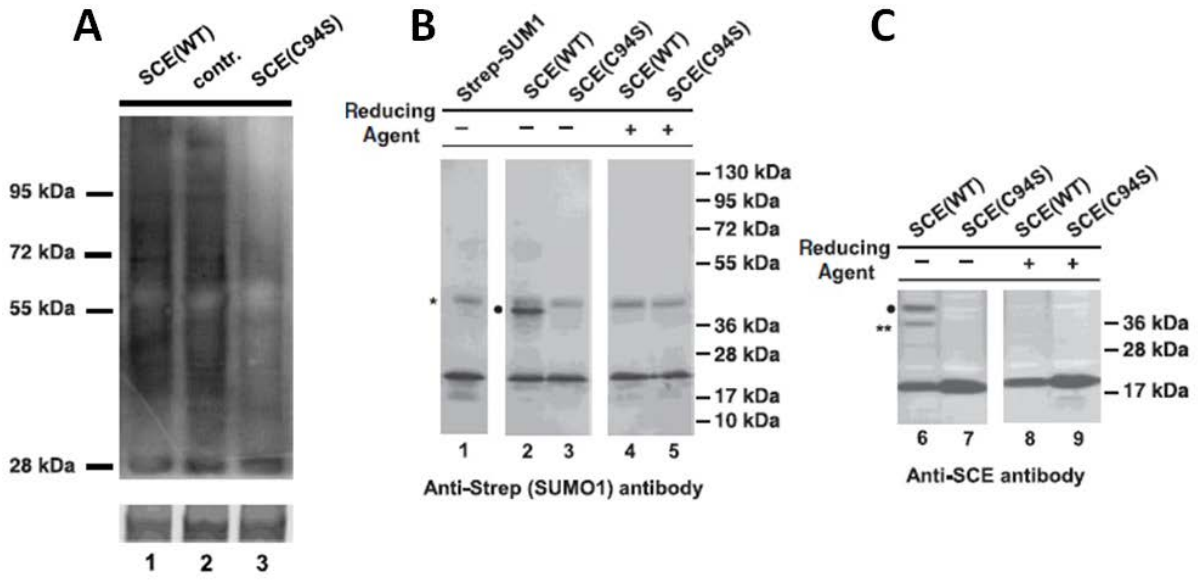


Figure 9. Effects of SCE1 C94S on SUMOylation *in vivo* and *in vitro*. A: Whole protein extract of plant leaves blotted with SUMO1 antibody. The leaves of AvrPto expressing *Arabidopsis* plants were infected with *Agrobacterium* strains bearing a construct overexpressing either SCE1 wild-type, or the SCE C94S mutant. The control plants were injected only with the infection solution. The wild-type overexpressing construct had no effect on the global SUMO conjugation levels (Lane 1), but the mutant transgene caused a decrease in SUMO conjugates. Below the blot is Coomassie stained RuBisCO large subunit as a loading control. B, C: Using the SCE1 wild-type and the SCE1 C94S in an *in vitro* thioester experiment showed that SCE1 formed a thioester (Lanes 2 and 6, dot) which was hydrolysed in the presence of reducing agent in the loading buffer (Lanes 4 and 8). The SCE1 C94S mutant could not form an oxyester (Lanes 3 and 7). One asterisk: a contamination in the recombinant SUMO1 preparation. Two asterisks: thioester SCE by SUMO1 with a proteolytically shortened amino terminus.

There are two possible mechanisms of action of the SCE1 C94S. It may be defect in accepting SUMO from the activating enzyme. Alternatively, the oxygen in the serine residue may form a strong oxyester with SUMO, prohibiting the transfer of SUMO to the substrate. Since SCE1 is the sole SUMO conjugating enzyme in plants, both mechanisms would explain the observed phenotype. To further investigate which is the case, a thioester experiment was performed (Figure 9 B, C). SUMO1, SAE and SCE1 were incubated in the presence of ATP for 30 minutes. The reaction was then stopped either with a reducing or with a non-reducing buffer and probed by Western blot. The wild-type SCE1 could form a thioester which was then cleaved by the reducing agent. The SCE C94S, however, could not form a thioester, and is therefore unable to accept SUMO from the activating enzyme.

3.3 PIAL1 and PIAL2 *in vitro*

3.3.1 Cloning of PIAL1 and PIAL2 for *in vitro* SUMOylation tests

The PIAL1 (At1g08910) protein coding sequence was isolated from total mRNA via RT-PCR. The cDNA for PIAL2 (At5g41580) was obtained from RIKEN (pda19847, RAFL16-52-I16), (Sakurai et al., 2005).

The only known feature of these two proteins was the SP-RING (also known as Zinc finger MIZ domain). Using proline and glycine residues as theoretical helix-breaking domain boundaries, three construct variants were created for each protein. One was the full-length protein, the second one contained the SP-RING only, and the last one was an intermediate fragment starting 28 amino acids before the SP-RING and ending 138 amino acids after it. The corresponding DNA stretches were amplified using the TaKaRa LaTaq, which has proofreading activity. Each fragment was ligated into the pCR 2.1 vector (Life Technologies). After confirming that the sequence was correct, plasmid DNA was prepared from the respective clones. The fragments were excised using BamHI and SalI and ligated into the pMAL-c2 vector (NEB), creating an MBP-PIAL fusion protein (Figure 10). All six fusion constructs expressed well in *E. coli* and a soluble protein could be purified.

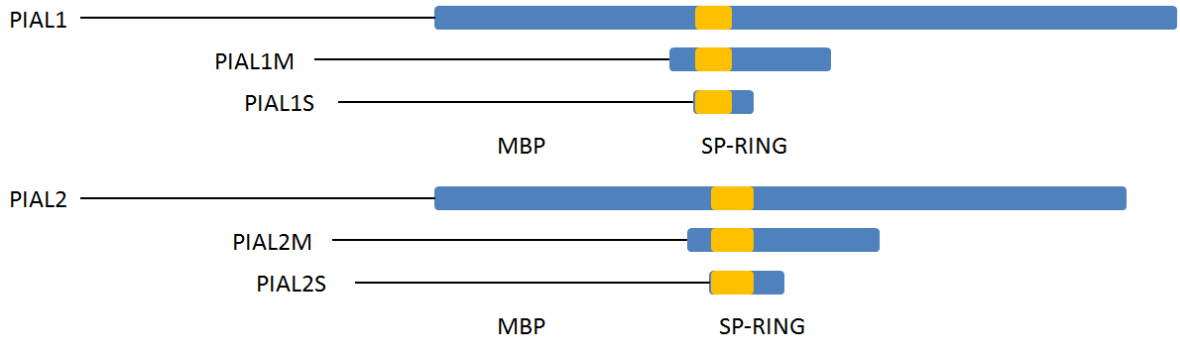
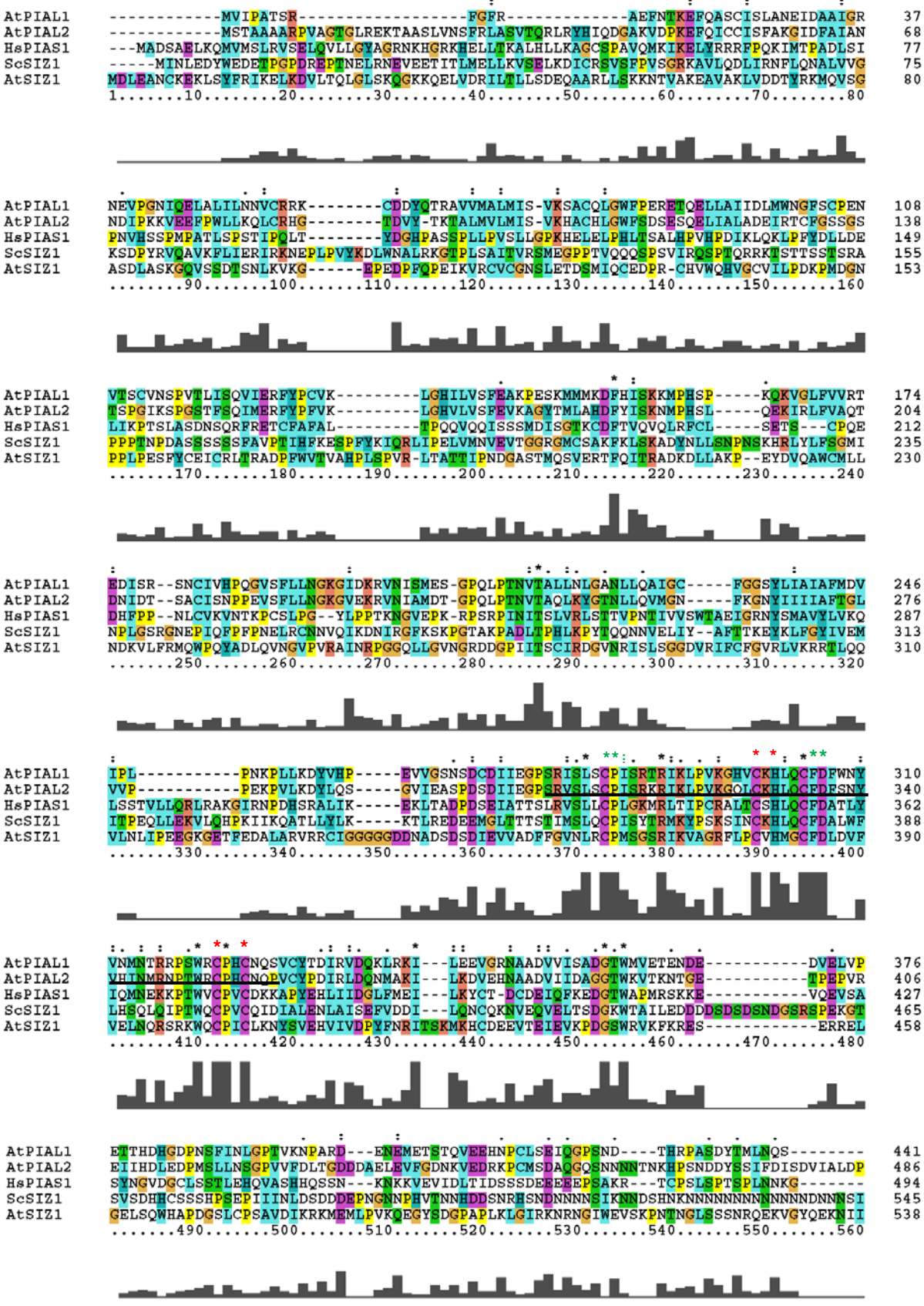


Figure 10. A scaled schematic representation of the PIAL1 and PIAL2 constructs created to study the *in vitro* SUMOylation. The PIAL parts of the constructs are shown as continuous blue bar, representing the lack of structural information available. The only known feature, the SP-RING, is shown in orange. The MBP N-terminal fusion partner is shown as a black bar.

A bioinformatic search using the PIAL1 or PIAL2 as a reference sequence yielded highly conserved sequences (Novatchkova et al., 2012) across a multitude of plant species. In the case of animal models, the only feature conserved was the SP-RING itself (Figure 11A). PIAL2 was more conserved than PIAL1, and it was constitutively expressed, whereas expression of PIAL1 was induced by stress. (R. Hermkes, A. Bachmair, personal communication). The bulk of this work is hence focused on PIAL2, assuming that PIAL1 should have similar mechanism of action, but the two proteins are differently regulated.

The TAIR homepage shows PIAL1 to be expressed in the embryo and in the leaf vasculature, but is not providing any information on stress regulation. It does not display any images about the PIAL2. The University of Toronto hosted Arabidopsis eFP browser does not contain any information about PIAL1. It does show, however, that PIAL2 is constitutively expressed at a moderate level (compared to alpha-tubulin) in all tissues and all developmental stages. During abiotic stress, PIAL2 is transiently induced, with the transcript levels returning to normal values during the adaptation period.

A



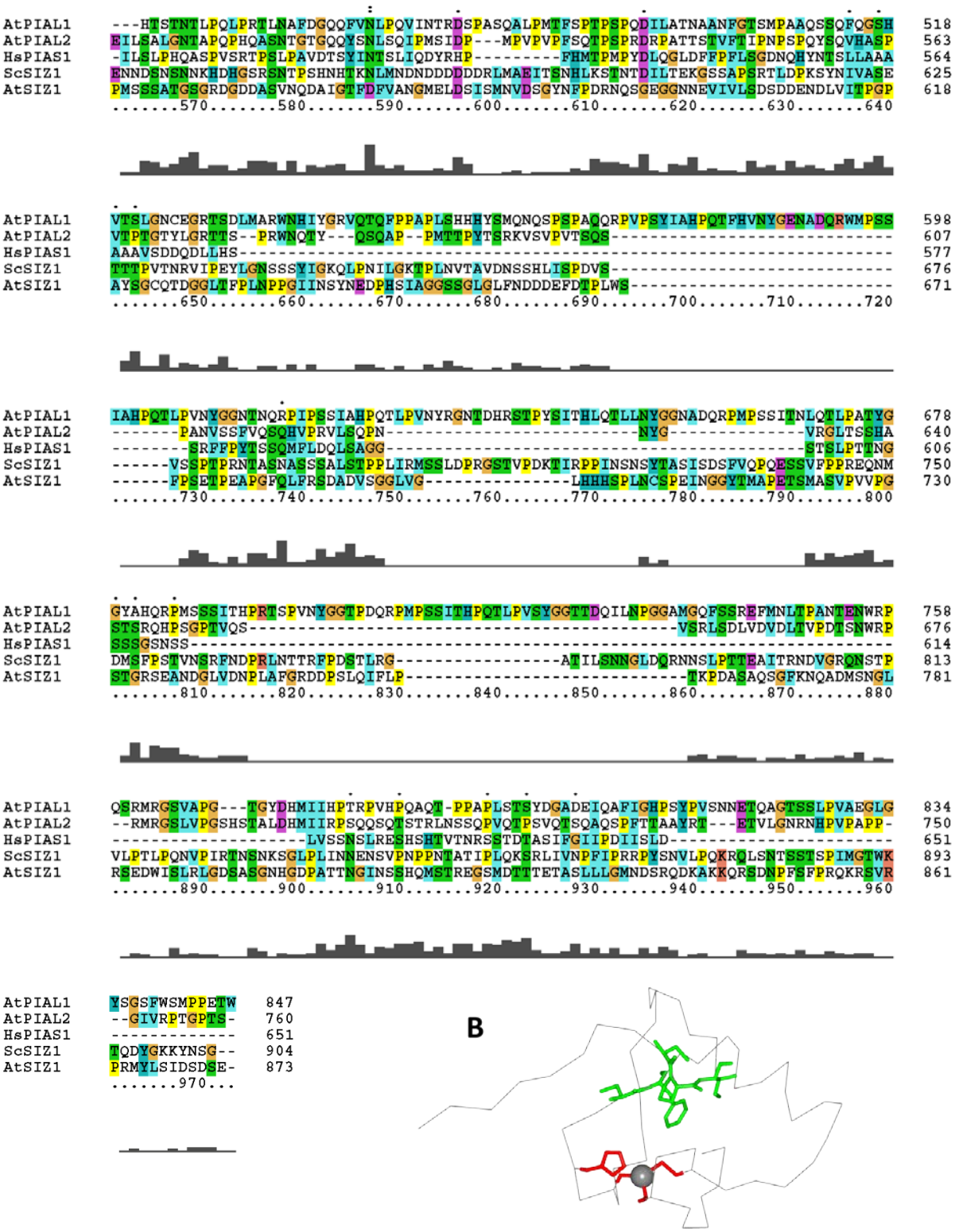


Figure 11. SP-RING SUMO ligases. A: A sequence alignment of SUMO ligases from *Arabidopsis*, baker's yeast and human reveals a high level of conservation throughout the SP-RING (underlined in black for PIAL2). The bars under the alignment show the degree of conservation. Black colons (:) show conserved traits, while black asterisks (*) denote identical residues. The SP-RING contains four conserved amino acids that coordinate one Zn^{2+} ion (red asterisks), and five other residues support the structure of the Zn-finger (green asterisks, green colon). B: A three dimensional structure model of the *Arabidopsis thaliana* PIAL2 SP-RING fold, based on the structure of ScSIZ1 (PDB ID 3E2D). The carbon backbone of the protein is shown as a grey line, the Zn^{2+} ion is a grey sphere, the four amino acids coordinating it are in red, and the five amino acids supporting the fold are coloured in green.

3.3.2 *In vitro* SUMOylation assay

The core assay was devised by Rebecca Hermkes and is described in her doctoral thesis, as well as in earlier publications (Budhiraja et al., 2009; Colby et al., 2006; Desterro et al., 1999). A 20 µl reaction was set up, containing 2 µM SAE, 1.75 µM SCE1 and 14 µM SUMO1 in the presence of Mg^{2+} and ATP. The reaction was incubated overnight at 30 °C and analysed with a Western blot. A reaction set up like this produced small free SUMO chains. In case a substrate was studied, it was added to the reaction in concentration of 7 µM. Such substrate is NAF, a nucleosome assembly factor, and it was used in the current work as a benchmark substrate.

3.3.3 PIAL1 and PIAL2 enhance the formation of SUMO chains *in vitro*

When added to the standard SUMOylation reaction, PIAL1M and PIAL2M fragments caused the increase of heavy molecular weight SUMO conjugates in increments indicative for SUMO chains. A mass spectrometry analysis of the bands showed branched SUMO peptides on the SUMO K10, K23 and K43. The presence of the full length PIAL1 or PIAL2 proteins caused the formation of even heavier chains, clustering as a smear on the gel, whereas the PIAL1S and PIAL2S constructs, containing only the SP-RING, did not have any visible effect on the reaction (Figure 12A).

3.3.4 PIAL1 and PIAL2 do not enhance monoSUMOylation

It has been previously established that NAF is an *in vitro* SUMOylation substrate (R. Hermkes, doctoral thesis). It is monoSUMOylated by the SCE1. When NAF was added to the SUMOylation reaction with a PIAL1 or PIAL2 functional fragment, its monoSUMOylation state was not affected by the ligase fragments. The fragments could, however, attach SUMO chains to NAF (Figure 12B, C).

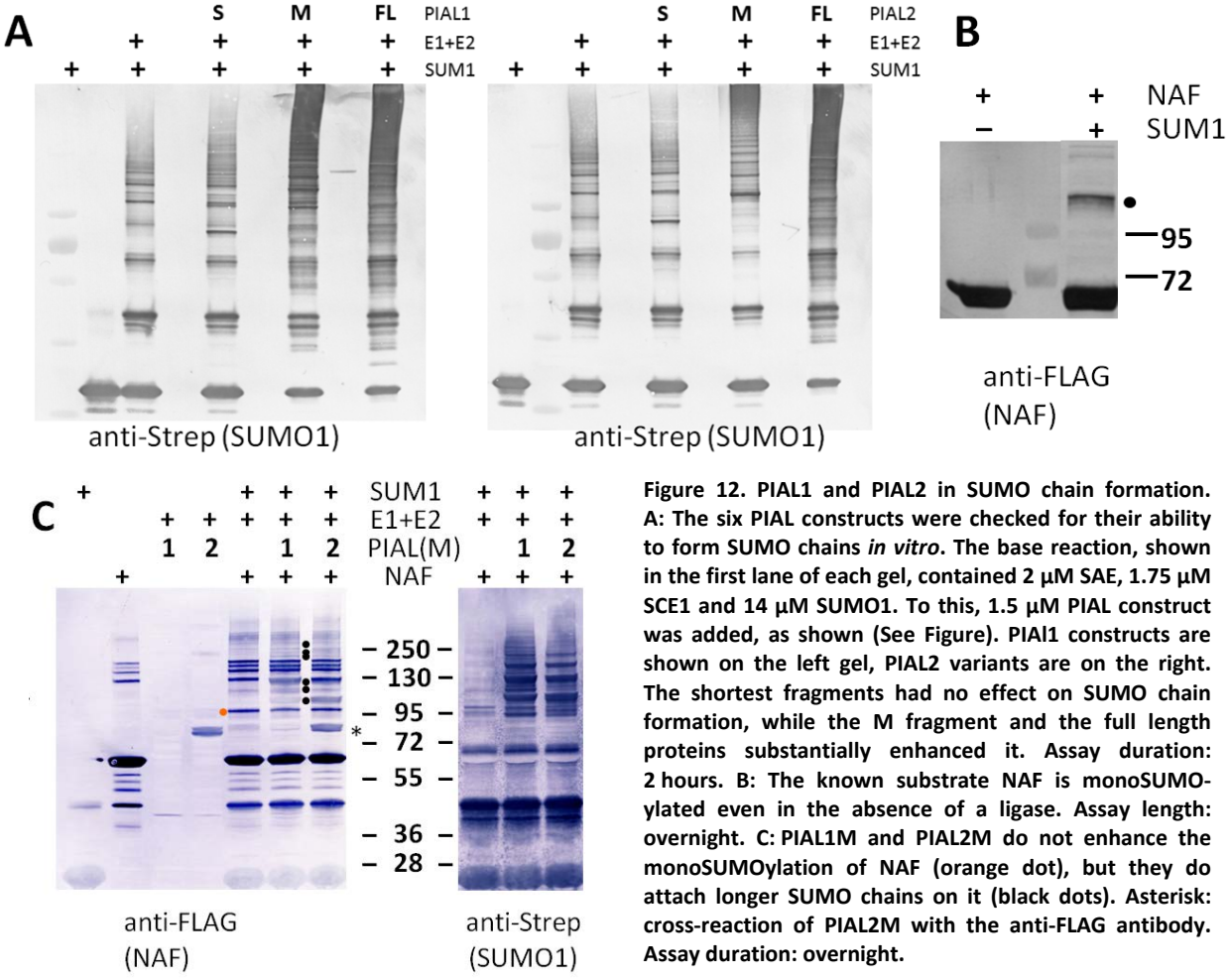


Figure 12. PIAL1 and PIAL2 in SUMO chain formation. A: The six PIAL constructs were checked for their ability to form SUMO chains *in vitro*. The base reaction, shown in the first lane of each gel, contained 2 µM SAE, 1.75 µM SCE1 and 14 µM SUMO1. To this, 1.5 µM PIAL construct was added, as shown (See Figure). PIAl1 constructs are shown on the left gel, PIAL2 variants are on the right. The shortest fragments had no effect on SUMO chain formation, while the M fragment and the full length proteins substantially enhanced it. Assay duration: 2 hours. B: The known substrate NAF is monoSUMOylated even in the absence of a ligase. Assay length: overnight. C: PIAL1M and PIAL2M do not enhance the monoSUMOylation of NAF (orange dot), but they do attach longer SUMO chains on it (black dots). Asterisk: cross-reaction of PIAL2M with the anti-FLAG antibody. Assay duration: overnight.

3.3.5 SCE1 is the limiting factor in PIAL2 mediated SUMO chain extension

It is known that the concentration of the SUMO conjugating enzyme determines the speed of the SUMOylation reaction (Klug et al., 2013). To test if the activity of the SCE1 is influenced by the presence of a ligase fragment, SUMOylation reactions were set up using serially smaller concentrations of SCE1 (Figure 13). At 0.35 µM SCE1, only a diSUMO band was visible after 2 hours, while no heavier chains could be seen (Lane 4). However, when the reaction was supplemented with 1.5 µM PIAL2M, there was a substantial increase in activity, evidenced by the appearance of SUMO conjugates with molecular weight higher than 100 kDa (Lane 8). Again, no increase was detected in the intensity of diSUMO chains, or monoSUMOylated SUMO, suggesting that PIAL2M had a more pronounced effect on larger SUMO chains. At the "standard" concentration of 1.75 µM SCE1, an increase in intensity was visible at triSUMO and heavier chains, while the diSUMO conjugates remained uninfluenced by the PIAL2M (Lanes 5 vs. 9). On the other hand, when the concentration of SCE1 was decreased to 0.07 µM, a band at the level of diSUMO appeared only when the PIAL2M fragment was present (lanes 3 vs. 7).

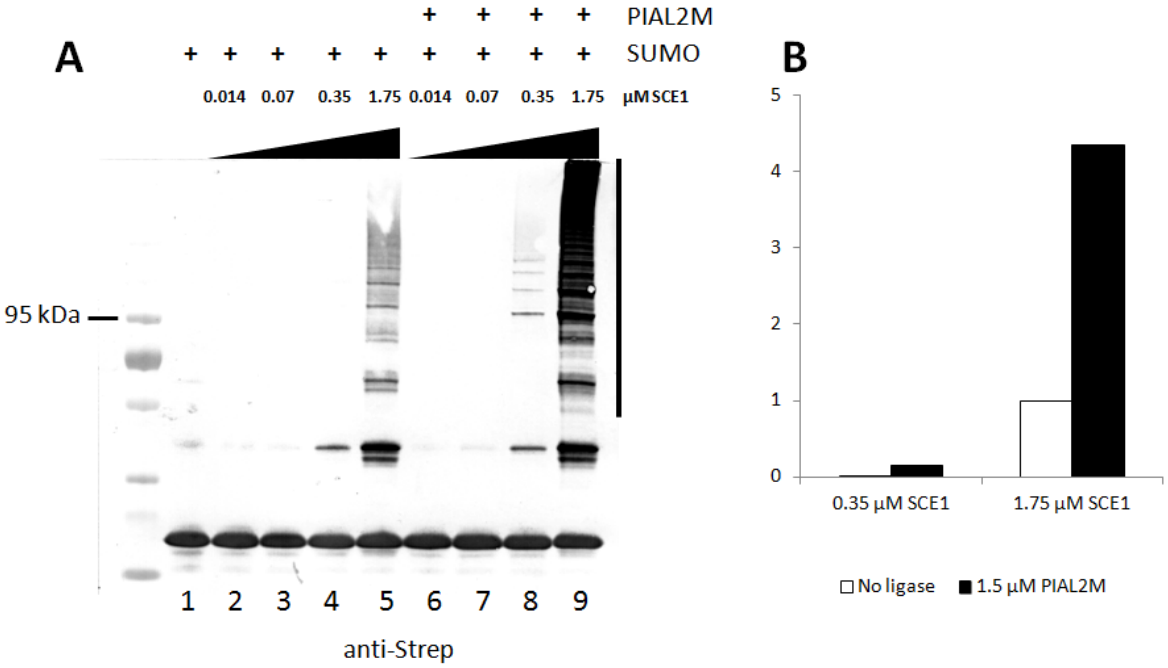


Figure 13. The concentration of SCE1 is critical for the reaction progress, while PIAL2M enhances the formation of heavy molecular weight SUMO chains. A: SUMO (14 µM) was incubated with 2 µM SAE and rising concentrations of SCE1 (0.014, 0.07, 0.35 and 1.75 µM) at 30 °C for 2 hours. When 1.5 µM PIAL2M was added to the reaction, the formation of heavy molecular weight SUMO chains was increased. The increase was observed for those reactions, where the concentration of SCE1 was enough to form a threshold of diSUMO, which could be extended by PIAL2M. The concentrations of diSUMO itself were not influenced by PIAL2M. B: Quantification of the band intensity. The heavy SUMO chains (A, vertical bar) were scored with ImageJ.

3.3.6 PIAL2 autoSUMOylates

When a SUMOylation reaction containing PIAL2M was separated and incubated with an anti-MBP antibody, additional bands appeared on the membrane, suggesting that the MBP-PIAL2M construct might itself be a substrate for SUMOylation. A mass spectrometry analysis identified two SUMO attachment sites, K372 and K448[3], named SAS1 and SAS2 (Figure 14A, B). When these lysines were substituted with arginines, using site-directed mutagenesis, the attachment of SUMO to the PIAL2 was not abolished (Figure 14C), but SUMO chains were formed even faster (Figure 14D).

[3] As numbered in the full-length PIAL2 ORF. The corresponding positions in the MBP-PIAL2 construct are K485 and K561.

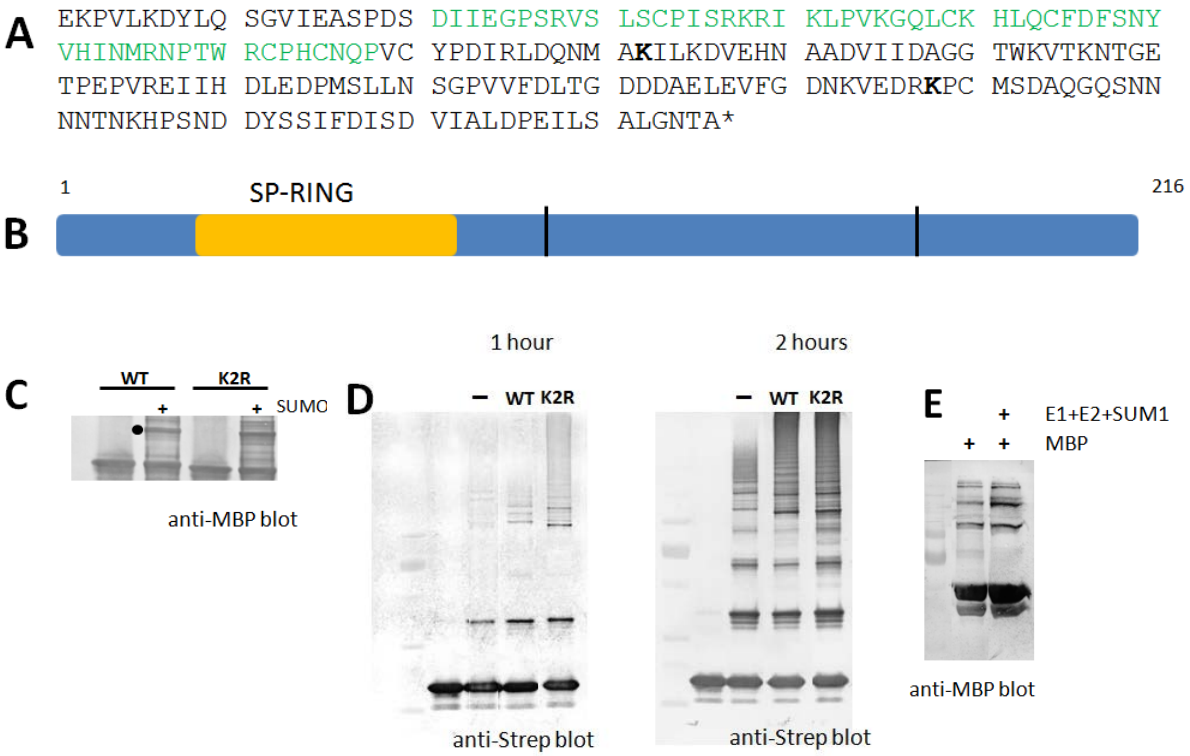


Figure 14. The SUMOylation sites of PIAL2. A: The sequence of the PIAL2M fragment (the MBP part is not shown). The SP-RING is coloured in green, while the two modified lysines, identified by mass spectrometry, are in bold typeface. B: Schematic representation of A. The two SUMOylation sites are depicted as black bars. C: When the two SUMOylated lysines were mutated to arginines, SUMOylation of the PIAL2M fragment was not abolished (black dot). Assay length: 2 hours. D: The PIAL2M K2R mutant enhanced the formation of SUMO chains even more than its wild-type counterpart. E: The maltose binding protein was not a substrate for E1 and E2. Assay length: overnight.

Both K372 and K448 are non-consensus sites, i.e. they do not have the ΨKXD/E sequence. A search with the SUMO sp v2.0 prediction software (Xue et al., 2006) identified a consensus SUMOylation site in the MBP part of the fusion construct. When tested as a substrate, MBP was not SUMOylated by SCE1 (Figure 14E). Since it was difficult to distinguish between SUMOylated MBP and SUMOylated MBP-PIAL2, an experiment testing if MBP-PIAL2 could SUMOylate MBP was not performed.

3.3.7 PIAL2 can be modified with rare SUMO isoforms

Arabidopsis SUMO1 and SUMO2 are the two SUMO isoforms that are predominantly used by the plant, and the two that can make SUMO chains. Of the other six isoforms, SUMO3 and SUMO5 transcripts have been identified (Novatchkova et al., 2004), but they cannot form chains and bind with a reduced affinity to the SCE1 (Castaño-Miquel et al., 2011). In an *in vitro* SUMOylation reaction, SUMO3, SUMO5 and SUMO7 can be attached to the PIAL2M fragment (Figure 15). As before, it is unclear whether this is an *in vitro* property of the PIAL2, working *in trans*, of the SCE1 with PIAL2 as a substrate, or a property of their combined action.

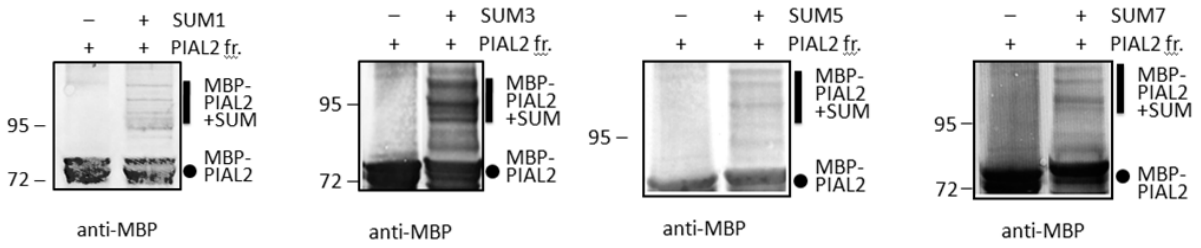


Figure 15. PIAL2 can be modified by rare SUMO isoforms *in vitro*. The PIAL2M functional fragment was incubated with the standard reagents of the *in vitro* SUMOylation reactions, but SUMO1 was substituted with SUMO3, SUMO5 or SUMO7. In all cases, PIAL2 (black dot) was modified with the available SUMO isoform (black bar). Assay length: overnight.

3.3.8 PIAL2 can build chains with SUMO3

In Arabidopsis, SUMO1 and SUMO2 are highly homologous and account for the vast majority of SUMOylation events. They can build chains and correspond to the human SUMO2/3 pair. In contrast, AtSUMO3 (which is functionally similar to HsSUMO1) does not make chains or is used as a chain terminator (Miller and Vierstra, 2011). However, when PIAL2M was added to an *in vitro* SUMOylation reaction using SUMO3, chains were visible on the Western blot (Figure 16).

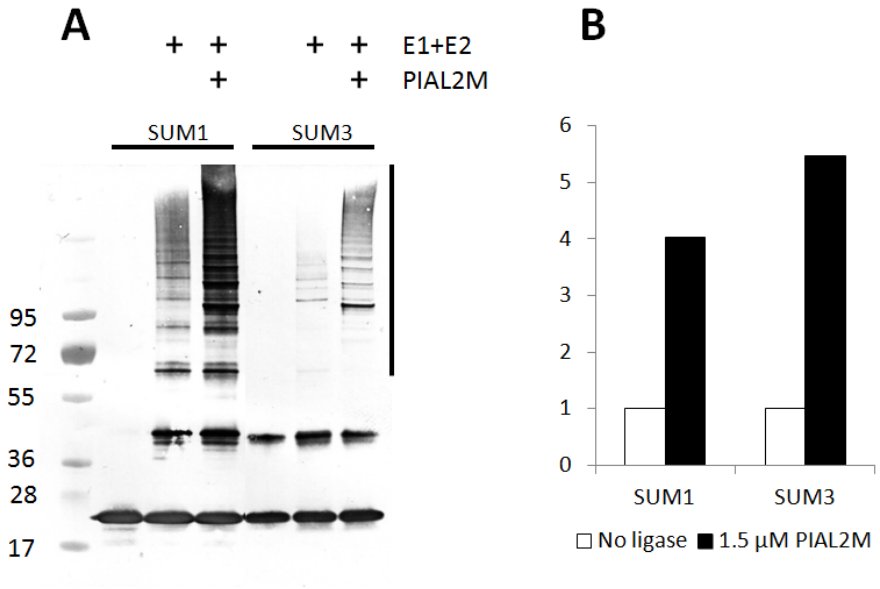


Figure 16. PIAL2M enhances SUMO3 chain formation. A: Western blot of *in vitro* SUMOylation reactions using either SUMO1 (left) or SUMO3 (right). Assay length: 2 hours. B: Quantification of the heavy molecular weight chains (A, vertical bar) using ImageJ.

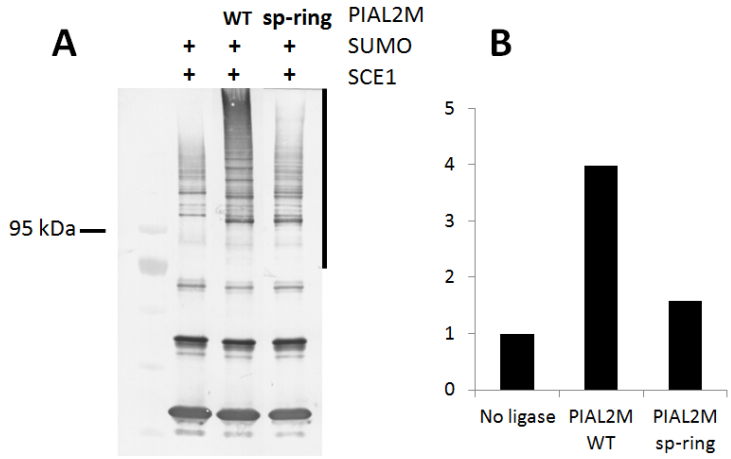


Figure 17. The SP-RING is not crucial for the chain formation activity of PIAL2M. A: An anti-SUMO1 Western blot, showing an increase in the molecular weight of the SUMO chains formed in the presence of PIAL2M. The PIAL2M *sp-ring* mutant (C329A, C355A), while less active then the wild-type fragment, was still able to enhance the SUMO chain formation. B: The intensity of the heavy molecular weight chains (A, black bar) was scored using ImageJ.

3.3.9 The SP-RING domain of PIAL2 is not critical for its activity

The SP-RING domain is the docking site for the E2 during the transfer of SUMO from the E2 to the substrate. It consists of three cysteines and one histidine which coordinate a single Zn^{2+} ion (Figure 11B). The interaction with the ion is supported by five other residues which stabilize the domain conformation (Ishida et al., 2012a). Mutating any of the Zn^{2+} coordinating residues in the *Saccharomyces cerevisiae* Siz1 SUMO ligase was reported to have a severe effect on SUMO conjugation (Yunus and Lima, 2009). Since the SP-RING domains are well conserved, a similar approach was undertaken for *A. thaliana* PIAL2 as well. When C329 and C355 were substituted with alanines, however, the PIAL2 was not perturbed in its activity (Figure 17).

3.3.10 PIAL2 contains two SUMO interaction motifs

SUMO interaction motifs, or SIMs, are short stretches of hydrophobic amino acids, flanked by polar ones. As their name implies, they can non-covalently bind to and interact with SUMO, thus recognizing SUMOylated proteins. An analysis of the PIAL2M sequence by Kay Hofmann identified one SIMb motif in the fragment, a Val-Phe-Asp-Leu stretch positioned between the two SUMO attachment sites. When these four amino acids were mutated to alanines, the chain formation activity of the PIAL2M fragment was increased, similarly to the PIAL2M K2R mutant variant, where the identified SUMOylation sites have been replaced with arginines (Figure 18).

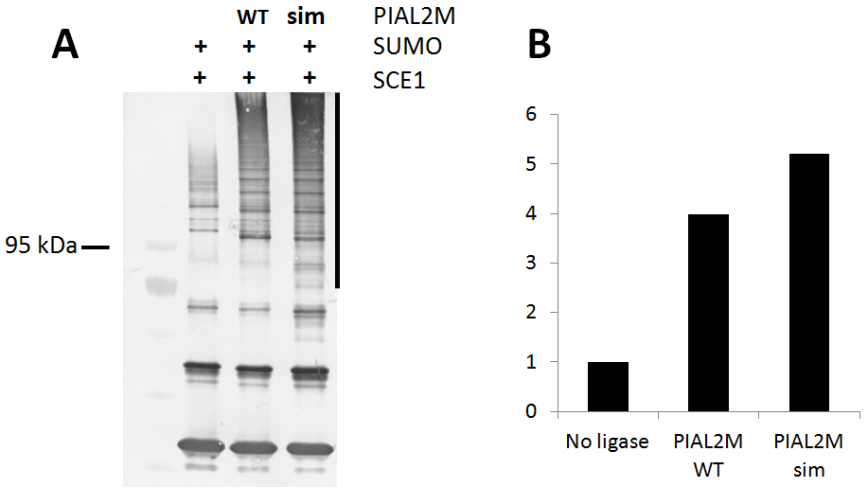


Figure 18. Mutating the SUMO interacting motif of the PIAL2M enhanced SUMO chain formation. A: An anti-SUMO1 Western blot, showing an increase in the molecular weight of the SUMO chains formed in the presence of PIAL2M. The PIAL2M *sim* mutant could substantially improve this effect. B: The intensity of the heavy molecular weight chains (A, black bar) was scored using ImageJ.

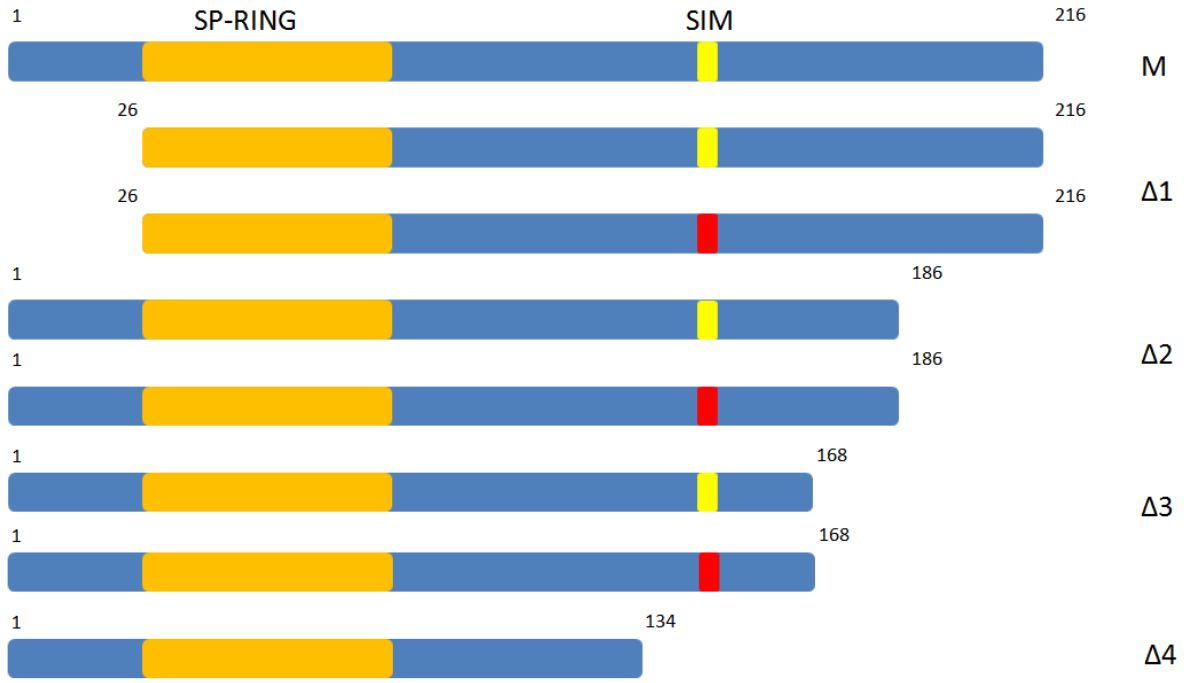
The second potential SIM was identified using a deletion approach. As PIAL2M is functional, but PIAL2S is not, a series of PIAL2M deletion constructs were created, using three evenly spaced proline residues as likely domain boundaries (Figure 19A). The constructs were amplified either from the wild-type PIAL2M or from the PIAL2M *sim* sequence. Like the original constructs, they were fused to MBP between the BamHI and the SalI restriction sites of the pMAL-c2 vector. The construct PIAL2MΔ1 had a deletion in the N-terminal part, effectively starting with the SP-RING. The other three constructs had intact N-terminal parts, but were C-terminally shortened. The shortest one, PIAL2MΔ4 was missing the SIM motif altogether (Figure 19B). When the constructs were assayed for their ability to form SUMO chains, the *sim* mutant variants did not perform any worse than their wild-type counterparts. They did, however, exhibit lowered SUMOylation levels of the PIAL2 fragment itself (Figure 19C, orange dots, Lanes 3 vs. 4, 5 vs. 6, and 7 vs. 8). Interestingly, the N-terminal deletion 1 showed reduced activity compared to the wild-type construct, and the *sim1* variant activity was even below the "baseline" activity of the E2 alone. Further sequence analysis identified an Ile-Phe-Asp-Ile stretch downstream of the second SUMO attachment site. Kay Hofmann suggested that this could be another SUMO interaction motif, involved in the SUMO chain formation. Thus, the VFDL and the IFDI motifs were dubbed SIM1 and SIM2, respectively (Figure 20A). When the Ile-Phe-Asp-Ile sequence of the SIM2 was substituted to alanines, thus creating the PIAL2M *sim2* mutant, the effect on the formation of free SUMO chains was similar to the one exhibited by the PIAL2M *sim1* mutant.

Interestingly, a *sp-ring/sim1* (C329A, C355A, VFDL 819-822 AAAA, Figure 20B) double mutant also had no SUMO chain formation activity. Furthermore, the levels of conjugated SUMO were even lower than the ones observed in the non-ligase control, implying that this mutant somehow perturbed the action of the SUMO activating and/or the SUMO conjugating enzyme (Figure 20C). This was not the case for the *sim1/sim2* (VFDL 819-822 AAAA, IFDI 869-872 AAAA), or for the *sp-ring/sim2* double mutant, which retained all (*sim1/sim2*) or part (*sp-ring/sim2*) of their activity. Additionally, the SUMO chains produced by the *sim1/sim2* mutant had a similar intensity to the ones made by the wild-type fragment, while the *sp-ring/sim2* resembled the *sp-ring* single mutant.

A

EKPVLKDYLQ SGVIEASPDS DIIEGPSRVS LSCPISRKRI KLPVKGQLCK HLQCFDFSNY
VHINMRNPTW RCPHCNQPVC YPDIRLDQNM AKILKDVEHN AADVIIDAGG TWKVTKNTGE
TPEPVREIIH DLEDPMSLLN SGPV**VFDL**TG DDDAELEVFG DNKVEDRKPC MSDAQGQSNN
NNTNKHPSND DYSSIFDISD VIALDPEILS ALGNTA*

B



C

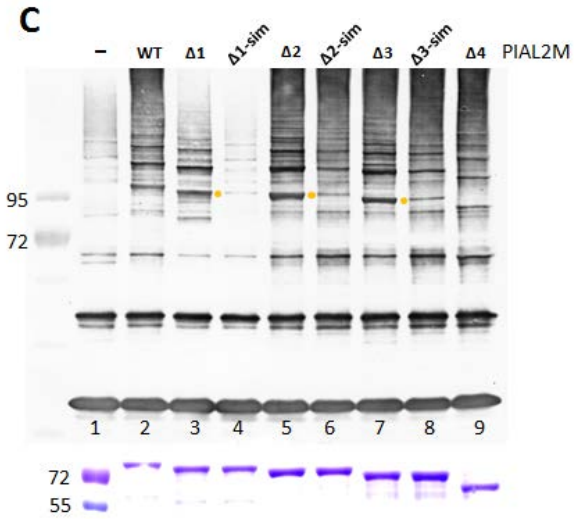
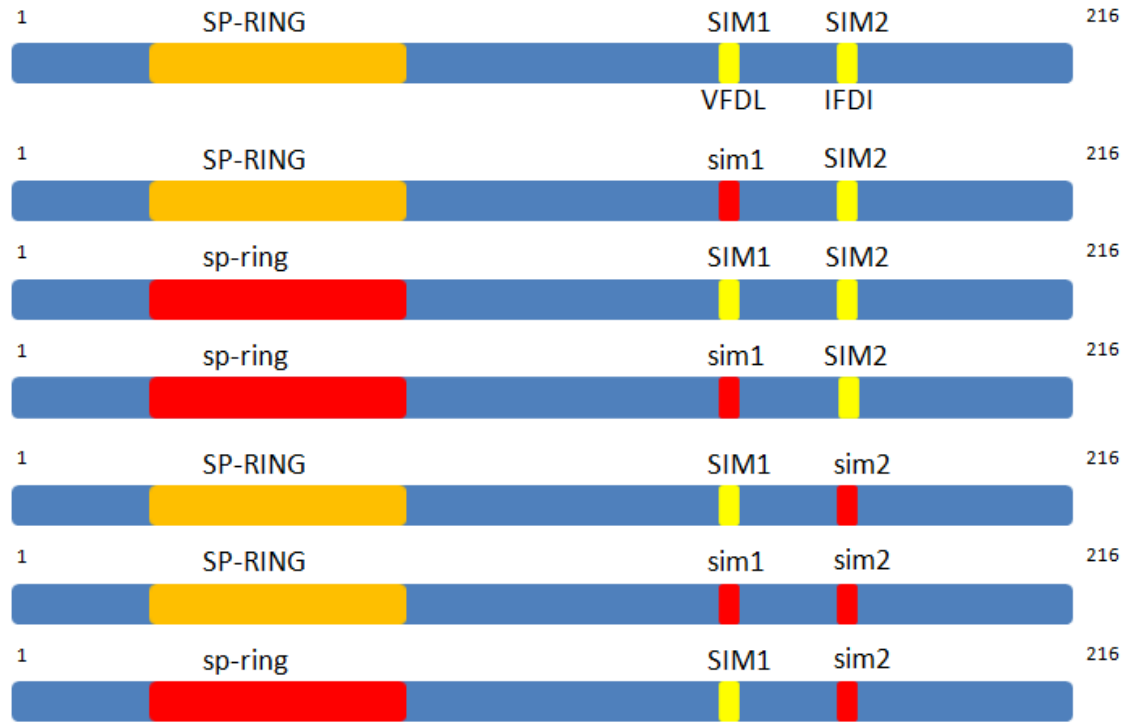


Figure 19. The deletion constructs used to map the second putative SUMO interacting motif. A: The sequence of the PIAL2M fragment (the MBP part is not shown). The SP-RING is coloured in green; the proline residues used as putative domain boundaries for deletions 2, 3 and 4 are underlined; the SIMb motif predicted by Kay Hofmann is shown in bold red. B: Scaled schematic representations of the deletion constructs. The first three deletions were created in either wild-type or *sim1* mutant background. The SP-RING is shown in orange; the SIM is shown in yellow for the VFDL and in red for the AAAA sequence. C: Anti-SUMO Western blot using the different truncation variants in comparison with the wild-type fragment. A reaction without a ligase was used as a background control. Assay duration: 2 hours. Orange dots: position of monoSUMOylated PIAL2 fragment in the WT and *sim1* mutant background deletion constructs.

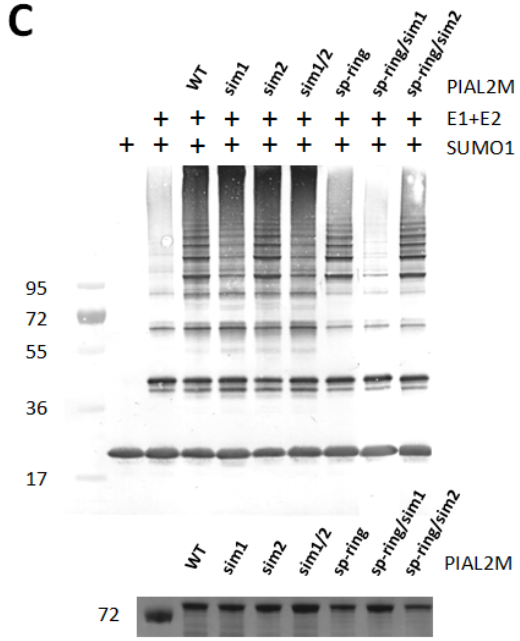
A

EKPVLKDYLQ SGVIEASPDS DIIEGPSRVS LSCPISRKRI KLPVKGQLCK HLQCFDFSNY
VHINMRNPTW RCPHCNQPVC YPDIRLDQNM AKILKDVEHN AADVIIDAGG TWKVTKNTGE
TPEPVREIIH DLEDPMSLLN SGPV**VFDL**TG DDDAELEVFG DNKVEDRKPC MSDAQGQSNN
NNTNKHPSND DYSS**IFDI**SD VIALDPEILS ALGNTA*

B



C



D

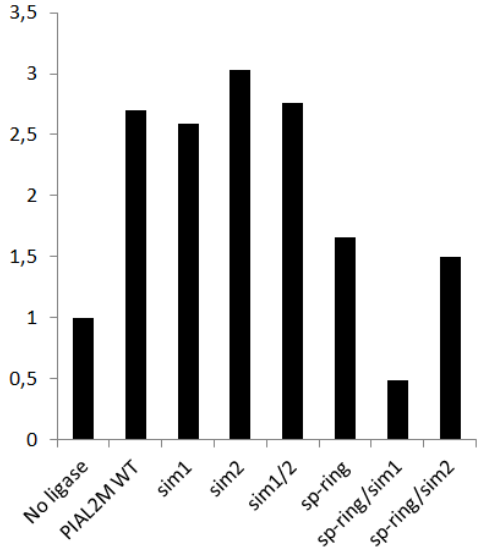


Figure 20. The SIM substitution variants of PIAL2M. A: The sequence of the PIAL2M fragment (the MBP part is not shown). The SP-RING is coloured in green; the SIM motifs predicted by Kay Hofmann are shown in bold red. B: Scaled schematic representations of the mutation constructs. The SP-RING is shown in orange for the wild type and in red for the CC329, 355AA variants; the SIMs are shown in yellow for the VFDL/IFDA and in red for the AAAA sequence. C: Anti-SUMO Western blot using the different mutation variants in comparison with the wild-type fragment. A reaction without a ligase was used as a background control. Assay duration: two hours. D: Quantification of the heavy (>70 kDa) SUMO chains in C.

3.4 SCE1 is a substrate for PIAL2

Finding a specific substrate for PIAL2 was an elusive task. Several putative substrates were tested, such as RRM1, Rfx3 and SVP, but this proved unsuccessful (data not shown). A SUMO chain formation experiment comparing the PIAL2M wild-type fragment to the PIAL2M *sim1* mutant, showed an extra band appearing at around 35 kDa, the combined apparent molecular weight of one SUMO protein and one SCE1 protein. Interestingly this band was only present in the reaction facilitated by the PIAL2M *sim1* mutant, but not with the wild-type ligase fragment or in the control reaction without a ligase.

A mass spectrometry analysis identified K15 as the SUMOylated residue of SCE1. When this lysine was mutated to an arginine, SUMOylation of the SCE1 was lost (Figure 21A, lanes 3 vs. 4, and 6 vs. 7). Surprisingly, K15 is a non-consensus SUMOylation site, while SUMOsp v2.0 suggested a consensus site at K28. To this date, no structure of the *A. thaliana* SUMO conjugating enzyme is available. The sequence of AtSCE1 was modelled with the ESyPred3D online three-dimensional structure prediction engine, based on the structure of the human SUMO E2, Ubc9 (PDB ID 2PE6). Both sites are far away from the active site cysteine, which sits at the bottom of a SUMO binding cleft. Lys-15 is positioned at the C-terminal part of the first N-terminal alpha-helix of the protein, while Lys-28 is slightly less exposed, positioned in a beta-sheet that forms the core of the enzyme (Figure 21B).

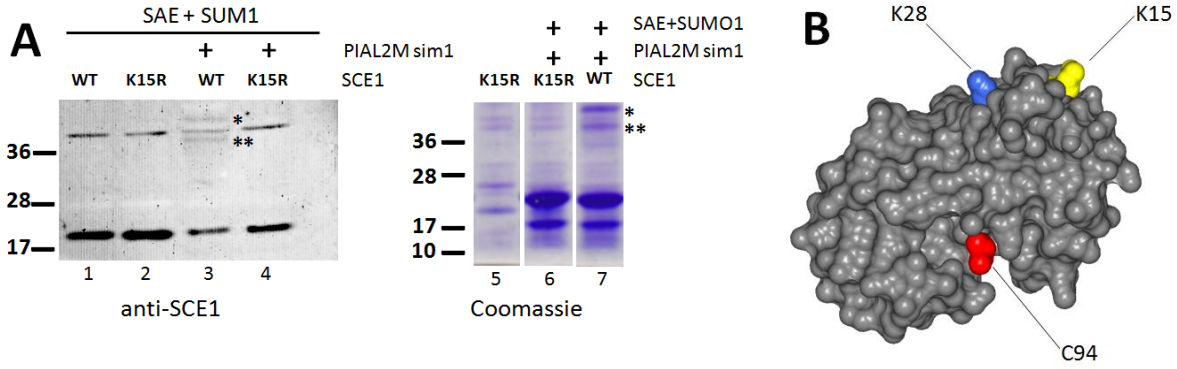


Figure 21. SCE1 is SUMOylated more abundantly in the presence of PIAL2. A: The presence of the PIAL2M *sim1* fragment in a standard SUMOylation reaction caused a band shift in the molecular weight of SCE1. Replacing K15 with arginine abolished the SUMOylation of SCE1. B: A three-dimensional structure prediction of *Arabidopsis thaliana* SCE1 based on the human homolog Ubc9 (PDB ID 2GRN). The active site cysteine, C94, is shown in red, while the SUMOylated K15 is yellow and the consensus K28 identified with the program SUMOsp 2.0 is blue.

3.4.1 SCE1 K15 is important for SUMO chain formation, but not for monoSUMOylation

Not only was SCE1 K15R not SUMOylated, but it had almost completely lost its SUMO chain formation activity. Only an excess of the mutated E2, together with the highly effective PIAL2M *sim1* fragment could partially reconstitute SUMO chain formation (Figure 229A). However, the benchmark substrate NAF was readily monoSUMOylated by both the wild-type SCE1 and by the mutant SCE1 K15R (Figure 22B).

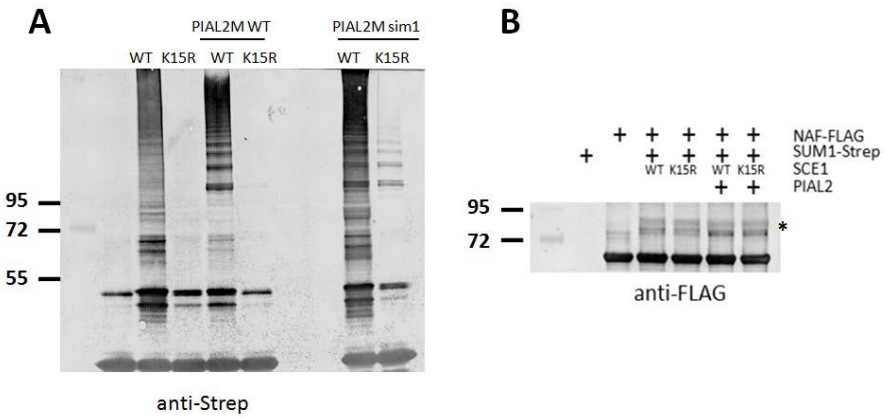


Figure 22. SCE1 K15R is partially functional as a SUMO E2 *in vitro.* A: An anti-Strep blot, showing SUMO and SUMO chains. SCE1 K15R could not produce SUMO chains, neither in a minimal reaction without a ligase fragment, nor with in the presence of PIAl2M. Only in a 5-fold excess of the mutated conjugating enzyme, in the presence of the highly efficient PIAL2M-*sim1* mutant, could some short chains be observed. B: An anti-FLAG blot, showing NAF, a known SUMO substrate. SCE1 K15R was not impaired in monoSUMOylating NAF (asterisk), and could do so even in the minimal reaction, where no ligase fragment was present.

Chapter 4: Discussion

4.1 Phenotypic analysis of pial1 pial2 siz1 mutants

Obtaining fertile seeds from mutant lines containing *siz1*, either alone or in combination with another ligase mutant, was impossible under normal conditions. This was conflicting with some other data (Ling et al., 2012) and the phenotype could only be partially rescued by supplying the plants with ammonium. A likely reason for this is the fact that the Arabidopsis nitrate reductase, the enzyme responsible for producing ammonium from the nitrate taken up by the roots, is a specific substrate for SIZ1 (Park et al., 2011). Perturbations in the nitrate pathway would limit the available ammonium and the biosynthesis of new amino acids, stunting the plant growth.

4.1.1 Global SUMO levels *in vivo*

Isolating SUMOylated species from plants proved to be a challenging task. When the isolated samples were stored, SUMO chains would degrade even at -80 °C and in 20 % (v/v) glycerol (data not shown). There are many SUMO proteases (Colby et al., 2006) active in the cells. The known ones have been described as cysteine proteases, but even after inhibiting all cysteine proteases using a protease inhibitor cocktail, supplemented with PMSF, some SUMO protease activity remained. This would suggest the existence of additional, hitherto uncharacterized SUMO proteases, for example the as of yet uncharacterised homologue of the yeast metalloprotease Wss1 (Mullen et al., 2010; Novatchkova et al., 2012). Furthermore, the protein isolation buffer would precipitate at low temperature, rendering even keeping the samples on ice useless. Hence, the separation of proteins would follow immediately after protein isolation. Ideally, the total protein concentration would be checked with a Coomassie stain, and then a new run would be set up with equal amounts of total protein. Because of the aforementioned sample decay, however, the fastest possible method for measuring protein concentration was chosen, the NanoDrop. This approach has its own pitfalls. It only shows reliable concentrations of pure proteins, something which was against the nature of the tested samples. It also requires the absence of nucleic acid impurities. Thus, purity was forgone in the interest of velocity, and after calculating the amounts needed for equal total protein concentrations, the samples were run in duplicates. One of the gels was stained with Coomassie Brilliant Blue R250, and the proteins from the other sample were transferred to a membrane for immunoblotting. Afterwards, the stain intensities were scored with the software ImageJ and the strength of SUMO stains was compared to the total protein levels. Although it is far from perfect, this method allowed for simple data analysis, and the trends exhibited by the various mutants could be reproduced in independent experiments. Another limitation of this approach was the Western blot itself. While low-intensity stained films were more or less linear, the high-intensity bands saturated the film and masked the real scope of the intensity. This can be avoided by using a chemiluminescent scanner that shows real-time image intensities. Unfortunately, such a machine was not available during this work.

The observed global SUMO levels of *siz1* mutant plants corresponded with their stunted growth phenotype. Although *pial1 pial2 siz1* triple knock-out displayed an even more severe phenotype, its global SUMO levels were, quite puzzling, comparable to the ones seen in wild type plants. Evidently, the action of the SUMO conjugating enzyme is sufficient to deposit SUMO on the substrates. Whether it is aided in this by HPY2 or by a yet undefined SUMO ligase, is still unclear. There are several factors that can fine-tune the SUMOylation state of the cell, which should be taken into account.

First, the availability of SUMO itself influences whether or not it can be used in a post-translational modification cascade. The Arabidopsis eFP browser (Winter et al., 2007) shows that SUMO1 is ubiquitously expressed throughout the whole plant. Upon short term stress, the expression of SUMO1 remains constant, whereas prolonged stresses can have differing effects. Cold (4 °C), salt (150 mM NaCl) and osmotic (300 mM mannitol) stress cause a slight decrease in the levels of

SUMO1, which corresponds to the effects of chronic osmotic stress observed in the current study. Drought, ultraviolet light and wounding do not have an effect on SUMO1 expression, while heat stress (37 °C) causes a temporal increase in the SUMO1 expression, which then subsides after 24 hours. Interestingly, the expression of SUMO1 is highest four hours after the start of the treatment, which is usually the time when elevated SUMO conjugation goes back to normal (Kurepa, 2003). SUMO2 is even more influenced by prolonged stress, showing the same trends as SUMO1, but with higher amplitude. These two SUMO isoforms are by far the most abundant ones, are capable of forming SUMO chains, and account for more than 90% of the overall SUMO conjugation (Castaño-Miquel et al., 2011)

The levels of SUMO3 are strongly influenced by cold and osmotic stress, especially in the roots, as well as by UV-B light in the shoots. However, due to the lower overall abundance of SUMO3, it is unclear which role this plays *in vivo*. Furthermore, the lack of suitably specific antibodies against SUMO isoforms other than SUMO1 and 2 did not allow their testing in the current study. According to the eFP browser, SUMO 5 was not influenced by any stress conditions tested, and there is no information available about the other isoforms. It is known that SUMO3 is involved in pathogen defence, and can be attached to AtPCNA, but it cannot substitute SUMO 1 and SUMO 2, as a knock-out of these two genes is embryonic lethal (Saracco et al., 2007). Not only is SUMO 3 evolutionarily more diverged than SUMO1/2, but also, according to a study, it cannot form chains *in vitro* (Colby, 2006). This discrepancy from the results presented here (Figure 16) might come from the fact that Colby *et al.* were investigating polySUMOylation of a specific substrate, whereas the SUMO3 chains described in the current study are free in solution. Apparently, this difference is critical enough *in vivo*, although the exact processes where a SUMO chain is indispensible have not been pinpointed yet. A study (Bruderer et al., 2011) has shown that polySUMOylated proteins are largely found in stress conditions, lending support to the idea that SUMO chains are of particular importance.

The SUMO ligase abundance and availability could also play a role in the effective levels of SUMO conjugates. Even though HPY2 is primarily involved in stem cell maintenance and the endocycle (the initiation of cell expansion and differentiation), it has also been proven to play a role in the heat-shock response (Ishida et al., 2012b). However, HPY2 and SIZ1 cannot substitute for each another (Ishida et al., 2012b), and a double knock-out is embryonic lethal. Therefore, a quadruple *pial1 pial2 siz1 hpy2* was not investigated in the current study. Since PIAL2 is expressed constitutively, it would seem a natural choice for an immediate response factor, capable of at least partially complementing SIZ1 in normal conditions (compare SUMO levels in *siz1, pial2 and pial2 siz1* mutants in chronic and shock stress, (Figures 6 and 7). Expression of PIAL1, on the other hand, would be first triggered by the stress conditions, and could contribute to the conjugation of SUMO to substrates at a later time point. Defining this moment precisely could be difficult for reasons discussed below.

All known SUMO ligases in *Arabidopsis* contain an SP-RING, which contacts the SUMO conjugating enzyme. However, there is at least one mammalian SUMO ligase which does not possess this domain. The RanBP2 nucleoporin contains an IR1-M-IR2 domain, which catalyzes the addition of SUMO1[4] to RanGAP1, thereby promoting the Ran nucleotide exchange cycle (Gareau et al., 2012). A plant homologue of RanBP2 has not been described yet, even though RanGAP1 is also SUMOylated in plants. Such a protein, if it existed, could explain the background SUMOylation activity in *pial1 pial2 siz1* triple knock-out plants.

Another consideration is the action of SUMO proteases. In *Arabidopsis*, there are eight known proteases divided in four classes, which participate in the SUMO turnover (Novatchkova et al., 2012). There might even be additional, unknown SUMO proteases, evidenced by the fact that blocking the action of cysteine proteases does not stop SUMOylated samples from degrading. Furthermore, the

[4] In human cells, SUMO1 cannot form chains, functionally corresponding to SUMO3 in *Arabidopsis*.

different proteases have a preference for different SUMO isoforms (Colby et al., 2006). Improper activation of a certain protease and the resulting imbalance in the SUMO turnover could be a possible explanation for the observed SUMO levels in the *pial1 pial2* double mutant and the *pial1 pial2 siz1* triple mutant. A model for the interplay between SUMO conjugation and deSUMOylation is proposed in Figure 23. Although all known ligases (and the SUMO conjugating enzyme) are taking part in SUMO conjugation, some of them have a stronger role than others. PIAL2, for instance, could strongly drive SUMOylation, but not have a pronounced effect in the subsequent SUMO turnover. On the other hand, PIAL1 might not have such a prominent role in SUMOylation, but in an as yet unclear manner trigger deSUMOylation and hence have a role in the maintenance of SUMO homeostasis. An *in vivo* tagging and tracking approach might shed more light on the conjugation, migration and deconjugation of SUMO in the living cells.

The redox state of the cell has also been shown to influence SUMOylation. SUMO proteases (Xu et al., 2007; 2009) and even the activating (E1) and conjugating enzymes (E2) (Bossis and Melchior, 2006) have been shown to be reversibly oxidized and inactivated at their active cysteines. A disulfide bridge between the active sites of the E1 and E2 has been described, sequestering the two enzymes from any active SUMOylation processes. If such bonds were formed in plants as well, this could be a very sensitive integrated signal for oxidative stress. For example, reactive oxygen species can damage DNA, and SUMOylation of the PCNA is required during the S-phase (Hoege et al., 2002; Leach and Michael, 2005). Thus, inactivation of the SUMOylation machinery could stop replication until the damage has been repaired and/or antioxidant mechanisms have been triggered.

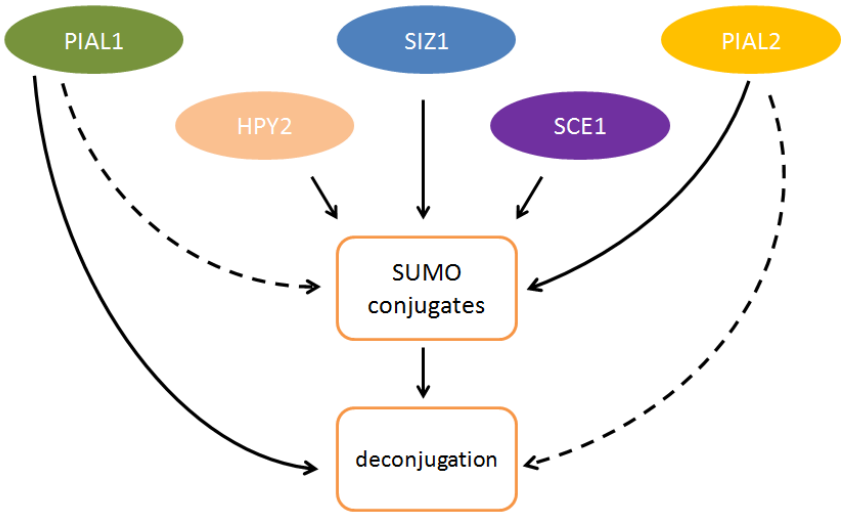


Figure 23. A model of the interplay between the currently known SUMO ligases, based on the stress phenotypes and global SUMO levels. The constitutively active PIAL2 promotes SUMO conjugation together with SIZ1 and the E2 (SCE1). Upon stress, PIAL1 is produced and helps the SUMO1 turnover by SUMOylating an unknown protein or, possibly, by binding an unknown DNA sequence (see below). Solid arrows denote strong positive influence, dashed arrows denote weak positive influence.

Having to survive various conditions without being able to evade them, plants have developed multiple adaptation strategies. Heat shock, for example, causes the rapid accumulation of SUMO conjugates in the nucleus (Saracco et al., 2007). The "panic" SUMO attachment is then gradually lost, as adaptation processes take place. One example of this is the *Arabidopsis* Heat-shock factor A2 (HsfA2). HSFs are conserved throughout eukaryotes (Sorger, 1991), but plants have quite a complex array of them (Tejedor-Cano et al., 2014). HsfA2 is activated upon heat-shock and is important in the establishment of thermotolerance, in other words in adaptation to heat (Cohen-Peer et al., 2010). When HSFs are SUMOylated, their affinity to corresponding DNA stretches is lowered, which

represses their transcription factor activities. Afterwards, they would have to be deSUMOylated, in order to be primed for a future emergency.

SUMOylation has been linked to actively transcribed chromatin regions. Interestingly, the presence of the mammalian Ubc9 and PIASy (SUMO E2 and E3, respectively) as a hallmark of actively ongoing SUMO conjugation was shown to repress gene expression (Neyret-Kahn et al., 2013). It is unclear if a similar parallel can be drawn to stress conditions, in other words if SUMOylation is needed to promote stress responses, would deSUMOylation be required to reset the cell in its original state. Nevertheless, it is certain that the interplay of SUMO ligases, SUMO proteases, and SUMO itself, is a finely tuned machine with far-reaching networking effects.

4.2 Mutating the active site cysteine of SCE1 (E2) has a dominant negative effect on SUMO conjugation

Substituting the active site cysteine with serine is an approach used to characterize ubiquitin conjugating enzymes. In some instances, the serine formed an oxyester with ubiquitin. This bond was stronger due to the larger electronegativity of the oxygen atom of serine compared with the sulfur of cysteine. In other cases, the smaller oxygen atom was too deep in the binding pocket to allow for proper ubiquitin binding. In this study, the SUMO E2 of Arabidopsis could not accept SUMO from the E1, as demonstrated by the thioester assay (Figure 9B, C). Since the C-terminal tail of SUMO has to enter a narrow cleft in the E2 (Figure 24) in order to access the active site cysteine, it may be that a C94S mutation renders the active site inaccessible. The SCE1 C94S mutant is thus inactive, but can still bind to the E1 and/or E3 ligases. When overexpressed *in vivo*, it is likely to compete with the endogenous SCE1, which would account for the observed growth defects (Figure 8). Overexpressing this dominant negative variant in a SCE1 knock-out background is impossible, because SUMOylation is an essential process, SCE1 is the only SUMO E2 in Arabidopsis and the mutant plants die during the embryonic development. Interestingly, even a transient expression in tobacco leaves was enough to influence the SUMO homeostasis (Figure 9A, Tomanov et al., 2013). Apparently, SUMO is involved in rapid response processes (such as stress perception) and perturbing its function can have immediate effects.

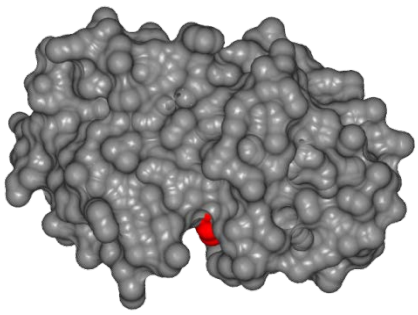


Figure 24. A three-dimensional structure of AtSCE1. Cysteine 94 is marked in red, sitting at the bottom of a cleft. The C-terminal diglycine motif of SUMO has to extend into this cleft, so that a thioester can be built. The structure was built with ESyPred, based on the structure of human Ubc9 (PDB ID 2GRN).

The stunted growth phenotype of the SCE1 C94S overexpressing plants mimicked the one exhibited by *siz1* (SUMO ligase) knock-out or ESD4 (SUMO protease) mutants. A recent study (Park et al., 2011) identified nitrate reductase as a substrate of SIZ1. The authors of the study watered the plants with ammonium, which partially alleviated the *siz1* dwarf phenotype. Watering the SCE1 C94S overexpressing plants with 5 mM $(NH_4)_2SO_4$ mitigated their growth defects. Therefore, SCE1 94S seems to be influencing the conjugation of SUMO to nitrate reductase, just like knock-out of SIZ1.

4.3 PIAL1 and PIAL2 *in vitro*

4.3.1 Cloning PIAL1 and PIAL2

As there was no cDNA of PIAL1 (At1g08910) available in the depositories (TAIR, RIKEN or SALK), the gene was amplified from total mRNA prepared from stressed plant tissue. Comparing the cDNA-amplified sequence of PIAL1 (At1g08910) to the one published in GenBank revealed two frame-shifts which, taken together, did not change the reading frame, but introduced an additional stretch of 17 amino acids just downstream of the SP-RING. In the TAIR database, this sequence was annotated as the first half of intron 15 (Figure 25A). The ARTADE database lists the same sequence as protein coding, and shows intron 15 correspondingly shorter. Whether this is a splice variant or an *in silico* annotation discrepancy is unclear. There is also an extra exon in the ARTADE model, splitting intron 14 of the TAIR sequence (and extending the SP-RING, which spans exons 13-15), but such a protein sequence was not obtained in this study.

The sequence of PIAL1 displays seven 23-amino acid long consecutive repeats in the region between amino acid 569 and 729 (Figure 25B). Whether or not these repeats have DNA binding properties was not tested. It is noteworthy, however, that the human protein ZNF821 was described to have four 23-amino acid long repeats, known as STPR domain, dispersed throughout the protein. These were shown to specifically bind a (5'-ATNTWTNTA-3') oligomer (Nonaka et al., 2010). Unfortunately, they are completely different from the repeats in PIAL1. ZNF821 is a Zn^{2+} finger domain protein of unknown function. Interestingly, another Zn^{2+} finger domain protein, ZNF451 was recently described as a SUMO ligase in humans. Since both the Arabidopsis SUMO ligase SIZ1 and its yeast homolog Siz1 have been reported to bind DNA, there is a possibility that PIAL1 can do so as well. More light on this will be shed when the subcellular localisation of the PIAL1 protein is known. The establishment of the relevant plant lines is still underway.

A cDNA for PIAL2 (At5g41580) did exist in the RIKEN repository and was used to create the fusion constructs. Even though PIAL1 and PIAL2 show high homology to each other, PIAL2 lacks the repeats that PIAL1 has. This difference is also the main reason for the size difference between the two proteins. As such repeats are also absent from SIZ1, it would appear that they are a unique feature of PIAL1, with an as yet unclear purpose.

4.3.2 *In vitro* SUMOylation: set-up and detection

In order to test the *in vitro* properties of the proteins forming the three-step cascade in Arabidopsis, they were expressed as recombinant constructs in *E. coli*, purified and incubated in a buffer containing magnesium and ATP. DTT was routinely used during the purification steps to protect the active site cysteines from oxidation. The reducing agent would then have to be removed before setting up the SUMOylation reaction, so that it would not cleave the thioester bonds between E1 (or E2) and SUMO. In initial experiments, especially before the addition of ligase fragments, the reactions were incubated overnight at 30 °C. Later, the reaction time was reduced to two hours, allowing for more physiologically relevant reaction conditions.

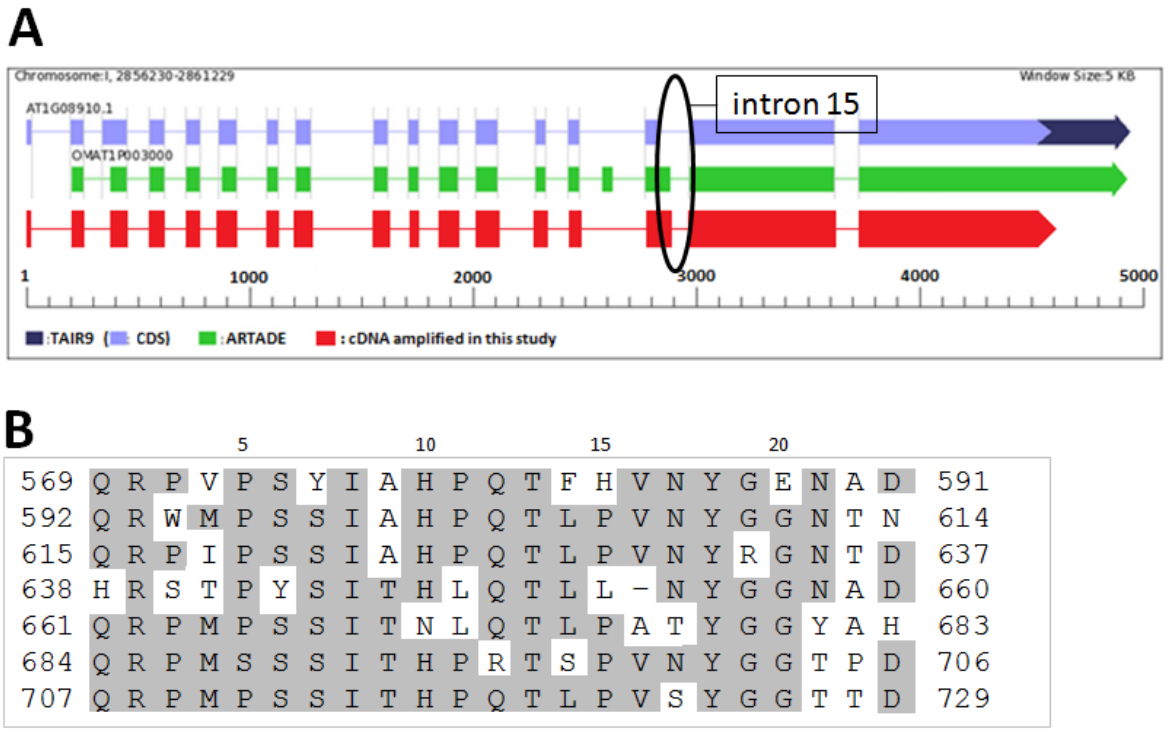


Figure 25. Some sequence features of PIAL1 (At1g08910). A: A gene map comparing the published exon-intron structures of PIAL1. The top one (blue) is taken from TAIR and shows a larger intron 15. The bottom one (green) is from the ARTADE project and assigns the first half of intron 15 as protein coding sequence, as obtained in this study. It also shows an extra exon in the middle of intron 14, which was not observed in this study. (Image withdrawn from http://matome.base.riken.jp/omat_gene_db/gene_info/gene_info.cgi?id=AT1G08910 and modified using http://wormweb.org/exonintron) B: Sequence of Gln551 to Asp710 of PIAL1. Seven 23-amino acid repeats are shown, with residues conserved in at least two repeats highlighted in grey (exception is position 22, where the conservation is evenly distributed between alanine and threonine).

Conjugation of SUMO to a substrate was routinely detected using a Western blot with antibody against a tag on SUMO and a tag on the substrate. Interestingly, SUMO1 always migrated to around 25 kDa, despite having a molecular weight of 18 kDa (11 kDa of the SUMO protein itself plus tags). When building SUMO chains, the band shifts were also in increments of 25 kDa. In some blots, there is an additional band under the main SUMO band. A mass spectrometry analysis identified this as an N-terminally shortened SUMO1, which could still take part in reactions, as evidenced by double bands at levels corresponding higher molecular weight. This band appeared even in pure SUMO samples after incubation in the SUMOylation reaction, suggesting that it was a spontaneous proteolysis effect. Fortunately, the cleavage site was within the tag region and the integrity of SUMO itself was not disturbed.

In the cases where the precise SUMOylation site had to be known, samples were submitted to the MFPL mass spectrometry facility and analysed by Dorothea Anrather. A SUMOylated protein would give a branched peptide after digestion with trypsin, where the C-terminal part of SUMO is attached to the ε-amino group of a lysine residue of the substrate protein. This lysine would be protected from trypsination and would be discoverable as an internal residue in a larger peptide. One problem with this approach was the relatively large mass of the ultimate C-terminal fragment of Arabidopsis SUMO1 (AEQTPDELDMEDGDEIDAMLHQTGG), added to the substrate peptide. This fragment would often degrade during flight, obscuring the results and often completely masking the substrate peptide. The problem was circumvented by substituting histidine 89 of SUMO1 with lysine, thereby yielding a much shorter QTGG signature branch. This allowed the identification of three SUMO attachment sites on SUMO1: K10, K23 and K42. K89 was also SUMOylated, but since this was an introduced mutation, it was disregarded. A study from 2010 also showed that K10, K23, and K43 are

SUMOylated *in vivo* (Miller et al., 2010). Furthermore, it showed that K23 is also ubiquitylated *in vivo*. Interestingly, K10 of SUMO1 is not conserved in SUMO3 (Novatchkova et al., 2004). The other two SUMOylation sites, however, are present in both SUMO1 and SUMO3, and K23 is conserved in all eight Arabidopsis SUMO isoforms. This could account for the discrepancy between the SUMO3 conjugation patterns reported by Colby et al. and the current study (see above).

Another recent finding in the ubiquitin field are the so called SUMO targeted ubiquitin ligases, or StUBLs, example of which is the human RNF4 (Prudden et al., 2007). This ubiquitin E3 ligase has an array of SUMO interacting motifs (SIM) which allow it to identify polySUMOylated proteins. It would then add ubiquitin to the SUMO chain, causing the original substrate protein to be degraded. Possible Arabidopsis StUBLs have been described (Elrouby et al., 2013), but their specific interaction with SUMO chains has not been proven yet.

4.4 PIAL2 acts as a SUMO E4

Ubiquitin is by far the best studied and most versatile post-translational modification protein. Apart from the classical activation (E1), conjugation (E2) and ligation (E3) enzymatic cascade, another class of ubiquitylation facilitating enzymes has been described (Koegl et al., 1999). The E4 ligases do not act on the original substrate. Rather, they "wait" for another E2/E3 pair to add one or a few ubiquitin moieties to a protein and then extend the ubiquitin chain. To draw a parallel from this, a specific substrate of PIAL2 could not be found despite numerous attempts. Furthermore, neither of the different PIAL2 constructs created in this study could increase the monoSUMOylation of a substrate (NAF). PIAL2 did, however, extend an already existing short chain on both NAF (Figure 12C) and on SUMO itself. This is well illustrated by Figure 13. Although the concentration of E2 was critical for the reaction, a minimal threshold amount of diSUMO, i.e. monoSUMOylated SUMO, was required for the PIAL2 to facilitate the chain formation. Interestingly, PIAL2 could also enhance the formation of the first isopeptide bond between two SUMO moieties (Figure 13A, Lanes 3 vs. 7). The presence of two closely spaced SUMO interaction motifs would lend additional support to the idea of PIAL2 being a SUMO E4, allowing the ligase to process a growing SUMO chain without having to interact with the original substrate.

4.4.1 PIAL2 is a promiscuous SUMO ligase that can build chains and be modified with rare SUMO isoforms *in vitro*

It was not clear whether the PIAL2M fragment was autoSUMOylating in *cis* or in *trans*, or whether it was being SUMOylated by the SCE1. The fact that PIAL2 can be modified with other, traditionally inert or simply rare, SUMO isoforms is indicative that PIAL2 may well be needed for its own modification. As follow-up mass spectrometry analysis with the rare SUMO isoforms was not done, it is still an open question whether the SUMOylation sites on PIAL2, occupied by SUMO3, SUMO5 and SUMO7 are the identical to the ones identified for SUMO1. As already mentioned, some of the lysine residues of SUMO1 that can be used to form chains are conserved in other SUMO isoforms, but a definitive proof in the form of a branched peptide identified by mass spectrometry is still lacking. Another issue is whether PIAL2 can allow these SUMO variants to build chains, something that they have not been reported to do. The functional fragment PIAL2M, used in this study, was able to extend SUMO3 chains *in vitro*, further supporting the idea of its E4 action. Whether it can do so *in vivo* and what functional significance this would have remains unknown. Moreover, it would be interesting to investigate whether PIAL2 could build mixed chains when several SUMO isoforms are present in the reaction mix and whether these would have any physiological relevance.

4.5 Several covalent and non-covalent interaction sites on PIAL2 allow for a precise control of its activity

As mentioned earlier, PIAL2 contains an SP-RING domain, which interacts with the E2 during SUMOylation reactions. This domain contains three cysteines and one histdine which coordinate a single Zn^{2+} ion, as opposed to the ubiquitin RING domain, which can accommodate two ions. These four residues are highly conserved (Figure 11A) and are thought to be critical for the activity of the ligase. Indeed, a study shows that mutating any one of the coordinating amino acids of the yeast Siz1 E3 ligase abolishes SUMOylation. However, when two of the cysteines of PIAL2 were substituted with alanines, this had only a moderate effect on its activity. While decreased, the activity of PIAL2 to enhance the formation of SUMO chains was still present. Only when combining this mutant with the *sim1* mutation (VFLD 819-822 AAAA), was the activity of PIAL2 abolished. Interestingly, the levels of SUMO conjugates were even lower than the ones observed with only E1 and E2 present in the reaction mix. As there was still one intact cysteine in the SP-RING of the PIAL2, one could hypothesise that this cysteine formed a disulfide bond to the active site cysteine of SCE1 (E2) or even of SAE (E1), sequestering some of them away from the reaction and thereby producing "below baseline" results. Unfortunately, a disulfide bridge formation between PIAL2 *sp-ring/sim1* and any other enzyme in the cascade has not been tested. The two SUMO interaction motifs are probably sufficient to bind and orient SUMO moieties in a way that facilitates the formation of a bond between them. In this sense, even a protein with an inactive SP-RING can still be a scaffold, enhancing the reaction. A model of how the SP-RING and the SIMs work with each other is shown in Figure 26.

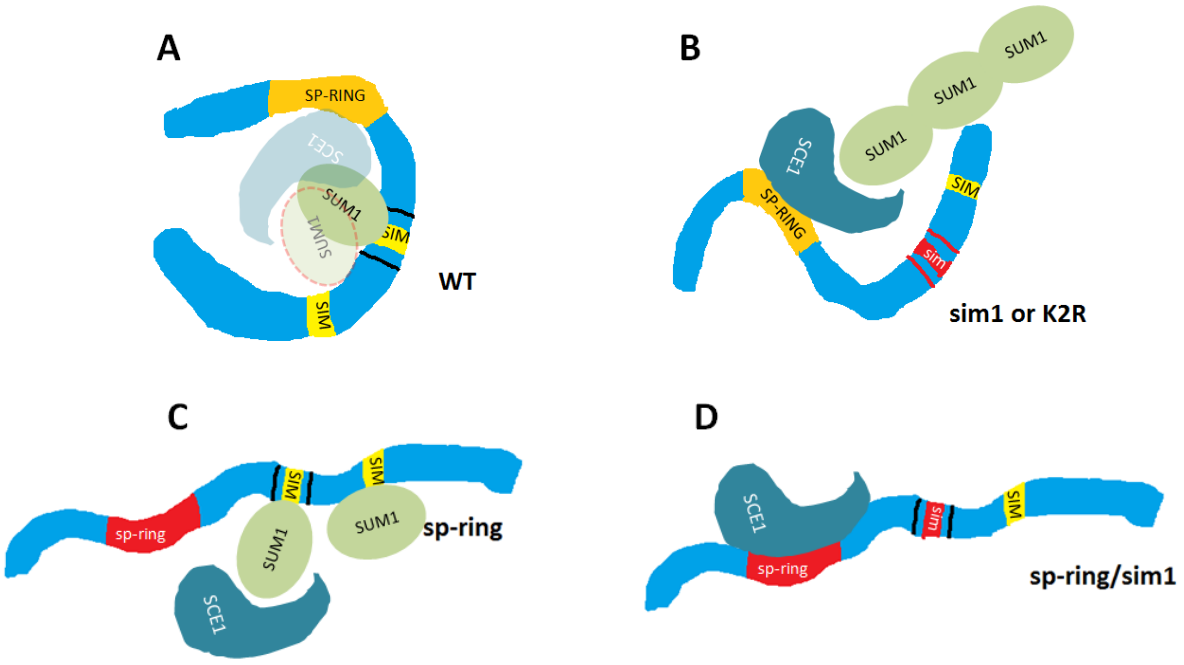


Figure 26. Model of SUMO chain formation by PIAL2. A: The wild-type PIAL2 fragment (cyan) can be SUMOylated (green) on either SUMO attachment site (black bars). This event may decrease access to SIM1 (yellow) and the next SCE1-SUM1 complex (transparent blue and red dashed line) would bind to the SP-RING (orange) with reduced affinity. B: If SIM1 or the SUMOylation sites are mutated (red), SCE1 does not have to compete with a covalently bound SUMO and can freely bind to the SP-RING, causing a rapid chain formation. It may be helped in this action by the presence of SIM2. C: If the SP-RING has been mutated (red), SCE1 cannot to it bind anymore, but the two SIMs are enough to present a scaffold for efficient SUMO chain formation. D: When both the SP-RING and SIM1 are mutated, there are not enough docking sites on the PIAL2 to promote SUMO chain formation. Furthermore, SCE1 itself may be trapped by the residual cysteine of the mutated SP-RING (See text), accounting for the below baseline SUMO chains (Figure 20).

Substituting the four residues of SUMO interacting motif (SIM1) with alanines had a very similar effect to the K2R substitution of the identified SUMO attachment sites (SAS). In both cases, the mutant fragments showed an increase in SUMO chain formation activity. The two SAS and SIM1 are in close proximity to each other with the SUMOylation sites flanking the interaction motif. Surprisingly, all constructs containing a *sim1* substitution showed a decreased SUMOylation of the PIAL2M fragment (Compare Figure 18, Figure 19C (orange dots) and Figure 20C). A *sim2* did not have this "defect". Interestingly, a *sim1/sim2* double mutant again had lowered PIAL2 SUMOylation, as if SIM1 was overpowering the effects of SIM2. It would appear that normally PIAL2 has a quite high SUMO chain formation activity, which is held in check by SUMO itself. Since the identified SUMOylation sites are close to one SUMO interacting motif, the modification of PIAL2 could disturb the binding of an activated SCE1 to it, effectively slowing down the reaction (Figure 26A vs. B). This would make sense in a situation where a sudden and drastic trigger of SUMOylation is needed in the cell. If the constitutively expressed PIAL2 is held at "resting state" by constant cycles of SUMOylation and deSUMOylation, a stress event would cause the conjugation of SUMO to a substrate by another SUMO ligase or even by SCE1 itself, thereby slightly lowering the available free SUMO. This could indirectly cause the deSUMOylation of PIAL2, freeing it to work in overdrive and extending numerous SUMO chains, which then in turn would signal downstream adaptation effects. The E4 mechanism of action of PIAL2 would ensure that only already prepared targets are SUMOylated, reducing the possibility of erroneous SUMO conjugation events. At the same time, more SUMO1 is being produced, triggered by the stress response. Eventually PIAL2 would also be SUMOylated, either on its own or by another ligase, and thus become primed for another stress event. Although this hypothesis would require the use of a lot of energy of futile SUMO-deSUMO cycles, it has been shown that such scenarios are normal for plants.

The ethene response is a fine example of futile cycling. ETR1, the receptor for the plant growth hormone ethene (ethylene) is a membrane protein bound to a dimeric protein kinase (CTR1). In the absence of ethene, the kinase phosphorylates the membrane anchored transcription factor EIN2, which is then targeted for 26S proteasomal degradation. When ethene binds to the receptor, the kinase is inactivated and the C-terminus of EIN2 is cleaved and can relocate to the nucleus, where it activates the downstream ethene response pathways (Ju et al., 2012). Another instance for sensor cycling are the hypoxia response genes (HRE1 and HRE2). Under normal oxygen levels, these proteins are constitutively synthesized and then degraded by the N-end rule pathway. In hypoxic conditions, the proteins are stabilized and help the plants to survive in low oxygen conditions (Gibbs et al., 2011).

4.6 The building of SUMO chains is a two-step process depending on SCE1

When the standard *in vitro* SUMOylation reaction was incubated with a PIAL2 functional fragment, the formation of SUMO chains was enhanced (Figure 12). This feature of PIAL2 was even more pronounced in the case of the *sim1* mutant, where a short Val-Phe-Asp-Leu stretch, predicted to be a SUMO interaction motif, was substituted with alanines. A mass spectrometry analysis of the SUMOylated species identified a SUMOylation event on K15 of SCE1. When this lysine was substituted with an arginine, SUMO chain formation was almost completely abolished and could only be rescued with an excess of PIAL2 (Figure 22). However, the conjugation of a single SUMO moiety to another substrate was unperturbed. An obvious conclusion was that the attachment of SUMO1 to the K15 of SCE1 was indispensable for SUMO chain formation.

On the other hand, wild-type SCE1 is able to form SUMO chains without the help of PIAL2. Therefore, it must be SUMOylated *in trans* even in the absence of the ligase. While contradicting itself at first

glance, this idea again supports the role of PIAL2 as an E4 ligase. At a "zero-stage", SCE1 is not SUMOylated and can only attach a single SUMO moiety to a substrate. When another SCE1 protein is modified with SUMO, it can start building SUMO chains. The mechanism of this switch is still unknown. And finally, when a short chain of three or four SUMOs has been made, PIAL2 can step in, presumably using its two (or more, unidentified) SUMO interacting motifs to co-ordinate the rapid extension of the SUMO chain. Whether PIAL2 actively caused the enhanced monoSUMOylation of SCE1 (Figure 21) or whether it merely acted as a scaffold, allowing SCE1 moieties to more effectively SUMOylate one another, could not be verified in this study.

Interestingly, K15 is on the opposite side of the protein relative to the active site C94 (Figure 21B). The mechanistic implications of this are unclear, but it could have an effect on the recognition of SCE1 by the SP-RING of a SUMO ligase for SUMO chain formation. It is also the basis for the proposed "*in trans"* model of autoSUMOylation of SCE1 (see above). The protein should be too small to "reach around" and SUMOylate itself *in cis*. The differentiation between mono- and polySUMOylation has been demonstrated in yeast (Klug et al., 2013). Klug *et al.* also state that not more than 1% of all SUMO E2 (Ubc9 in yeast and human) is modified with SUMO *in vivo*. Nevertheless, this fraction is important, since losing it caused meiotic defects. A similar observation was difficult to make in Arabidopsis (P. Schlögelhofer, personal communication).

Conclusion

Modern science is a puzzle, not only for the reason that it constantly pushes the boundaries of the unknown, but also because newly found data have to fit together with other information, creating a continuous pattern of knowledge. The results of the current study shed additional light on the paradigm of SUMOylation in plants. As already discussed, the initial question, how the specificity of SUMOylation to numerous substrates achieved, cannot be answered in the simple terms that this or that ligase specifically SUMOylates this or that protein. Nevertheless, the identification of two additional SUMO ligases with stress-related functions contributes to the delicate and multifaceted interplay that governs SUMOylation. Additionally, the proposed E4 activity of the ligase PIAL2, if confirmed *in vivo*, can generate a whole new set of exciting questions to tackle.

Another interesting *in vivo* study is the physiological relevance of the SUMOylation of SCE1. Do plants bearing a K15R variant of the SUMO conjugating enzyme survive under normal conditions? How do they behave when subjected to stress? Moreover, are there plant SUMO ligases without an SP-RING? Why are there so many SUMO isoforms in Arabidosis? These and many other challenging questions await future exploration.

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Appendix A: Abbreviations

ABA – abscisic acid

AP – alkaline phosphatase

ARTADE – Arabidopsis tilling-array-based detection of exons

At – *Arabidopsis thaliana*

ATP – adenosine triphosphate

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

CaMV – Cauliflower mosaic virus

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

GAP – GTPase activating protein

GTP – guanidine triphosphate

FLC – flowering locus C

HCSM – hydrophobic cluster SUMOylation motif

HPY – high ploidy

HRP – horseradish peroxidase

Hs – *Homo sapiens*

IMAC – immobilised metal ion affinity chromatography

LB – lysogeny broth
MBP – maltose binding protein

MES – 3-(N-morpholino)-ethanesulphonic acid

MOPS – 2-(N-morpholino)-propanesulphonic acid

MS – Murashige-Skoog plant medium

NAF – nucleosome assembly factor

NBT – nitro blue tetrazolium

NDSM – negatively charged amino acid-dependent SUMOylation motif

OX – overexpressor

PBS – phosphate buffered saline

PDSM – phosphorylation dependent SUMOylation motif

PEG – polyethylene glycol

PIAL – protein inhibitor of activated STAT-like

RNS – reactive nitrogen species

ROS – reactive oxygen species

SAE – SUMO activating enzyme, E1

SCE – SUMO conjugating enzyme, E2

SDS – sodium dodecyl sulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

SIZ – SAP and Miz finger domain

SP-RING – Siz/PIAS-Really Interesting New Gene

STAT – signal transducers and activators of transcription

SUMO – small ubiquitin-related modifier

TAE – Tris-acetic acid buffer

TAIR – The Arabidopsis Information Resource

TBS – Tris buffered saline

T-DNA – transfer DNA

TE – Tris-EDTA

TEMED - tetramethylethylenediamine

TSS – transformation and storage solution

YEB – yeast extract broth

βME – 2-mercapto-ethanol

Appendix B: Constructs

Name	Description
pBIN-UBI::PIAL1-YFP	C-terminal YFP fusion construct used to detect the subcellular localisation of PIAL1
pBIN-UBI::PIAL2-YFP	C-terminal YFP fusion construct used to detect the subcellular localisation of PIAL2
pET42c-NAFa	Nucleosome Assembly Factor, a known SUMOylation substrate, Flag-tagged
pET9d-SAE1c2corr	Dicistronic construct for the expression of both subunits of SUMO E1 in *E. coli*, His-tag on SAE2
pET9d-SCE1	A construct for the expression of SUMO E2, no tags
pET9d-SCE1 C94S	A dominant negative mutant of SCE1, no tags
pET9d-SCE1 K15R	A mutant of SUMO E2 where the SUMOylation site, identified by mass spectrometry, was substituted with arginine. This construct is defective in SUMO chain formation, but retains monoSUMOylation activity.
pET-Tag3-SUM1	The SUMO1 construct predominantly used for *in vitro* SUMOylation assays, carries a Strep-3xHA-8xHis tag
pET-Tag3-SUM1 H89K	SUMO1 used for mass spectrometry analysis, His 89 substituted with Lys to give a QTGG fragment after tryptic digest
pET-Tag3-SUM3	SUMO3, used for *in vitro* SUMOylation assays, carries a Strep-3xHA-8xHis tag
pET-Tag3-SUM5	SUMO5, used for *in vitro* SUMOylation assays, carries a Strep-3xHA-8xHis tag
pET-Tag3-SUM7	SUMO7, used for *in vitro* SUMOylation assays, carries a Strep-3xHA-8xHis tag
pMAL-PIAL1	N-terminal MBP fusion construct for expression and Western blot detection of the full-length PIAL1
pMAL-PIAL1M	N-terminal MBP fusion construct for expression and Western blot detection of the N264-P445 fragment of PIAL1
pMAL-PIAL1S	N-terminal MBP fusion construct for expression and Western blot detection of the S290-A353 fragment of PIAL1
pMAL-PIAL2	N-terminal MBP fusion construct for expression and Western blot detection of the full-length PIAL2
pMAL-PIAL2M	N-terminal MBP fusion construct for expression and Western blot detection of the E281-A496 fragment of PIAL2. Most of the experiments were done using this construct
pMAL-PIAL2M-K2R	A mutant of PIAL2M where the two SUMOylation sites identified by mass spectrometry (K372, K448) were substituted with arginines to test their influence on SUMO chain formation
pMAL-PIAL2M-*sim1*	A mutant of PIAL2M where the SIM interacting motif 1 (VFDL 425-428) was substituted with alanines to test its influence on SUMO chain formation
pMAL-PIAL2M-*sim1/sim2*	A double mutant of PIAL2M, combining the VFDL 425-428 AAAA and the IFDI 475-478 AAAA
pMAL-PIAL2M-*sim2*	A mutant of PIAL2M where the SIM interacting motif 2 (IFDI 475-478) was substituted with alanines to test its influence on SUMO chain formation
pMAL-PIAL2M-*sp-ring*	A mutant of PIAL2 where two Zn^{2+} coordinating cysteines (C329, C355) of the SP-RING were substituted with alanines to test its influence on SUMO chain formation
pMAL-PIAL2M-*sp-ring/sim1*	A double mutant of PIAL2M, combining the C329A, C355A and VFDL 425-428 AAAA mutations
pMAL-PIAL2M-*sp-ring/sim2*	A double mutant of PIAL2M, combining the C329A, C355A and IFDI 475-478 AAAA mutations
pMAL-PIAL2M-Δ1	A truncation of PIAL2M, encompassing S307-A496, used in the search for a putative SIM

pMAL-PIAL2M-Δ1-*sim1*	A truncation of PIAL2M, encompassing S307-A496, used in the search for a putative SIM, cloned in the *sim1* background
pMAL-PIAL2M-Δ2	A truncation of PIAL2M, encompassing E281-P466, used in the search for a putative SIM
pMAL-PIAL2M-Δ2-*sim1*	A truncation of PIAL2M, encompassing E281-P466, used in the search for a putative SIM, cloned in the *sim1* background
pMAL-PIAL2M-Δ3	A truncation of PIAL2M, encompassing E281-K448, used in the search for a putative SIM
pMAL-PIAL2M-Δ3-*sim1*	A truncation of PIAL2M, encompassing E281-K448, used in the search for a putative SIM, cloned in the *sim1* background
pMAL-PIAL2M-Δ4	A truncation of PIAL2M, encompassing E281-D414, used in the search for a putative SIM
pMAL-PIAL2S	N-terminal MBP fusion construct for expression and Western blot detection of the S307-A388 fragment of PIAL2
pTCSH1-SCE1 C94S OX	A binary vector construct used for the *in vivo* overexpression of the dominant negative mutant of SUMO E2
pTCSH1-SCE1 OX	A binary vector construct used for the *in vivo* overexpression of SUMO E2

Appendix C: Oligonucleotides

Name	Sequence 5'-3'	Purpose
438A-SIZ1endup	CCG AGT CAA TGG AGA GGT ACA TC	Genotyping of SIZ1 *in planta,* detection of WT allele
441A-PIAS1endup	TAT TAC TAA CGG GAT AGC TTG GGT	Genotyping of PIAL1 *in planta,* forward primer for detection of 1c allele
443A-PIAS1startdn	CAT TAT CTC CCC CGG AAA ACT TCT	Genotyping of PIAL1 *in planta*, forward primer for detection of 1a and WT allele
444A-PIAS1midup	GGA GAA ACC ACA TAT GCA CAA TAC T	Genotyping of PIAL1 *in planta*, reverse primer for detection of WT allele
459A-SIZ1accdn	GTG GAG GTG GAG ATG ATA ATG CC	Genotyping of SIZ1 *in planta,* detection of 1a and WT allele
479A-PIAS2endup	CCC TTG TTC TAG CCT CTC AAG TTC T	Genotyping of PIAL2 *in planta,* forward primer for detection of 2a, 2b and WT allele
480A-PIAS2startdn	GTC CGG TGG CTG GAA CTG GCT TAC	Genotyping of PIAL2 *in planta,* reverse primer for detection of WT allele
560A-o8760	GGG CTA CAC TGA ATT GGT AGC TC	Detection of GABI-KAT T-DNA insertions, binds left border of insertion
709A-Garlic LB1	GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C	Detection of Garlic/SAIL (Syngenta) T-DNA insertions, binds left border of insertion
770A-PIAS1midup2	CAT CTG CAG CAT TAC GTC CCA CTT	Genotyping of PIAL1 *in planta,* forward primer for detection of WT allele
853A-malE	GGT CGT CAG ACT GTC GAT GAA GCC	Forward primer for sequencing MBP fusion constructs, binds at insert start
1127-SALK LBa1	TGG TTC ACG TAG TGG GCC ATC G	Detection of SALK T-DNA insertions, binds left border of insertion
1128-GFPup1	GCT CTT GAA GAA GTC GTG CCG CTT C	Reverse primer for sequencing GFP/YFP fusion constructs
1286-SUMO1dn2	CCG CCC GGT ACC CAT ATG TCT GCA AAC C	Forward primer for PCR mutagenesis of SUM1 His89 to Lys for MS analysis of SUMO chains
1363-PIL1startdn2	CCC CTC GAG GGA TCC ATG GTT ATT CCG GCG ACT TCT AGG TT	Forward primer for isolation of full-length PIAL1 cDNA, has a BamHI site
1395-PIL1middn3	CCC GCC GGA TCC AAC AAA CCG TTA CTG AAA GAT TAT GTT C	Forward primer for cloning the PIAL1M construct, has a BamHI site
1396-PIL1middn4	CCC GCC GGA TCC TCG AGG ATA TCT CTC AGT TGT CCT	Forward primer for cloning the PIAL1S construct, has a BamHI site
1397-PIL1endup1	CCC GCC GTC GAC TCA CCA TGT CTC AGG AGG CAT TGA CC	Reverse primer for isolation of full-length PIAL1 cDNA, has a SalI site
1398-PIL1up2	CCC GCC GTC GAC TCA CTG TGG	Reverse primer for cloning the

	TAG TGT GTT GGT TGA AGT AT	PIAL1M construct, has a SalI site
1400-PIL2startdn1	CCC GCC GGA TCC CAT ATG TCT ACG GCG GCA GCG GCT	Forward primer for cloning the full-length PIAL2 cDNA, has a BamHI site
1401-PIL2dn2	CCC GCC GGA TCC GAA AAA CCG GTT CTT AAA GAT TAC C	Forward primer for cloning the PIAL2M construct, has a BamHI site, also used as forward primer for PIAL2M Δ2, Δ3 and Δ4
1402-PIL2dn3	CCC GCC GGA TCC TCA CGA GTA TCT CTC AGT TGC CCT	Forward primer for cloning the PIAL2S construct, has a BamHI site, also used as forward primer for PIAL2M Δ1
1403-PIL2endup1	CCC GCC GTC GAC TCA AGT TCT CCA TCA AGA TGT CGG TC	Reverse primer for cloning the full-length PIAL2 cDNA, has a SalI site
1404-PIL2up2	CCC GCC GTC GAC TCA CGC AGT GTT TCC CAA AGC AGA T	Reverse primer for cloning the PIAL2M construct, has a SalI site, also used as reverse primer for PIAL2M Δ1
1405-PIL2up3	CCC GCC GTC GAC TCA AGC ATC GAT GAT TAC ATC AGC	Reverse primer for cloning the PIAL2S construct, has a SalI site
1422-SUM1HtoKup2	CCG CCC GCG GCC GCT CAG CCA CCA GTC TGT TTG AGC ATC GCA TCG ATC	Reverse primer for PCR mutagenesis of SUM1 His89 to Lys for MS analysis of SUMO chains
1424-PIL1up4	CCC GCC GTC GAC TCA AGC AGA GAT AAC CAC ATC TGC AGC ATT	Reverse primer for cloning the PIAL1S construct, has a SalI site
1425-T7term	GCT AGT TAT TGC TCA GCG G	Reverse primer for sequencing of inserts in the pET vectors
1445-PIL1middn5	CTC AGC CGA ACG CGT ATC AAA CT	Forward primer for sequencing across exon 14 of PIAL1
1473-M13F	CCA GGG TTT TCC CAG TCA CG	Reverse primer for sequencing MBP fusion constructs and inserts in the pCR 2.1 vector
1474-M14R	CGG ATA ACA ATT TCA CAC AGG	Forward primer for sequencing inserts in the pCR 2.1 vector
1479-SAS1f	GAT CAA AAC ATG GCC AGG ATA TTA AAA GAT G	Forward primer for site directed mutagenesis of PIAL2 K372R for removal of the SUMOylation site
1480-SAS1r	CAT CTT TTA ATA TCC TGG CCA TGT TTT GAT C	Reverse primer for site directed mutagenesis of PIAL2 K372R for removal of the SUMOylation site
1481-SAS2f	GGT TGA GGA CCG GAG GCC CTG TAT GTC TG	Forward primer for site directed mutagenesis of PIAL2 K448R for removal of the SUMOylation site
1482-SAS2r	CAG ACA TAC AGG GCC TCC GGT CCT CAA CC	Reverse primer for site directed mutagenesis of PIAL2 K448R for removal of the SUMOylation site
1483-SIMf2	CTT ATT AAA CTC TGG TCC TGT TGC TGC AGC TGC TAC GGG GGA TGA TGA TGC	Forward primer for site directed mutagenesis of PIAL2 VFDL 425-428 AAAA for removal of SUMO interacting motif 1
1483-SIMr2	GCA TCA TCA TCC CCC GTA GCA GCT GCA GCA ACA GGA CCA GAG	Reverse primer for site directed mutagenesis of PIAL2 VFDL 425-428

	TTT AAT AAG	AAAA for removal of SUMO interacting motif 1
1511-P2SPRING1	GTC AAG GGC CAG TTA GCT AAA CAT CTT CAG	Forward primer for site directed mutagenesis of PIAL2 C329A for inactivation of SP-RING
1512-P2SPRING2	CTG AAG ATG TTT AGC TAA CTG GCC CTT GAC	Reverse primer for site directed mutagenesis of PIAL2 C329A for inactivation of SP-RING
1513-P2SPRING3	CGC TGC CCG CAT GCT AAT CAA CCT GTT TG	Forward primer for site directed mutagenesis of PIAL2 C355A for inactivation of SP-RING
1514-P2SPRING4	CAA ACA GGT TGA TTA GCA TGC GGG CAG CG	Reverse primer for site directed mutagenesis of PIAL2 C355A for inactivation of SP-RING
1541-PIL2up4	CCC GCC GTC GAC TCA ATG TTT ATT TGT GTT GTT ATT ATT AGA TT	Reverse primer for cloning of PIAL2M Δ2
1542-PIL2up5	CCC GCC GTC GAC TCA CTT CCG GTC CTC AAC CTT GTT GTC	Reverse primer for cloning of PIAL2M Δ3
1543-PIL2up6	CCC GCC GTC GAC TCA GTC TTC TAG ATC ATG AAT GAT CTC	Reverse primer for cloning of PIAL2M Δ4
1564-PIAL1Apa-stdn	CCC ACC GGG CCC ATG GTT ATT CCG GCG	Forward primer for inserting PIAL1 behind a ubiquitin promoter, fused with YFP
1565-PIAL1Not-dst	CCC ACC GCG GCC GCG CCA TGT CTC AGG AGG	Reverse primer for inserting PIAL1 behind a ubiquitin promoter, fused with YFP
1566-PIAL2Apa-stdn	CCC ACC GGG CCC ATG TCT ACG GCG GCA G	Forward primer for inserting PIAL2 behind a ubiquitin promoter, fused with YFP
1567-PIAL2Not-dst	CCC ACC GCG GCC GCG AGA TGT CGG TCC AGT	Reverse primer for inserting PIAL2 behind a ubiquitin promoter, fused with YFP
1614-PIAL2sim2F	TCA AAC GAT GAT TAC TCT TCG GCA GCT GCT GCC TCT GAT GTG ATC GCA CTT GAC	Forward primer for site directed mutagenesis of PIAL2 VFDL 475-478 AAAA for removal of SUMO interacting motif 2
1615-PIAL2sim2R	GTC AAG TGC GAT CAC ATC AGA GGC AGC AGC TGC CGA AGA GTA ATC ATC GTT TGA	Reverse primer for site directed mutagenesis of PIAL2 VFDL 475-478 AAAA for removal of SUMO interacting motif 2
1618-SCE1K15R-fwd	CGT TTA GCT GAA GAG AGG AGA TCG TGG AGG AAG AAT CAT	Forward primer for site directed mutagenesis of SCE1 K15R for removal of SUMOylation site
1619-SCE1K15R-rev	ATG ATT CTT CCT CCA CGA TCT CCT CTC TTC AGC TAA ACG	Reverse primer for site directed mutagenesis of SCE1 K15R for removal of SUMOylation site

Appendix D: Fresh weight of seedlings grown on different stress conditions, raw data

Untreated plants

Col-0	siz1	pial1a	pial1c	pial2a	pial2b	pial1a pial2b	pial1c pial2a	pial1a siz1	pial1c siz1	pial2a siz1	pial2b siz1	pial1a pial2b siz1	pial1c pial2a siz1
18,7	9,6	11,7	17,1	20,6	13,9	5,2	6,5	8,6	27,8	4,1	21,3	18,0	4,4
5,3	8,8	6,4	20,0	10,2	27,5	16,9	13,0	9,4	7,9	2,3	23,3	14,0	23,1
29,8	17,1	6,2	7,3	25,4	12,4	6,3	12,9	3,2	19,6	3,0	15,6	12,4	1,7
26,4	11,8	19,9	9,4	6,3	17,0	5,9	6,2	5,4	9,4	12,1	11,5	3,2	14,7
8,2	14,4	3,7	9,6	2,8	5,8	4,8	4,0	8,0	8,1	2,6	11,4	8,7	7,4
13,3	11,3	21,0	24,7	4,6	14,8	2,4	5,2	5,8	8,4	6,3	16,7	6,2	12,7
14,6	13,8	24,1	6,7	3,7	30,5	9,7	6,3	29,6	20,3	11,0	6,1	14,9	26,7
6,0	11,5	4,9	5,6	3,8	5,4	8,1	10,9	18,9	3,1	4,9	4,8	5,2	2,3
31,4	6,8	6,7	4,6	11,6	1,4	16,4	5,7	5,8	7,9	8,1	12,0	11,4	21,8
6,5	13,1	5,1	2,6	15,9	8,2	2,5	3,5	6,1	4,0	3,1	16,3	11,3	23,1
7,2	16,8	3,3	8,2	7,4	7,3	3,8	4,2	22,1	7,2	2,9	14,3	20,0	23,5
8,5	14,7	24,4	10,9	3,2	5,2	6,8	8,3	7,0	1,4	4,8	11,7	5,9	2,8
9,6	7,7	6,4	7,9	2,5	14,0	15,5	8,7	5,1	2,9	4,5	4,4	4,3	20,2
6,3	24,5	5,5	19,6	3,0	11,0	17,7	5,4	7,9	14,2	7,6	1,3	8,9	11,7
6,6	4,1	11,2	9,2	2,8	6,0	8,4	13,2	5,2	2,6	6,5	2,3	8,9	5,5
20,6	6,2	20,4	7,5	1,1	10,8	3,5	11,6	7,7	9,3	4,2	7,4	4,5	9,4
9,0	2,7	17,1	7,8	0,6	10,6	8,2	9,0	5,0	15,0	4,0	1,2	12,1	6,5
8,5	23,1	14,3	3,9	4,0	5,7	7,3	21,3	2,4	6,4	2,3	17,2	13,5	21,5
25,0	20,4	6,5	9,9	2,0	2,9	11,5	9,0	2,0	6,4	2,3	17,2	13,5	21,5
10,8	3,2	8,0	7,1	3,6	4,1	5,9	6,3	2,2	5,1	10,0	16,0	4,1	12,2
11,7	14,1	4,4	11,4	4,6	14,4	8,5	14,5	2,5	7,5	9,2	13,4	9,6	8,3
6,2	5,2	10,7	8,8	5,6	2,5	13,7	4,3	1,4	12,2	7,2	11,2	11,4	11,9
31,8	5,8	7,6	4,3	14,5	8,1	9,4	1,7	2,0	11,4	9,8	17,9	3,1	5,9
22,3	17,3	11,7	19,0	15,5	15,4	6,6	4,2	4,1	5,1	2,3	2,8	1,5	10,6
9,1	7,5	4,4	6,2	6,1	14,3	24,1	30,7	7,5	4,9	10,5	4,1	13,5	4,4
2,3	11,9	16,2	13,0	7,8	17,8	5,6	10,2	6,6	7,1	4,3	4,6	3,8	9,0
22,5	5,1	5,6	8,8	4,1	10,3	17,8	19,4	9,1	5,4	3,4	10,0	3,7	11,6
29,9	10,6	17,7	9,4	12,5	6,6	5,9	13,9	1,5	4,9	1,4	2,9	4,7	11,7
7,5	5,0	6,2	3,8	7,4	13,1	15,5	10,3	12,0	16,8	4,2	8,6	10,5	3,2
4,0	1,9	6,1	8,5	17,3	7,3	3,4	4,3	6,8	0,7	0,8	22,4	7,8	2,0
9,1	10,2	3,8	5,8	15,1	10,2	21,7	1,9	7,1	2,7	1,7	19,0	4,5	1,7
6,6	6,1	4,2	9,2	15,5	8,0	2,5	15,4	13,7	9,0	5,0	1,8	9,3	8,8
7,2	7,8	9,0	7,4	9,9	8,6	14,3	7,0	2,0	8,9	2,0	3,3	7,2	12,1
5,2	0,8	11,4	15,3	10,1	8,0	3,1	5,5	4,0	1,5	6,0	9,0	13,7	23,6
6,9	5,8	6,5	6,9	14,8	14,7	3,6	8,2	10,7	17,0	6,8	8,8	1,1	8,9
30,3	7,1	7,5	11,3	5,6	8,5	13,3	9,3	15,2	3,8	13,9	8,9	1,9	3,8
20,9	24,3	6,2	5,6	10,9	10,9	2,5	4,6	8,4	11,4	2,1	6,9	21,6	1,8
11,6	11,2	12,4	11,3	21,3		8,1	1,3	15,3	5,5	8,1	11,6	0,8	2,1

12,6	10,3	10,6	14,3	7,3	7,4	3,6	9,6	4,8	3,1	3,0	2,4	5,9
7,0	16,3	26,5	2,2	11,3	4,3	4,2	5,1	11,0	3,5	4,1	9,9	5,5
9,7	18,0	10,7	6,7	3,5	11,5	14,8	6,4	2,6	6,6	16,9	3,2	12,5
5,0	8,9	6,5	1,4	2,8	4,4	4,5	8,9	5,8	12,1	4,6	1,7	5,6
3,1	5,3	10,0	6,3	2,3	3,0	7,8	14,3	10,5	2,2	1,4	3,3	5,2
7,4	1,7	5,6	12,2	14,1	12,3	6,8	6,3	2,3	3,0	20,6	1,4	20,1
16,2	11,3	1,5	6,1	17,7	12,0	2,3	10,4	3,0	8,5	14,3	24,1	3,3
9,9	12,1	1,6	10,0	9,3	2,2	12,4	6,6	2,2	2,3	8,8	9,1	10,6
4,1	15,8	0,5	16,4	10,7	3,0	2,1	8,9	10,7	7,0	6,5	11,1	7,5
20,3	11,1	17,7	9,6	14,7	5,4	7,3	12,1	2,2	8,9	1,0	3,4	7,0
12,3	22,5	1,7	17,2	19,6	1,1	3,0	17,2	5,7	4,8	12,6	6,9	4,2
9,7	1,8	6,6	6,8	12,8	19,4	20,9	8,3	10,6	4,5	7,9	10,4	9,3
1,3	2,4	11,9	7,8	6,1	11,1	11,6	5,3	11,4	12,0	13,8	10,8	7,5
5,1	10,5	2,6	6,7	16,7	7,3	5,7	21,4	5,1	8,3	7,9	7,1	11,0
3,9	7,7	13,1	8,6	1,8	10,0	11,6	5,3	1,9	6,5	15,4	8,6	2,0
2,8	6,5	7,8	15,5	2,8	3,3	6,6	5,3	3,5	5,3	7,2	2,0	11,1
8,4	7,3	10,3	6,0	1,1	2,5	1,7	4,6	9,4	6,2	12,0	1,7	18,4
13,1	2,7	6,4	16,5	5,5	6,7	4,6	1,0	4,1	7,0	4,4	8,4	5,6
5,3	12,6	8,2	7,7	0,8	8,4	9,5	13,6	12,9	2,6	5,0	23,7	12,6
4,2	9,1	6,0	4,8	6,3	9,2	9,0	7,8	5,9	11,4	14,0	27,4	3,5
11,3	5,5	8,5	4,8	3,2	8,5	3,8	11,2	4,3	2,7	11,2	21,6	7,8
9,6	8,4	3,3	11,1	2,1	7,3	6,6	1,9	2,1	1,7	4,3	3,3	2,8
8,8	5,6	7,2	9,0	9,3	23,1	0,8	12,7	3,8	6,0	2,7	8,6	5,0
	19,0	7,8	16,8	4,6	11,1	5,3	12,0	7,7	1,6	7,2	8,4	7,1
	3,6	3,9	7,8	13,1	4,5	12,8	5,1	4,1	2,4	13,3	1,5	31,6
	8,2	5,3	14,6	2,9	2,5	11,2	5,2	4,1	3,0	7,4	14,5	16,1
	4,0	7,2	13,1	4,5	3,1	3,6	8,6	3,5	7,3	4,1	13,4	14,3
	11,0	13,6	9,4	6,2	1,8	8,6	7,3	14,5	6,8	0,8	8,7	6,0
	9,0	13,8	9,8	10,0	13,1	6,1	2,3	14,2	7,3	4,6	9,5	20,5
	7,3	5,7	2,0	12,7	7,5	5,8	15,2	20,9	7,1	3,4	12,4	6,7
	4,7	4,2		12,3	7,0	6,0	6,3	1,8	5,0	6,8	1,7	12,6
	11,9	3,1		4,0	15,8	7,8	5,0	9,8	6,4	11,5	0,9	7,0
	4,5	1,1		2,6	8,2	9,8	13,0	12,0	5,0	6,0	4,5	3,3
	11,9	0,9		10,4	16,6	4,8	13,8	2,8	8,6	1,3	4,4	12,8
	11,7			1,6	11,7	5,9	12,6	3,0	10,3	6,8	2,1	8,7
	20,3			2,6	16,1	7,6	6,0	2,4	12,5	2,4	2,8	8,5
	7,3			3,2	11,2	1,5	5,5	7,0	3,3	1,2	6,5	16,6
	12,0			10,8	10,4	1,4	2,7	4,1	6,5	3,3	1,5	4,3
	7,1			6,3	24,4	4,6	3,8	5,0	8,7	7,9	17,3	2,4
	5,3			1,6	3,1	11,9	2,4	2,9	11,3	8,2	2,4	15,4
	3,8			2,8	18,7	5,5	1,6	2,3	5,7	9,0	23,0	5,8
	5,9			17,8	3,6	8,4	4,6	1,6	4,1	7,8	18,2	9,4
	10,1			4,3	6,6	7,3		1,2	6,2	1,9	3,5	18,2
	4,9					9,8		3,3	6,8	5,4	13,5	10,0
	3,0					2,0		1,7	7,8	11,7	3,6	14,4
	4,1					4,5		2,4	4,8	0,6	6,3	2,6

9,4	5,5	10,5	11,0	7,2	3,5	13,8
2,8	4,2	4,5	13,9	5,3	6,4	9,9
	2,3	0,9	12,7	8,3	2,7	4,0
	3,2	29,2	11,6	12,8	0,6	24,4
	7,7	2,8	8,2	4,9	3,0	14,0
	1,3	9,0	7,5	5,5	2,9	4,7
	1,9	16,8	7,1	5,5	7,8	7,6
	9,5	10,0	5,6	6,5	4,0	5,5
	2,1	6,5	4,7	11,3	4,5	6,7
	1,8	1,4	5,7	5,0	2,0	10,1
	1,9	1,5	3,9	15,3	1,0	2,1
	1,4	0,1	9,7	6,5	1,3	16,8
	3,0		10,5	3,8	1,0	10,3
	4,7		6,4	5,3	4,4	17,4
	6,9		8,7	3,7	1,4	10,0
	2,2		3,7	3,5	2,0	4,7
			2,8	7,5	5,7	5,5
			1,5	5,0		0,7
			5,2	2,9		13,2
			5,5	9,2		4,5
			2,5	6,0		12,8
			2,1	6,7		9,0
			7,2	7,5		3,5
			13,2	2,0		6,5
			10,0	3,8		6,4
			6,7	1,4		10,7
			13,3	6,7		5,9
			5,7	2,1		3,7
			3,2	1,8		3,8
			2,1	1,7		10,6
			9,0	1,3		11,4
			6,3	1,3		16,6
			4,3	0,4		5,4
			10,7	0,3		11,8
			9,5			7,3
			6,0			6,2
			2,7			7,1
			6,2			2,4
			3,1			3,9
			7,1			9,1
			3,8			9,3
			1,8			8,8
			1,6			7,3
						11,5
						4,6
						5,4

													3,4
													12,0
													11,7
													3,4
													5,4
													3,3
													8,3
													11,7
Non-germinated seeds:													
31	24	44	30	45	20	36	38	18	29	28	43	57	40
Plants:													
60	85	71	67	80	36	80	99	79	95	126	117	100	137

150 mM NaCl

Col-0	siz1	pial1a	pial1c	pial2a	pial2b	pial1a pial2b	pial1c pial2a	pial1a siz1	pial1c siz1	pial2a siz1	pial2b siz1	pial1a pial2b siz1	pial1c pial2a siz1
18,4	44,1	10,1	28,7	17,5	3,0	30,2	61,3	16,6	13,4	4,3	22,6	3,6	19,3
16,7	24,5	6,4	34,2	56,7	37,3	36,2	53,1	28,4	12,6	14,6	13,9	3,2	14,1
2,2	26,8	12,2	15,3	53,4	18,2	23,1	23,3	10,7	11,8	5,1	4,4	1,7	11,1
35,6	10,4	14,5	13,3	24,6	48,9	49,5	40,7	15,9	6,3	17,1	20,2	2,2	10,5
15,2	25,0	29,9	15,9	19,4	25,2	35,4	25,6	3,0	9,2	6,8	13,2	8,1	5,3
27,9	15,4	2,5	15,5	32,3	40,6	31,3	15,5	9,8	8,1	35,0	16,6	11,5	11,4
6,5	19,4	16,4	11,6	13,4	45,6	15,5	38,8	5,1	2,0	14,1	11,7	7,8	23,1
4,9	18,7	11,4	33,5	29,3	21,1	13,2	20,6	7,5	12,8	8,8	15,7	7,1	10,1
11,3	17,8	24,9	21,6	35,2	48,4	35,3	31,6	10,5	1,2	1,5	9,1	2,0	11,1
6,5	39,3	11,2	28,9	40,2	65,3	44,8	4,8	2,1	7,5	28,4	3,2	8,7	14,9
25,0	35,3	30,2	16,3	32,5	40,1	32,4	33,3	1,6	5,4	18,2	8,6	14,9	19,1
7,1	37,3	10,0	12,1	9,9	29,1	41,3	8,9	2,3	16,2	3,2	1,9	13,1	9,9
42,4	13,7	10,1	14,6	27,8	10,2	32,3	27,1	14,5	3,1	12,7	14,6	3,0	9,0
33,6	7,7	4,8	24,3	46,5	34,7	24,7	23,5	4,9	21,8	19,5	4,1	2,2	11,5
21,1	3,5	27,8	12,7	11,7	7,0	41,1	30,2	18,7	5,8	6,7	5,9	11,9	19,2
3,8	19,0	17,2	38,8	9,4	37,9	57,0	43,0	6,6	9,9	13,0	13,9	1,5	12,4
6,0	10,7	33,4	13,0	18,2	16,5	11,9	45,5	9,3	6,1	19,4	20,2	7,7	18,9
9,3	4,5	14,2	13,3	64,3	32,1	24,0	13,6	6,0	18,1	19,4	15,5	13,0	9,4
7,6	27,8	19,5	20,4	14,4	75,4	22,7	27,5	28,0	14,5	20,3	16,6	12,0	10,6
7,8	11,8	13,3	8,9	19,4	60,2	28,4	41,4	20,7	19,7	15,2	9,3	7,3	14,9
10,6	35,9	3,1	22,4	34,4	39,5	29,0	30,8	10,1	14,7	22,4	20,1	16,5	5,9
14,7	25,8	6,1	21,7	20,5	64,4	20,8	44,0	24,6	10,7	13,9	7,4	6,0	4,2
2,3	21,5	3,8	23,4	40,3	30,5	35,6	44,0	15,1	4,5	19,6	20,4	5,6	14,5
16,4	24,3	9,0	25,8	4,7	25,7	24,5	29,7	17,0	24,5	15,0	7,1	3,0	5,3
18,0	27,6	9,3	13,1	7,4	35,3	25,5	24,8	21,0	7,0	1,7	9,7	4,0	4,8

53,6	34,8	4,3	27,5	14,5	44,8	46,6	19,0	6,7	14,9	17,4	12,2	1,3	2,3
21,8	49,7	19,6	40,4	44,6	65,9	17,5	47,9	15,7	20,2	14,6	3,8	1,2	3,7
24,3	1,0	4,3	10,3	34,6	57,4	43,0	20,7	14,8	10,8	12,7	13,8	7,4	13,1
1,6	0,5	10,3	3,3	56,3	34,6	34,6	24,7	9,1	17,1	9,1	8,7	3,6	2,3
3,8	9,3	5,3	38,2	21,3	44,5	15,2	32,7	17,8	13,8	6,8	16,3	2,2	6,6
6,0	13,8	8,0	34,3	36,2	16,0	10,7	40,1	19,2	17,6	22,0	4,7	0,6	4,6
13,9	16,6	7,7	12,8	20,6	50,1	29,7	14,7	7,3	12,9	16,5	7,5	3,3	8,3
7,3	15,1	29,5	55,7	24,6	14,3	21,1	23,2	26,3	11,3	3,7	16,1	5,9	12,4
16,5	17,0	5,8	30,6	27,8	46,5	9,4	28,7	10,6	7,3	9,6	10,2	2,9	12,1
13,3	1,5	10,1	26,8	11,5	40,5	5,6	20,7	16,6	9,2	22,0	17,1	3,4	15,7
22,1	6,6	10,3	27,9	1,3	18,1	21,2	25,5	15,3	8,4	23,0	4,6	1,8	8,1
13,0	1,1	14,8	3,1	15,6	29,7	24,1	27,5	6,3	4,8	22,3	12,5	0,9	9,2
7,8	13,3	14,3	9,1	37,3	31,5	6,5	10,9	10,5	25,1	12,2	1,1	9,2	2,8
9,6	3,0	7,3	25,1	26,0	27,6	6,5	25,6	7,4	5,0	4,5	17,1	8,2	14,6
3,5	21,2	1,0	18,5	61,5	3,8	27,7	20,4	5,8	8,6	8,6	21,2	1,4	6,9
3,1	7,6	11,5	15,3	53,0	6,8	14,0	38,8	11,1	23,5	12,5	19,6	0,9	14,7
1,3	1,0	10,1	20,0	27,7	32,8	32,8	24,6	9,9	11,3	15,4	18,8	2,7	11,1
1,0	21,5	15,2	22,2	30,3	31,7	43,9	24,3	12,4	10,3	21,8	12,4	0,7	13,0
0,7	5,6	5,3	20,5	14,4	9,8	24,4	37,6	9,2	5,0	7,4	6,6	0,8	13,0
1,4	16,9	8,0	23,5	33,6	30,7	33,6	37,9	9,5	5,0	2,6	5,1	1,2	11,7
1,2	31,7	20,4	29,7	0,8	17,1	17,1	28,8	10,2	10,8	16,4	17,8	4,1	6,6
1,6	30,6	28,7	1,5	9,2	14,6	21,3	6,2	10,0	14,1	16,4	12,5	1,3	10,4
1,3	14,4	3,4	31,8	27,3	3,6	41,0	13,1	16,5	17,9	7,8	20,1	0,8	10,6
0,9	12,4	9,3	12,8	52,7	6,3	39,3	18,8	12,4	19,1	8,3	14,4	0,5	8,9
0,9	0,9	28,7	51,2	13,6		25,0	22,9	13,5	11,7	20,3	18,2	1,5	17,3
1,1	19,4	27,8	43,3	44,4		14,0	34,5	8,2	19,6	12,6	14,2	1,3	14,6
1,7	15,2	7,7	12,3	61,9		23,0	33,8	15,1	11,4	6,4	22,3	0,1	15,4
	1,3	11,5	26,8	4,5		28,1	29,8	29,5	4,1	12,6	13,7	0,8	16,6
	6,2	52,2	3,8	26,7		40,5	29,1	8,0	8,6	15,1	18,6	0,7	7,2
	15,2	1,5	18,1	14,0		28,3	32,0	16,7	3,9	1,3	21,8	0,2	13,4
	3,9	2,4	11,9	26,4		39,4	21,5	20,8	18,4	7,0	13,1		12,5
	3,0	17,4	11,3	9,5		22,7	15,9	9,0	5,7	12,2	17,0		13,7
	1,0	7,6	25,3	0,5		19,0	27,3	18,3	13,1	22,5	7,7		11,5
	1,2	8,2	33,2	0,3		49,3	17,0	6,5	12,9	14,5	8,7		10,7
	1,1	9,0	7,2			46,0	16,0	14,2	19,5	15,1	15,5		12,5
		3,2	10,7			11,0	15,7	9,3	12,7	4,8	4,3		2,8
		9,3	11,3			22,4	17,1	0,8	21,1	3,3	8,8		9,5
		1,1	1,5			32,7	27,7	12,2	9,8	8,6	13,8		10,4
		11,3	13,1			21,5	23,6	7,0	12,0	7,0	9,1		12,0
		12,5	3,2			26,0	25,2	7,9	15,8	6,7	8,1		8,4
		16,4	21,5			41,4	18,9	13,8	9,8	14,2	11,0		11,6
		4,0	4,5			18,5	3,8	6,2	37,1	4,3	2,2		5,7
		0,9	24,0			15,9	12,5	20,4	12,7	6,5	1,5		9,6
		1,1	9,3			10,1	19,2	9,4	4,3	14,0	13,1		14,8
		1,3	25,8			41,3	25,3	6,3	6,2	8,0	8,9		5,8
		0,7	2,3			31,2	3,5	1,1	23,1	1,0	8,9		6,7

17,9	30,6	24,7	0,8	14,6	15,4	6,9	7,3
19,7	48,4	15,2	6,1	8,6	11,2	1,4	7,5
21,8	31,3	3,8	15,7	17,1	14,5	6,0	12,8
21,5	9,8	16,3	13,6	15,6	8,5	6,3	5,8
9,2	3,3	23,6	23,6	13,7	15,8	0,5	0,8
9,1	8,8	42,4	6,2	8,0	7,2	3,4	16,3
18,9	15,3	26,0	12,8	6,5	24,4	7,0	14,5
13,4	19,5	37,4	15,8	5,0	22,9	9,7	2,4
21,3	34,0	19,6		5,0	2,0	9,9	6,4
27,7	43,7	43,9		13,2	1,8	12,0	7,4
25,3	21,9	35,5		16,2	10,0	8,7	10,5
	13,1	21,2		21,2	0,8	6,2	7,9
	13,1	30,3		11,3	7,5	4,5	13,5
	15,9	18,7		11,0	13,5	3,8	1,2
	14,7	32,1		11,0	9,6	5,3	13,1
	22,7	5,1		14,3	13,5	12,2	11,7
	31,1	27,3		17,4	12,7	7,0	6,1
	10,9	28,6		15,3	9,6	12,3	9,4
	22,0	12,2		8,7	5,0	19,8	8,5
	4,1	15,1		15,3	5,5	12,6	3,3
	35,5	27,0		14,4	17,8	14,4	8,5
		8,7		14,2	2,4	11,5	16,7
		44,8		15,5	9,7	7,5	18,0
		36,4		15,4	7,1	5,8	9,8
		53,0		14,9	6,8	4,7	11,7
		26,2		11,4	8,9	12,8	8,9
		23,5		13,5	12,1	7,0	11,8
		21,5		13,6	11,1	13,9	8,9
		32,2		10,8	3,3	18,9	11,2
		16,1		11,3	12,2	7,0	10,0
		36,0		1,0	10,6	9,8	8,8
		17,1		10,5	11,3	12,1	9,2
		30,8		11,7	15,6	7,5	5,0
		22,1		6,1	10,7	9,4	2,1
		16,2		10,1	7,6	9,1	1,5
		28,6		19,9	16,3	6,9	1,5
		7,1		14,0	15,8	8,7	3,6
				8,7	2,2	14,6	3,0
				4,3	13,5	0,5	1,7
					10,5	4,8	1,4
					4,5	4,2	1,2
					10,3	10,7	2,4
					5,1	1,8	
					3,0	4,6	
					5,7	9,5	
					4,8	14,5	

6,7	13,7
11,9	10,9
5,0	16,8
15,2	1,8
5,2	2,5
4,5	9,9
10,6	6,0
9,4	2,3
2,5	1,1
9,7	7,1
1,1	4,8
2,5	2,1
20,5	1,4
11,8	0,2
6,0	
10,9	
1,4	
14,5	
12,4	
9,1	
13,7	
5,7	
7,8	

Non-germinated seeds

39	29	54	51	49	35	34	23	36	31	29	46	60	41

Plants:

51	59	70	81	58	48	91	107	78	109	139	130	54	112

Notes:

Genotypes 1-7 had branched roots, 8-14 had a single root

Genotype 13 had tiny leaves and almost all of the biomass was comprised by the root

300 mM mannitol

Col-0	siz1	pial1a	pial1c	pial2a	pial2b	pial1a pial2b	pial1c pial2a	pial1a siz1	pial1c siz1	pial2a siz1	pial2b siz1	pial1a pial2b siz1	pial1c pial2a siz1
7,5	0,6	6,2	4,0	1,7	1,3	7,3	2,6	0,3	0,4	3,4	1,6	1,1	2,6
8,4	4,0	4,5	6,1	5,4	2,2	5,7	6,4	1,4	2,5	4,8	1,5	2,1	2,4
7,2	2,1	7,5	4,6	5,1	3,5	4,2	3,8	5,3	2,1	4,2	5,1	3,1	2,2
4,1	2,4	2,5	2,8	0,1	4,2	3,2	6,1	2,3	3,6	3,5	2,0	2,4	3,1
5,3	1,9	5,5	6,4	5,1	3,2	1,1	2,1	1,1	1,0	2,5	2,8	3,6	1,8

5,5	1,8	3,3	4,8	7,9	6,2	1,7	3,7	0,5	2,6	2,4	3,4	1,0	1,4
6,3	2,4	5,8	6,6	3,5	4,4	3,1	3,2	0,4	1,7	0,2	0,9	0,4	2,1
8,1	3,6	0,5	5,4	3,3	5,6	3,2	4,1	2,3	3,6	2,8	0,5	1,0	2,5
8,4	1,9	4,3	1,2	3,1	1,5	2,0	2,6	2,2	1,8	3,5	0,6	0,1	3,0
8,6	2,4	4,0	7,2	3,7	4,6	2,9	4,6	0,7	1,6	2,3	0,2	1,4	2,8
2,4	2,1	2,7	5,1	1,6	3,5	1,3	5,3	1,3	2,4	0,6	0,3	1,3	2,3
6,4	1,9	4,6	3,6	6,7	2,0	1,5	1,0	4,5	2,6	4,4	1,8	3,9	4,0
4,5	1,4	0,3	7,2	3,0	0,2	0,3	4,3	1,7	0,8	2,3	0,5	2,1	1,2
1,9	1,9	0,4	1,9	2,5	2,0	0,2	4,2	0,8	1,2	1,9	1,5	1,7	3,2
6,5	2,8	0,2	4,5	2,9	3,7	2,6	1,5	1,9	1,9	3,3	1,3	2,1	3,1
2,9	2,9	0,7	4,0	1,3	2,5	3,7	0,7	3,9	3,2	1,9	0,7	1,3	2,7
2,9	2,9	0,2	2,2	4,6	3,0	1,6	0,3	3,4	0,3	4,3	0,7	3,3	2,7
6,0	2,4	0,2	5,8	7,7	4,6	1,5	2,3	2,3	3,1	2,2	0,3	1,1	1,1
1,4	2,8	4,2	3,6	3,1	5,0	1,4	3,0	3,3	3,4	2,8	0,9	0,2	1,8
8,0	3,5	5,2	6,7	5,4	2,8	2,1	2,1	0,3	1,8	5,4	0,4	0,9	2,0
6,0	4,6	6,0	5,5	3,9	5,4	3,8	2,8	0,4	4,0	2,2	1,3	1,2	3,1
6,3	2,8	3,4	3,3	4,6	2,1	2,9	1,6	0,4	1,0	1,8	1,0	0,9	1,5
9,5	2,2	1,3	5,8	0,7	1,9	4,3	2,8	3,5	2,2	2,2	1,8	1,1	2,4
5,8	2,2	3,6	8,5	3,1	2,4	5,3	1,2	3,1	0,6	0,2	1,5	1,5	1,3
2,5	3,5	6,9	5,8	3,3	1,5	5,1	1,3	1,0	2,0	2,9	0,4	0,9	0,5
3,7	2,4	6,7	4,9	0,8	1,8	4,9	1,1	0,6	1,6	3,2	1,6	0,1	2,3
5,0	4,0	3,0	5,4	1,8	1,5	4,9	4,0	2,5	2,1	2,9	1,9	0,6	2,6
8,3	1,0	4,3	3,8	1,5	1,7	4,2	1,9	1,7	1,1	2,9	1,6	1,2	0,9
5,9	2,7	2,4	3,4	2,5	1,2	2,3	0,9	3,2	2,0	2,0	0,2	0,5	1,8
8,1	2,2	1,2	3,1	2,8	2,6	2,5	3,2	2,5	1,1	0,1	1,3	3,1	2,8
5,1	2,7	9,3	7,2	2,8	1,4	5,3	1,7	3,8	1,5	3,0	1,6	0,2	2,5
8,0	3,1	3,8	3,3	3,9	3,2	3,8	9,3	2,7	9,4	2,1	2,3	9,5	1,6
0,6	3,3	4,5	3,2	2,4	4,3	4,6	3,4	0,3	1,6	3,3	1,2	1,1	1,1
2,0	3,5	6,2	3,9	1,8	3,3	2,2	0,5	1,4	1,2	2,7	0,5	0,8	2,7
6,4	3,7	4,7	7,6	1,2	2,5	0,3	0,3	0,7	1,7	3,4	0,7	0,9	1,6
8,5	2,1	6,5	1,4	8,5	7,0	1,6	0,2	3,2	1,1	1,7	4,9	1,3	1,0
6,1	3,8	4,6	5,7	2,0	1,2	3,2	2,0	1,0	1,2	3,0	3,4	1,2	1,6
4,4	2,4	2,6	2,4	2,6	3,7	2,8	1,2	1,2	1,8	3,1	2,1	0,2	2,0
4,5	4,7	2,6	3,7	5,8	2,6	1,7	2,0	0,1	1,6	2,5	1,4	1,1	1,0
5,7	2,7	1,7	1,2	5,1	3,3	1,2	6,3	2,0	0,8	4,8	1,0	0,4	2,6
6,6	3,6	3,5	3,5	1,4	5,0	3,6	2,2	1,3	0,9	3,1	0,4	1,1	0,2
6,9	0,7	3,5	3,8	4,0	3,5	1,2	2,2	0,2	0,5	2,8	0,7	0,5	1,6
7,7	1,5	4,9	5,5	3,1	1,2	1,9	1,3	2,6	1,7	2,6	2,4	1,5	1,1
0,4	3,5	4,6	6,1	5,1	1,0	3,4	1,2	1,4	0,6	2,9	1,3	1,2	1,8
1,8	2,7	4,8	6,8	3,6	0,7	3,4	3,4	2,2	1,6	1,7	1,2	1,3	2,4
0,5	3,0	5,7	3,3	3,3	3,3	1,3	1,3	1,0	1,5	1,7	1,1	0,9	2,3
0,8	5,3	2,6	1,4	6,6	3,2	2,3	2,5	2,8	1,3	0,8	5,1	1,6	1,6
1,1	5,2	0,6	7,3	1,3	3,9	2,7	2,5	2,0	1,0	1,8	0,7	0,8	0,9
	4,4	1,8	3,0	6,4	3,0	1,5	0,5	2,3	2,5	4,9	2,0	0,6	1,2
	5,8	4,5	5,1	5,2	5,0	3,1	2,3	0,6	1,0	2,2	1,8	0,3	0,4
	1,4	4,1	2,8	1,5	4,5	2,4	2,0	1,2	1,8	2,7	1,8	0,7	0,2

3,3	4,9	3,2	2,0	2,7	0,4	1,7	1,1	1,2	2,2	3,1	0,9	1,7
5,1	4,0	2,5	0,3	4,2	6,2	1,9	4,4	1,0	1,7	2,7	1,0	1,8
3,1	3,4	1,6	2,7	3,4	3,9	4,1	0,5	0,1	5,3	1,1	0,8	3,2
2,1	1,1	2,3	3,6	8,1	5,7	1,3	1,6	1,3	3,9	1,8		1,1
4,1	1,9	1,2	0,1	1,2	4,6	4,2	0,8	0,1	1,5	0,8		0,9
3,2	0,9	0,5	1,9	4,4	2,9	2,4	2,3	0,2	1,9	1,6		0,9
3,9	3,6	2,3	1,9	4,3	0,3	1,8	1,8	0,2	1,5	2,2		2,5
4,3	4,4	1,5	2,9	0,6	0,4	0,7	1,9	1,4	3,6	2,8		0,7
2,9	3,4	2,8	2,8	2,1	2,7	3,3	2,3	0,1	2,8	1,3		1,2
4,7	3,6		4,9	3,8	1,9	3,4	0,1	3,4	2,8	1,7		1,7
1,1	5,2		4,1	3,5	0,8	4,0	2,8	1,7	2,6	1,0		1,3
1,9	1,8		3,4	1,9	4,7	3,0	0,4	1,6	4,1	0,2		0,3
4,0	5,1		2,2	2,7	5,5	2,0	2,9	1,9	2,3	0,9		1,0
2,4	7,7		1,1	2,1	2,1	0,4	2,3	1,2	0,5	2,7		1,2
4,7	6,9		1,6	1,4	1,2	0,1	1,2	0,3	1,7	0,2		0,8
2,7	5,6		1,5	2,6	4,7	0,7	1,8	0,6	0,8	1,2		0,3
2,5	4,1		1,6	0,4	4,3	3,3	1,1	0,1	2,1	3,4		2,0
1,0	2,0		0,5	0,2	2,1	0,9	1,2	1,5	2,1	2,8		2,2
3,4	1,4		2,0	0,9	0,9	1,5	0,5	1,1	1,5	1,3		1,0
	2,3		0,9	0,1	3,0	1,6	0,2	0,2	1,7	1,5		0,2
	2,2		0,8		2,8	1,1	0,5	2,3	2,1	0,7		1,2
	1,1		0,8		4,3	0,2	0,1	0,2	2,1	1,2		2,8
	0,4		1,1		4,7	1,2	1,5	0,5	1,3	3,2		1,0
	0,2		0,2		1,9	0,2	1,4	1,4	1,7	0,9		1,1
	0,4		0,3		0,3	2,7	0,6	1,9	1,5	1,1		0,7
	0,5		0,5		1,3	1,4	2,3	0,7	0,9	0,9		0,9
	0,7		0,7		2,4	1,8	0,8	0,9	1,2	1,2		1,3
	0,3		0,2		1,6	2,7	1,0	1,2	2,7	1,3		1,8
	0,2		0,7		3,8	0,3	1,1	0,7	2,0	1,0		0,5
	0,4		0,5		5,5	2,3	0,1	0,5	1,1	0,5		0,1
	0,5		0,9		3,0	3,0	1,3	0,3	2,9	0,8		0,3
	0,6		0,3		0,9	3,1	1,6	1,1	1,8	1,2		0,4
	0,3		0,4		3,6	1,0	0,3	0,9	3,1	1,1		1,4
	0,6		0,9		3,9	1,5	0,6	1,6	2,7	0,5		1,0
	1,5		0,8		1,1	3,7	1,4	1,1	2,6	0,3		1,2
			1,1		1,2	3,1	1,9	0,7	2,2	0,6		0,7
			0,5		2,0	3,1	0,8	0,5	1,8	0,2		1,3
			0,6		2,3		0,4	1,9	1,7	1,0		1,1
			0,5		0,5		0,3	1,3	2,4	0,7		1,7
			0,4		0,3		3,3	0,8	0,1	0,6		1,6
			1,0		4,8		0,2	1,2	2,2			1,9
			0,8		3,8		1,4	1,1	3,2			0,4
			1,7		1,4		0,5	1,0	1,6			1,1
			1,5		1,3		0,6	1,1	3,5			0,1
			4,3		1,0		1,9	1,4	1,2			1,4
			2,8		1,6		0,1	0,3	0,3			1,6

2,6	1,4	2,3	2,4	1,9
4,7	3,3	1,0	3,5	0,1
2,4	2,8	0,9	1,3	1,3
	0,1	0,8	0,3	0,2
	2,3	1,1	1,0	1,4
	2,4	0,5	3,7	2,5
	1,2	0,4	3,3	0,1
	1,8	1,7	1,7	1,7
	1,4	0,2	2,0	1,6
	0,9	0,3	1,3	1,5
	3,9	0,5	1,1	3,7
	1,1	0,8	3,5	0,9
	2,9	0,5	3,0	2,5
	1,6	0,1	2,4	2,1
	3,5		2,9	2,2
	1,3		2,8	0,3
	0,2		4,1	0,1
	1,7		1,5	0,3
	4,2		1,2	1,0
	2,1		3,5	0,6
	1,6		2,8	0,2
	1,8		0,3	2,8
	0,4		0,5	4,0
	1,7		0,3	0,8
	0,6		1,1	0,1
	0,2		1,8	0,2
	0,3		2,4	1,2
	1,6		0,6	2,1
	4,0		2,7	3,1
	0,2		2,4	2,8
	3,2		2,6	1,3
	1,2		1,5	1,1
	2,2		3,2	1,5
	1,1		2,1	0,1
	2,9		0,5	2,6
	1,7		2,3	4,8
	0,8		2,1	0,6
	0,7		3,0	0,6
	0,3		1,8	1,1
	1,3		1,9	1,7
	0,9		2,0	0,5
	0,3		1,3	0,1
	0,1		1,9	0,8
	0,1		2,4	1,3
			2,9	0,3
			2,1	0,7

1,8	1,2
2,6	0,3
1,0	1,2
2,6	1,4
1,1	1,0
1,6	1,0
1,6	0,2
6,0	0,4
0,4	1,3
4,3	0,5
1,8	1,5
4,4	0,7
0,8	0,3
2,1	0,5
0,2	0,2
1,3	0,6
0,9	0,1
0,2	0,2
0,9	0,2
1,3	1,0
1,0	
2,1	
0,9	
1,5	
0,3	
0,4	
0,5	
0,2	
0,9	
0,3	
0,3	
0,9	
0,2	
0,5	
1,1	
0,9	
1,5	
0,7	
0,6	
2,5	
1,9	
1,8	

Non-germinated seeds:

29	42	56	36	55	47	72	31	45	49	89	43	78	64

Plants :													
48	70	86	60	100	71	141	88	111	97	179	91	54	163

Notes:
Genotypes 1-8 are fragile, with leaves and roots tearing off easily, genotypes 9-14 are even more fragile. Genotypes 13 and 14 have very long roots, usually growing on the surface of the medium.

2.5 µM ABA

Col-0	siz1	pial1a	pial1c	pial2a	pial2b	pial1a pial2b	pial1c pial2a	pial1a siz1	pial1c siz1	pial2a siz1	pial2b siz1	pial1a pial2b siz1	pial1c pial2a siz1
3,9	5,0	9,0	2,7	7,4	5,0	6,1	1,2	1,1	1,2	2,0	2,0	0.3	0.2
1,8	5,0	2,8	5,1	3,5	6,1	6,9	1,5	1,9	0,2	0,6	1,0	0.2	0.3
9,8	1,9	3,9	3,0	3,5	4,8	2,9	2,8	1,4	1,3	1,8	1,1	0.3	0.3
2,7	3,2	4,0	7,6	2,3	1,5	5,2	2,5	0,3	2,0	0.6	1,6	0.1	0.4
5,3	3,0	4,9	4,4	2,4	3,2	4,8	3,3	2,9	2,2	0.9	0,8	0.1	0,9
5,3	2,2	3,3	2,6	4,4	2,3	4,9	0,2	1,2	0,7	0,1	0,9	0.3	0.3
4,8	2,5	7,4	4,6	2,1	1,6	5,4	1,5	1,4	0,8	0,4	0,6	0.3	0.3
7,2	0.5	2,8	3,6	4,6	2,5	7,9	1,7	0,8	1,1	0,9	1,3	0.3	0.4
6,9	1,3	4,2	6,0	1,2	1,6	5,0	1,3	1,7	1,0	0,1	1,2	0.4	0.3
6,6	1,3	2,0	4,9	2,9	3,5	5,3	2,2	0,8	0,8	0.2	1,3	0.2	0.3
6,8	0,4	1,8	10,6	3,4	3,5	4,3	1,3	1,2	1,2	0,2	1,5		0.3
6,2	1,4	1,8	3,0	0,8	1,6	2,4	0,9	1,3	1,3	0,3	0,9		
7,3	1,9	4,8	4,8	1,2	0,5	6,0	1,8	0,5	0,8	1,0	1,2	143	176
10,5	1,2	1,6	3,8	2,6	2,1	9,7	2,4	1,3	0,2	0,8	1,1		
2,8	1,4	3,8	4,5	2,9	3,5	0,3	1,7	0.8	1,0	1,2	2,5		
5,8	2,3	3,3	10,5	4,1	6,1	5,4	2,1	0.7	1,7	0.4	1,6		
4,4	2,0	5,4	4,9	1,1	2,5	5,5	1,5	0,7	1,0	0.6	0,3		
3,8	1,1	6,0	9,2	0,1	5,0	4,5	1,7	0,8	2,7	0.9	0,4		
3,0	1,5	3,7	0,9	5,9	6,1	1,7	3,1	2,1	0,8	0,1	1,2		
5,6	0,9	2,1	6,3	0,7	1,6	3,3	1,2	0,7	0,9	1,2	1,3		
2,0	1,8	4,2	8,9	1,6	2,4	4,8	3,8	0.6	1,2	0.5	0,8		
1,6	2,5	1,2	7,9	0,5	3,8	0,6	2,9	0,4	0,2	0.1	1,0		
1,0	0.2	4,3	9,5	1,6	2,9	3,8	3,1	0,6	1,3	0.8	1,5		
0,7	0.4	1,8	6,0	1,4	2,0	2,7	1,0	0,4	0,6	0.3	0,3		
2,3	2,1	5,3	6,3	4,1	1,9	5,3	2,7	0,6	1,3	0,7	1,5		
9,6	1,8	6,4	5,1	1,0	3,1	4,5	1.4	0,7	0,2	0.2	0,9		
4,4	1,6	3,3	1,9	1,0	3,3	5,1	1,8	0,1	1,2	0.1	1,0		
4,0	1,1	5,5	8,9	1,6	6,1	4,2	1,7	0,6	0,5	0,7	0,4		
3,5	1,9	3,4	6,3	3,4	5,6	1,8	3,0	0,6	0,3	0,6	1,8		
5,2	0,5	4,2	8,6	0,8	2,0	5,0	2,1	0,5	1,5	0,1	1,5		
5,2	1,1	6,9	6,1	0,9	2,3	4,5	1,1	0,1	1,5	0,2	0,3		
3,4	1,4	3,3	3,5	2,2	1,1	5,6	3,0	1,4	0,2	0,1	1,2		
2,4	0,1	0,1	2,3	4,8	3,3	3,2	2,5	1,1	0,1	0,7	0,7		

3,1	2,0	2,8	4,1	1,2	2,5	2,1	1,0	0,7	0,9	0,5	1,2
9,1	1,8	4,8	6,8	4,9	6,0	3,1	2,7	0,7	0,5	0,6	1,3
3,5	0.2	1,2	5,1	1,7	1,5	6,0	4,7	1,4	1,0	0,3	0,6
5,5	0.6	2,3	8,5	3,0	3,1	5,0	2,6	0,9	1,4	0,2	0,7
1,1	0,8	5,1	7,0	2,2	1,7	1,6	1,5	1,0	1,1	1,0	0,1
4,6	1,1	2,3	7,7	4,6	1,6	2,6	1,9	1,2	0,6	0,5	0,5
2,4	1,2	5,7	5,7	0,5	2,3	3,4	2,4	0,4	0,6	0,4	1,7
7,6	0.6	3,6	4,0	3,0	1,6	3,7	4,1	2,6	0,8	0.4	0,1
3,0	1,2	1,7	3,3	1,7	2,9	7,5	4,5	2,2	0,9	0.6	1,0
5,6	0,5	3,6	3,0	2,9	1,7	5,5	0,9	1,6	0,8	0.2	1,0
1,3	1,1	1,2	1,4	3,5	2,3	6,4	1,6	1,1	1,5	0.4	2,0
2,2	2,3	1,4	3,9	5,4	2,0	1,5	1,2	0,5	0,6	0.6	1,9
5,0	0.7	2,8	6,5	1,0	2,3	2,5	2,1	1,3	0,3	0.1	1,4
4,7	1,8	3,3	5,7	1,1	0.3	4,3	3,0	0,8	1,1	0.2	0,4
9,5	2,4	2,8	7,7	0,7	0.9	1,7	3,1	0,8	0,9	0.3	0,8
7,3	2,0	2,7	3,1	2,0	1,1	5,6	1,2	0,1	0,6	0.1	0,8
5,8	1,1	3,6	2,5	6,5	1,2	1,3	1,6	1,1	0,4	0.2	1,3
6,3	0.8	4,8	4,4	0,9	1,6	1,6	0,9	2,6	0,1	0.7	1,6
2,3	1,0	5,2	4,9	3,1	1,6	1,6	1,4	1,8	0,1	0.9	1,0
3,6	1,3	1,7	3,3	3,5	3,1	5,1	2,0	0.6	1,2	0.5	1,9
4,2	1,8	2,6	2,9	3,4	0,1	8,6	6,6	0,8	0,9	0.9	1,5
2,9	1,5	4,1	9,9	3,0	3,5	4,2	1,3	0.1	1,5	0.1	1,3
2,6	0.2	2,9	6,0	3,8	2,6	3,4	1,8	0,3	1,3	0.1	1,2
1,9	2,0	0,3	4,2	1,3	3,2	3,0	0,4	1,4	0,7	0.3	1,4
5,8	0,3	2,0	4,6	2,1	3,3	4,9	0,7	0,6	1,3	0.4	1,4
0,5	2,3	1,1	7,1	1,8	2,0	2,5	2,8	0.2	1,2	1.2	1,6
3,2	1,1	3,8	6,5	1,8	1,1	4,6	1,4	0,7	0,9	0.3	1,4
1,9	1,5	1,6	3,4	2,5	3,4	6,1	1,3	0,3	1,0	0.4	1,2
2,0	0.2	5,4	4,7	2,1	0.8	7,6	1,0	0.5	0,8	0.6	1,6
1,5	0,4	3,4	7,0	2,4	1.1	4,9	2,8	0.3	1,2	0.1	1,3
4,3	0.2	1,3	6,7	0,1	0.3	5,1	1,1	1.0	1,1	0.3	1,0
3,6	0,7	2,0	5,3	3,5	0.1	8,1	0,6	0.7	0,4	0.7	1,7
1,8	0,7	2,1	5,0	2,7	0.1	4,4	1,5	0.4	1,3	0.9	0,2
6,0	0,8	0,7	6,3	3,0		6,9	1,3	0.6	1,4	0.9	0,9
6,4	0,9	1,6	3,0	2,4		3,4	1,7	0.2	0,5	0.7	1,5
7,3	1,1	3,4	4,3	2,0		2,8	1,6	0.6	0,6	0.5	1,6
2,6	1,3	0,2	4,1	0,5		4,5	2,6	1.1	1,1	0.4	1,7
6,3	1,3	1,1	6,6	1,6		5,8	1,2	0.9	0,2	0.9	1,2
2,6	0,5	1,5	1,6	4,0		3,8	1,3	0.8	0,9	0.3	1,3
3,1	1,0	2,0	2,9	2,1		4,2	2,9	0.1	1,2	0.2	1,0
1,9	0.3	0,9	7,1	3,0		3,5	1,0	0.4	0,7	0.8	0,9
3,4	1,4	1,3	1,4	3,8		5,0	1,8	0.6	1,0	0.7	1,0
6,2	1,8	1,2	5,0	2,6		2,4	1,6	0.3	1,4	0.7	1,4
7,4	0,7	1,4	4,4	2,6		4,1	1,7	0.3	1,2	0.8	0,7
4,4	1,2	1,7	8,5	2,5		2,6	2,3	0.5	1,1	0.1	1,2
2,8	1,7	1,4	5,6	1,8		6,0	1,8	0.1	0,7	1.0	1,5

1,5	1,4	1,1	6,7	1,5	6,0	2,0	0.2	0,3	0.9	1,6
3,2	1,3	2,0	7,1	2,4	2,5	2,0	0.6	1,6	0.6	1,3
2,6	0,2	0,0	0,2	2,3	6,9	2,2	0.4	1,2	0.6	0,3
2,4	1,1	1,5	5,4	1,1	3,7	2,7	0.6	0,5	0.7	1,1
4,1	0.5	1,5	3,3	1,8	6,8	1,6	0.3	1,2	0.8	1,2
5,2	0.6	1,8	4,8	1,3	5,1	1,7	1.0	1,3	0.7	1,1
4,5	0.4	1,4	5,1	2,2	4,6	5,2	0.5	1,1	0.5	1,2
1,5	0.4	1,9	4,0	3,4	9,7	1,4	0.7	1,1	0.4	1,7
5,0	0.7	2,1	5,1	3,1	8,8	1,3	0.1	1,7	0.6	0,6
2,3	0.3	1,8	4,3	0,1	3,7	1,9	0.7	0,4	0.8	1,5
1,3	0.2	1,6	5,7	2,5	2,5	1,6	0.8	0,1	0.9	0,3
4,4	0.2	1,9	7,7	2,6	5,4	2,5	0.6	0,3	0.2	1,2
2,4	0.1	1,3	6,0	1,1	2,6	1,0	0.5	0,2	0.3	1,7
1,2	0.2	1,1	5,8	1,1	3,0	2,6	0.8	0,9	0.6	1,2
2,9	0.3	2,7	5,6	1,5	2,9	1,6	0.9	1,1	0.6	0,7
2,2	0.5	2,3	6,0	5,2	3,6	2,8	1.2	0,3	0.5	1,2
2,9	0.9	1,5	2,6	3,0	2,6	2,1	0.6	0,9	0.1	1,3
1,6	1.0	1,6	5,7	3,1	7,2	0,7	0.6	0,2	0.2	0,6
1,9	0.6	1,1	7,0	1,0	3,0	4,8	0.3	1,2	0.3	0,5
2,3	0.7	1,0	4,6	1,3	5,2	1,5	1.2	0,7	0.2	1,1
1,6	0.4	1,2	8,0	1,8	5,2	3,4	0.9	0.1	0.6	1,6
3,0	0.3	2,1	5,1	2,2	4,8	1,7	0.3	1,9	0.4	1,3
1,2	0.3	3,0	0,7	3,6	5,4	1,8	0.7	0,3	0.9	1,2
1,5	0.1	2,0	1,0	1,8	5,5	1,5	0.7	0,5	0.2	1,4
2,5	0.9	1,8	4,9	1,4	4,3	3,2	1,0	1,1	0.3	1,5
2,0	0.7	0,2	5,6	4,2	4,1	4,0	0.6	1,0	0.6	1,7
1,1	1,5	1,5	1,5	2,7	1,8	2,8	0.2	0,2	0.7	1,2
0,3	0.6	0,7	2,6	2,4	4,0	3,4	0.7	1,2	0.8	1,1
0,9	0.1	2,8	2,3	0,8	4,3	1,3	0.8	0,3	0.9	0,9
1,6	1,3	2,3	2,2	2,2	1,4	1,5	0.9	0,4	0.6	1,7
1,2	0,5	0,9	5,4	0,1	5,5	0,9	0.4	1,1	0.7	1,3
1,3	1,0	1,3	3,8	0,8	4,2	1,4	0.3	1,3	1.0	1,5
1,0	0.3	1,7	4,3	1,5	4,0	3,8	0.5	1,7	1.1	1,2
	0.2	1,6	2,5	1,4	4,9	2,1	0.2	0,8	0.2	1,6
	1,2	2,0	3,8	4,0	5,0	1,8	0.2	1,2	0.3	1,1
	0.5	1,3	3,3	2,6	5,1	1,8	1.1	0,3	0.1	1,0
	0.6	0,9	5,4	1,6	2,6	0,3	0.9	0,5	0.4	0,4
	2,5	1,2	6,0	2,4	3,1	0,5	1.3	1,2	0.6	1,5
	0.7	1,4	4,1	3,2	2,8	4,0	0.1	1,4	0.5	1,6
	2,0	1,0	4,4	3,0	2,4	2,5	0.2	1,1	0.7	1,3
	0.6	1,9	5,2	2,1	4,2	2,1	0.1	0,7	0.8	1,2
	0.3	1,5	2,1	0,7	4,3	1,1	1.4	0,6	0.8	1,9
	0,8	1,7	2,5	1,0	1,7	3,8	0.7	1,2	0.1	0,8
	1,0		1,2	1,1	7,8	1,6	0.3	0,9	0.5	0.1
	0.4		3,1	0,9	2,8	1,7	0.9	1,3	0.4	0.3
	1,5		2,6	0,4	4,2	1,2	0.8	1,1	0.6	0.4

1,4	3,0	1,7	9,1	1,7	0.4	0,4	0.9	0.7
0.3	1,9	3,0	1,5	1,8	1.0	1,3	1.1	0.1
0.7	1,7	3,2	3,8	1,0	0.8	1,4	1.3	0.9
1,2	0,9		4,8	1,6	0.8	1,5	1.0	1.1
1,8	1,3		2,8	4,0	0.7	0,2	1.2	0.3
2,0	0,4		3,9	3,1	0.1	1,3	0.3	0.4
0.6	4,4		2,1	2,5	0.8	0,7	0.5	0.5
0.5	3,2		1,8	2,2	0.6	1,5	0.6	0.7
0.4	3,5		1,5	3,4	0.3	1,2	0.2	1.3
0.4	6,1		2,4	2,1	0.2	1,1	1.0	1.5
0.4			4,0	1,9	0.1	0,2	0.9	1.1
0.6			4,2	0,8	0.3	0,1	0.3	1.0
0.7			6,1	1,4	0.4	0,3	0.5	0.3
0.3			5,0	2,2	0.2	0,9	0.6	1.0
0.1			3,8	2,3	1.0	1,7	0.7	1.2
0.4			4,6	2,6		1,9	0.4	0.7
0.3			2,3			0,9		0.3
1,0			4,4			1,4		0.2
0.5			2,5			1,5		0.1
0,4			3,0			0,6		0.2
0.4			3,4			1,6		0.3
1,1			1,7			0.5		0.3
0.9			4,4			0.1		1.0
1,0			3,2			0.2		0.9
0.5			6,7			0.6		0.9
0.7			2,3			0.5		1.1
0.9			2,8			1.1		0.8
0.3			2,0			1.0		0.3
0.2			9,0			0.1		0.1
0.4			0,8			0.2		0.2
0.7			2,7			0.2		0.2
1,4			4,0			0.1		1.0
1,0			7,2			0.3		0.7
1,1			5,8			0.6		0.6
0.7			6,0			0.9		0.3
0.4			2,0			0.8		0.9
0.8			8,8			1.1		0.8
0.3			4,0			0.6		1.3
0.4			4,7			0.7		0.3
0.3			5,6			0.3		1.4
0.2						1.2		1.0
0.5						0.5		0.7
0.2						0.9		0.6
0.4						1.1		0.9
0.7						0.2		0.3
0.2						1.3		0.9

0.4	1.4	0.7
0.3	0.6	1.2
0.2	0.3	0.3
	0.8	0.5
	0.6	0.1
	0.2	0.6
	0.4	0.4
	0.5	0.9
	1.1	0.3
	0.7	1.4
	0.4	1.1
	0.3	1.2
	0.1	1.5
	0.1	0.5
	0.1	0.4
	0.4	0.7
		0.1
		0.2
		1.1
		0.3
		0.4
		0.2
		1.0
		0.7
		0.5
		1.5
		0.3

Non-germinated seeds:

60	64	79	28	62	35	47	30	28	52	43	35	83	48

Plants:

112	174	122	135	128	66	165	140	140	187	141	198	153	187

Notes:

Seedlings not as fragile as mannitol seedlings

Very long roots relative to leaf size

Anthocyanins in hypocotyl

Numbers given as x.y (as opposed to x,y) denote plants where the green colour has been completely overwhelmed by red or brown. These plants were scored as dead. Since all triple mutant plants, except one, were dead, their total number was given, highlighted in grey.

Total numbers of stressed plants

Genotype	Col-0	pial1	pial2	siz1	pial1 pial2	pial1 siz1	pial2 siz1	pial1 pial2 siz1	Total
No stress	61	140	118	86	181	176	245	239	1246
150 mM NaCl	52	153	108	60	200	189	271	168	1201
300 mM mannitol	49	148	173	71	231	210	278	219	1259
2.5 µM ABA	112	257	194	174	305	327	339	340	1762
Total	274	698	593	391	917	902	1133	966	5468

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Statement

I hereby state that the work presented in this dissertation is my own, unless excplicitly stated otherwise. All of the used sources and materials have been cited accordingly. All figures and tables have been compiled by me, and in the cases when publicly available online databases have been used, these have been mentioned accordingly. This dissertation has not been made public prior to the defense, with the exception of certain cited publications.

Vienna, 17 March 2014

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Education and training:

2009: Master's Degree Project at Phadia AB, Uppsala, Sweden: Identifying cross-reactive allergens between timothy grass and wheat

2007 – 2009: Uppsala University, Master's Program in Molecular Biology

2007: Bachelor's Degree Project at Uppsala University: Literature Studies on DNA Replication in Archaea

2004 – 2007: Uppsala University, Bachelor's Degree in Molecular Biology

2003 – 2004: Sofia University, Bachelor's Program in Molecular Biology

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Conferences attended:

01.10.2013 – 05.10.2013: EMBO conference: ubiquitin and ubiquitin-like proteins, Riva del Garda, Italy, poster talk

09.10-2013: Vienna Plant Network Meeting, Klosterneuburg, Austria, lecture

18.09.2012 – 21.09.2012: Zomes VII, International Conference and annual meeting of the DFG-SPP1365, Ubiquitin-like proteins and their cognate PCI complexes, München, Germany, poster talk

28.02.2012 – 02.03.2012: 25. Tagung Molekularbiologie der Pflanzen, Dabringhausen, Germany, poster talk

25.09.2010 – 28.09.2010: 7th Trinational Arabidopsis Meeting, Salzburg, Austria, poster talk

13.04.2010 – 16.04.2010: Annual Meeting of the DFG Priority Program SPP 1365 Ubiquitin Family Network, Berlin, Germany