Universitat Autònoma de Barcelona

SUMOylation at the crosstalk between development and defense response

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Barcelona, 2022

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SUMOylation at the crosstalk between development and defense response

Doctoral Thesis presented by Diana Fuertes Bailón to opt for the doctor title from the Autonomous University of Barcelona

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Barcelona 2022

Don't say it's impossible, rather say you haven't done it yet.

Japanese proverb.

In memory of María Coca

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Introduction

1. Post-translational modifications as key players in cell signaling

The complexity of life relays in the temporal and spatial coordination of mechanisms involving DNA transcription and RNA translation. These mechanisms end with the synthesis and folding of proteins. Each gene contains condensed and highly complex genetic information whose destiny is diverse according to cellular requirements. Starting from a relatively small genome, the cell is able to achieve a wide variety of functions by expanding the proteome, since several proteoforms can arise from a single gene. One of the main mechanisms generating the wide variety of the proteome are the post translational modifications (PTMs).

Any protein can be modified after or during translation, thereby increasing the repertoire of different cellular proteins and, consequently, generating a huge source of variation beyond that determined by DNA or the variation of RNA. PTMs are modifications generally produced by specialized enzymes that can increase the diversity of the proteome up to 50-100 times. PTMs induce changes in protein activity, turnover, subcellular localization, and interactions with other molecules, which enables a fine-tuning of cellular responses. In a cell, we can find more than 200 different PTMs and these modifications can take place through the addition of small molecules of different nature, such as phosphate groups, acetyl groups, ubiquitin lipids, etc (Jensen, 2006; Kerscher *et al*., 2006; Vertegaal, 2011, Sharma *et al*., 2021). This mechanism of rapid protein regulation in response to intra and extracellular signals is essential for the correct functioning and balance of the proteins, particularly during sudden changes in environmental factors, in which physiological responses often occur rapidly and reversibly. Therefore, understanding how, when, and why these mechanisms occur is essential for the proper function of organisms (Bayer *et al*., 1998; Kerscher *et al*., 2006; Vierstra, 2012; Maupin-Furlow, 2014).

2. Ubiquitination and SUMOylation

In the second part of the 1970s, ubiquitin was discovered as a novel modifier of proteins that constituted a signal for protein degradation. In the 1980s, the discovery of the enzymatic cascade involved in ubiquitin conjugation highlighted that this modification was a highly regulated process. Ubiquitin received its name because of its ubiquitous location (in different tissues and species). This protein, which consists of 76 amino acids and a molecular weight of about 8.5 kDa, is highly conserved among eukaryotes (Goldstein *et al*., 1975; Schlesinger and Goldstein, 1975). Following the discovery of ubiquitin, other modifiers sharing the same tertiary structure of ubiquitin were identified (Ubiquitin-Like proteins) and constitute the so-called ubiquitin family. Up to 12 different UBL proteins have been characterized and postulated to regulate various aspects of cellular activity by modulating the structure and function of their substrates (Hochstrasser, 2009; Vierstra, 2012). Ubiquitin is the most prominent representative

of post-translational modifiers with a conserved structure known as β-grasp fold or Ub-fold (UFD), consisting of a β-sheet of 5 strands and a helix between strands 2 and 3 (typic ββαβββ fold) (**Figure I.1**). Ub conjugation is reversible and consists of an enzymatic cascade known biochemically as ubiquitination. Although other roles have been described, the binding of ubiquitin to the protein substrate functions as a mark for degradation via de proteosome (Hershko *et al*., 2000; Kerscher *et al*., 2006; Oh *et al*., 2018).

The second most prominent member of the Ub-family is SUMO (Small Ubiquitin-like Modifier) which was identified 20 years after Ub. The SUMO protein is a reversible posttranslational modification which plays an important role in a wide range of cellular pathways. It is conserved in all eukaryotes and perform essential functions in most of organisms (Bayer *et al*., 1998; Pichler *et al.,* 2017). This small polypeptide, composed of around 100 amino acids, belongs to the superfamily of UBL modifiers. It was discovered in 1996 when studying the function of active nuclear transport by means of the RanGAP protein in mammalian cells. By using specific antibodies that recognized RanGAP, two forms of this enzyme were identified displaying a difference of about 20kDa. The characterization of the two isoforms confirmed that the larger RanGAP isoform contained other peptide sequences not previously identified, which finally corresponded to SUMO (Matunis *et al*., 1996).

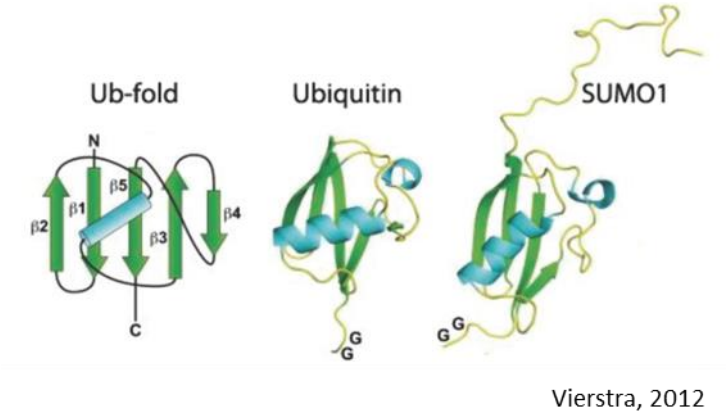


Figure I.1 – Diagram of the three-dimensional structure of Ubiquitin and SUMO. *On the left, the schematic structure of the Ub-fold domain (UFD), ββαββ, is shown, in which the β sheets are arranged around the diagonally oriented α helix. Next, the structure of human Ubiquitin and SUMO is shown.*

The amino acid sequence of SUMO differs from those of ubiquitin with 18% homology but share the same well-defined tertiary structure termed Ub-Fold (UFD) and a C-terminal di-glycine motif. This UFD domain is highly conserved among UBLs to the extent that the three-dimensional structures of SUMO and ubiquitin overlap (**Figure I.1**).

SUMO, unlike ubiquitin, consists of 10-30 additional amino acids at the amino terminus providing a flexible N-terminal tail that appears to be intrinsically disordered. This flexibility and possibility of exposure to the solvent facilitates a potential binding to other proteins and, also, provides an additional surface to accept other PTMs. Ubiquitin and SUMO have a hydrophobic core, while the surface charge differs significantly among both, which may influence their mechanism of action (Bayer *et al*., 1998; Augustine and Vierstra, 2018). The conjugation to protein targets involves the formation of a covalent bond between the exposed C-terminal glycine of SUMO and a lysine residue in the substrate, forming a peptide bond. Ubiquitin and SUMO conjugation occurs through an equivalent enzymatic cascade, although the enzymes involved are unique for each system (Kerscher *et al*., 2006; Augustine and Vierstra, 2018). Despite having a similar structure and conjugation cycle, SUMO and ubiquitin display distinct molecular roles. While ubiquitin is related to the regulation of protein half-life, the function of SUMO is variable and dependent on the modified protein. SUMO is involved in a large variety of processes including chromatin organization, DNA repair, DNA replication, subcellular transport, transcriptional regulation, pre-mRNA splicing, protein degradation and cell signaling (Hay, 2005; Pichler *et al*., 2017).

3. SUMO cycle conjugation and deconjugation

SUMOylation is mechanistically similar to the process of ubiquitin conjugation. All SUMO proteins are expressed as immature precursors and must be processed by SUMO proteases to expose the conserved C-terminal motif (Gly-Gly), which is necessary for conjugation. This step is mediated by ULPs (Ubiquitin-like proteases, SUMO-specific cysteine-containing proteases) (Johnson and Gupta, 2001; Pichler *et al.,* 2017).

Analogous to ubiquitination, SUMO conjugation is carried out by the sequential action of three enzymes. First, SUMO is activated by the heterodimeric E1 activating enzyme, SAE1/SAE2. SUMO activation begins with an ATP-dependent reaction producing adenylated SUMO (SUMO-AMP). In a second step, AMP is released from the adenylated SUMO and a thioester bond is formed between the carboxyl group of the C-terminal glycine residue of SUMO and the sulfhydryl group of the active cysteine residue of the E1, located in the large subunit SAE2 (SUMO-SAE2/SAE1) (Johnson, 2004; Lois and Lima, 2005). Then, SUMO is transferred to the catalytic cysteine present in the E2 conjugation enzyme through a transesterification reaction (SUMO-E2). Finally, SUMO binds to the target protein forming an isopeptide bond between the C-terminal glycine residue of SUMO and the ε-group amino of a Lys residue in the substrate (Rodriguez *et al.*, 2001; Bernier-Villamor et al., 2002; Pichler *et al*., 2017). In most cases,

SUMOylation takes place in a lysine residue contained in the consensus sequence ΨKxE/D (Ψ aliphatic residue (I, V L); k: lysine; x: any amino acid, E/D: glutamate or aspartic).

In contrast to the ubiquitin E2s, the SUMO E2 is competent for transferring SUMO to the substrate, although the SUMO ligase enzymes (E3) facilitate this final step of ligation to the target protein. This property is experimentally advantageous for reconstitution of SUMO conjugation *in vitro*, which is only dependent on recombinant E1, E2 and SUMO proteins (Bernier-Villamor *et al*., 2002; Castaño-Miquel *et al.,* 2011).

The modification by SUMO is reversible and deSUMOylation or deconjugation occurs by the action of the same ULPs that are involved in the maturation of SUMO (Johnson and Gupta, 2001; Bernier-Villamor et al., 2002; Pichler *et al.,* 2002). At this stage, SUMO is recycled and can reenter the SUMOylation cycle (**Figure I.2**).

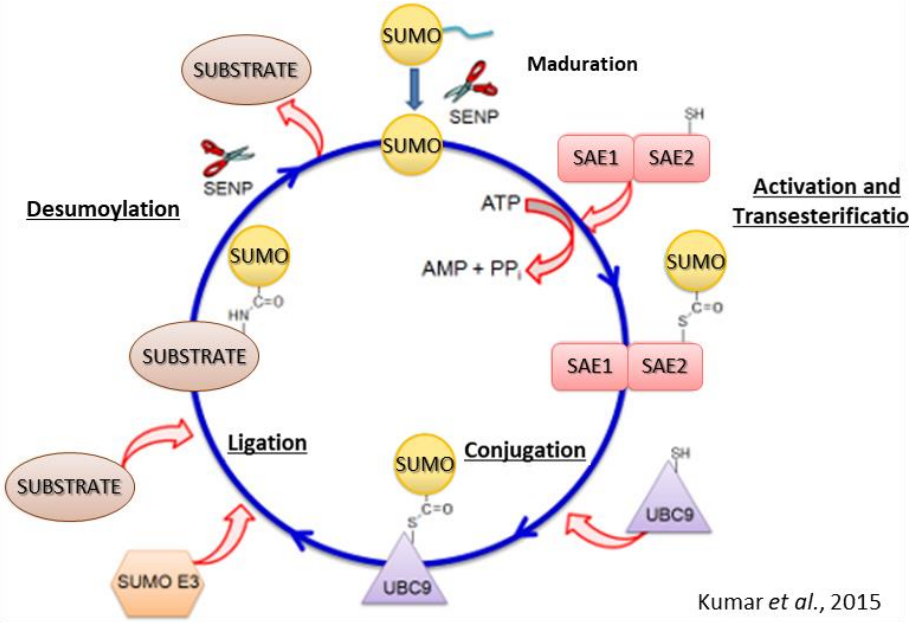


Figure I.2 – SUMOylation and deSUMOylation cycle. *SUMO is processed at its C-terminal tail by the specific ULP proteases setting free a mature SUMO form with a Gly-Gly motif in its C-terminal. Consequently, SUMO is activated by the heterodimeric E1-Activating Enzyme, SAE1/SAE2, transferred to the E2-Conjugating Enzyme (SCE) and, finally, attached to a target lysine in the substrate. This final step is facilitated by E3-Ligase Enzymes, which interact with the SUMO-charged E2 and the substrate. SUMOylation is a reversible modification and the same cysteine proteases involved in the maturation process can catalyze the SUMO excision from the substrate (Park et al., 2011; Kumar et al., 2015).*

In addition to the four canonical amino acids that constitute the SUMOylation consensus motif, the following variants have been identified: the inverted consensus motif, the hydrophobic SUMOylation motif, the phosphorylation-dependent SUMO consensus motif

(PDSM), and the negatively charged amino acid-dependent SUMO motif (NDSM) (Yang *et al.*, 2006; Gareau and Lima, 2010; Matic *et al*., 2010; Vertegaal, 2011).

SUMOylation machinery in Arabidopsis

The sequencing of the *Arabidopsis* made it possible to identify the genes encoding for the components of the SUMOylation system, confirming its conservation in *Arabidopsis*. The biochemical study of the enzymes involved in this pathway and the gain/loss function studies have elucidated the regulatory mechanisms of this modification and some of its biological implications.

SUMO

In yeast, diptera and nematodes there is only one gene codifying for SUMO, while in vertebrates and plants there is a family of genes encoding this molecule. In the *Arabidopsis thaliana* genome, there are up to eight genes codifying for the different SUMO isoforms, although significant expression levels have only been detected for AtSUMO1, 2, 3 and 5. AtSUMO1 and AtSUMO2 are highly conserved, displaying 89% sequence identity; while AtSUMO3 and AtSUMO5 have 42% and 30% of sequence identity with AtSUMO1, respectively (Kurepa *et al*., 2003; Lois, 2003).

Sequence alignments with their human orthologous, yeast and Drosophila show that the most conserved region is in the UFD region and the most divergent corresponds to the N-terminus of SUMO (Kurepa *et al.,* 2003; Lois, 2003). The *in vivo* expression of AtSUMO1/2 and AtSUMO3 has been demonstrated using antibodies that recognize both proteins (Kurepa *et al.,* 2003; Lois *et al*., 2003), while AtSUMO5 has only been detected in vivo in overexpressing plants of AtSUMO5 (Budhiraja *et al.,* 2009).

The study of SUMO paralogs in *Arabidopsis* has shown that they have acquired different molecular and functional properties. In plants, AtSUMO1 and 2 can form SUMO chains while AtSUMO3 and 5 do not contain the SUMO attachment site to allow chain formation (Colby, 2006; Budhiraja *et al*., 2009; Castaño-Miquel *et al.,* 2011). In addition, SUMO proteases have a low isopeptidase capacity against AtSUMO3 and a high capacity to deconjugate AtSUMO1 and 2 conjugates (Chosed *et al.,* 2006).

Through genetic analysis, the essential role of AtSUMO1 and 2 has been determined, since the double mutant *atsumo1/atsumo2* is lethal causing growth arrest during the early stages of embryo development, indicating that AtSUMO3 and 5 do not complement the biological function of AtSUMO1 and 2 (Saracco *et al.,* 2007). In addition, a differential accumulation of conjugates of the SUMO paralogs has been observed in response to different types of stress (like

thermal stress, hydrogen peroxide and ethanol), observing a rapid increase in conjugates of AtSUMO1 and 2, while this is not observed with AtSUMO3 (Kurepa *et al*., 2003; Pichler *et al*., 2017). This differential specialization of SUMO paralogs in *Arabidopsis* is reinforced by a different spatio-temporal localization and abundance of the proteins (Van den Burg *et al*., 2010). Consistent with this biological differentiation, AtSUMO1, 2, 3 and 5 display distinct conjugation rate as determined in biochemical studies. AtSUMO1/2 are the isoforms that are conjugated with higher efficiency, while AtSUMO3 conjugation rate presents a four-fold reduction and AtSUMO5 is the most inefficiently conjugated (Castaño-Miquel *et al*., 2011)

The non-covalent interaction between SUMO and its cognate E2-conjugating enzyme is a characteristic of the SUMOylation system. Molecular specialization between SUMO isoforms has also been observed regarding their capacity to interact non-covalently with the SUMO E2-conjugating enzyme AtSCE1. The isoforms with a higher conjugation rate, AtSUMO1/2, are competent to interact with AtSCE1, but this ability is not conserved in either AtSUMO3 or AtSUMO5 (Castaño-Miquel *et al.*, 2011).

SUMO Activating Enzyme (E1)

The SUMO activator enzyme (E1) is responsible for carrying out the adenylation reaction, which allows the activation of SUMO and its subsequent transfer to the conjugating enzyme. Independent crystallization studies of Ubiquitin E1, the SUMO E1 and the Nedd8 E1 revealed the existence of a conserved structural model for E1 enzymes (Walden *et al.*, 2003; Lois and Lima, 2005; Lee and Schindelin, 2008). In contrast to the ubiquitin-activating enzyme that is a monomer, SUMO and Nedd8 E1s are heterodimers, but all three conserve the same structural and functional domains.

The large subunit of the SUMO E1 (SAE2) consists of 4 functional domains: the adenylation domain, the catalytic cysteine domain, the ubiquitin-fold domain (UFD) and a C-terminal domain (**Figure I.3**). Except for the C-terminal tail, these domains play an essential role in the SUMO activation. The adenylation domain, which is responsible for the recognition and adenylation of the SUMO C-terminus, is flanked by the catalytic cysteine and the UFD domains.

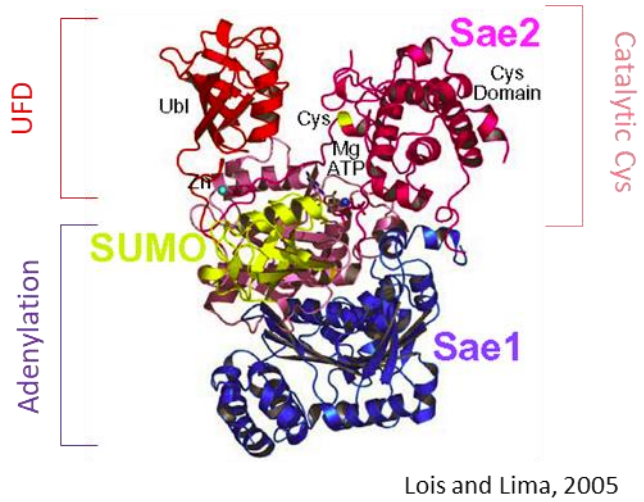


Figure I.3 – Structure of the SUMO activator enzyme (E1). *Diagram of the SAE1/SAE2-SUMO-Mg·ATP complex. The SAE2 domains are represented in different colors: red for the UFD domain, magenta for the catalytic cysteine domain, and pale pink for the adenylation domain. The active catalytic cysteine is highlighted in yellow. The small subunit SAE1 is represented in blue and finally SUMO in yellow.*

The catalytic cysteine domain is necessary for the formation of the E1-SUMO thioester bond, a reaction that is preceded by a conformational change of E1 to bring the adenylated SUMO closer to the catalytic cysteine (Olsen *et al*., 2010). The transfer of SUMO from the activating enzyme (E1) to the conjugating enzyme (E2) requires prior recognition of the E2 by the E1, through the UFD domain (Lois and Lima, 2005). It has been proposed the catalytic cysteine domain has a minor role in this recognition by bringing the two cysteine residues of E1 and E2 closer together, an essential process for the transfer of SUMO from E1 to E2 (Wang *et al.,* 2007).

In *Arabidopsis*, there are two forms of the activating enzyme, E1a and E1b, which differ in the composition of the E1 small subunit, *At*SAE1A and *At*SAE1B. SAE1 isoforms display 81% of sequence similarity and modulate SUMO activation rate, providing a regulatory mechanism to control SUMOylation efficiency. In *Arabidopsis*, the lethal phenotype of *sae2* mutants is consistent with the essential role of SAE2 during embryo development (Saracco *et al.,* 2007).

SUMO Conjugating Enzyme (E2)

The SUMO conjugating enzyme (E2) plays a central role in the SUMOylation cycle by interacting with all the enzymes in the pathway. This enzyme is initially recognized by the UFD domain and the catalytic cysteine of E1, facilitating the transfer of SUMO from E1 to E2. Once the thioester bond is formed between the C-terminal glycine of SUMO and the catalytic cysteine of E2, the SUMO-loaded E2 catalyzes the binding of SUMO to the substrate directly or in cooperation with E3 ligases.

This enzyme confers substrate specificity by recognizing, through non-covalent interactions, the consensus SUMOylation motif of the substrate, bringing the target protein closer to SUMO covalently bound to E2 (Lin *et al.,* 2002). The surface responsible for this recognition is adjacent to the catalytic cysteine of E2 (Gareau and Lima, 2010).

In addition to forming the intermediate thioester bond with SUMO, the SUMO E2-conjugating enzyme is able to form a complex with SUMO through non-covalent interactions. In mammals, the non-covalent interaction between E2 and SUMO promotes the formation of SUMO chains (Knipscheer *et al*., 2007).

Unlike the ubiquitin system, in which different E2 enzymes have been identified, only one exists in mammals, yeast, and *Arabidopsis*, suggesting that the SUMO conjugating enzyme plays a role in substrate specificity together with E3 ligases (Gareau and Lima, 2010). The genome of *Arabidopsis* contains a pseudogene and an active gene encoding the SUMO-conjugating enzyme, *At*SCE1b and *At*SCE1a, respectively. There is only one isoform of E2 encoded by *At*SCE1a and the inactivation of this gene is lethal (Saracco *et al.,* 2007). The expression of E2 has been detected in all *Arabidopsis* tissues, and it shows a nuclear enrichment (Lois *et al.,* 2003).

SUMO ligases (E3)

These enzymes have three properties: they interact with the SUMO conjugating enzyme, with the substrate, and facilitate the transfer of SUMO from E2 to the target protein.

SUMO E3 ligases identified in eukaryotes are characterized by the presence of the SP-RING domain, similar to the RING domain that contains most ubiquitin E3 ligases (Johnson, 2004). The SP-RING domain was the first discovered family of SUMO E3 ligases and is essential for the interaction with the SUMO conjugating enzyme and has ligase activity (Pichler *et al*., 2017). In addition, other different domains can be present in SUMO E3 ligases, including SAP, PINIT, SIM and the C-terminal rich in serine and threonine domains. The SAP (Scaffold Attachment Factor (SAF)-A/B, Acinus, PIAS) domain located at the amino terminus is necessary for binding to DNA. The PINIT domain contributes to substrate recognition. And the SIM (SUMO Interacting Motif) domain allows non-covalent interaction with SUMO (Sharrocks, 2006; Rytinki *et al.,* 2009).

So far, two SUMO E3 ligases have been identified in *Arabidopsis*, while up to 3 and 15 have been described in the yeast and mammalian system, respectively (Miura and Hasegawa, 2010; Wilkinson and Henley, 2010).

The first to be identified was SIZ1 (PIAS SUMO E3 ligase). SIZ1 belongs to the PIAS family (protein inhibitor of activated STAT), which are the best characterized and most abundant SUMO

E3 ligases. *At*SIZ1 conserves the four functional domains of PIAS and contains the additional PHD domain (Plant HomeoDomain), which interacts with SCE1 and has ligase activity (Garcia-Dominguez *et al.,* 2008).

The second SUMO E3 ligase was identified by two independent groups and named MMS21 and HPY2 (Methyl Methanesulfonate-Sensitivity protein 21, High Ploidy 2), respectively (Huang *et al.,* 2009; Ishida *et al.,* 2009), which we will refer to as HPY2. In contrast to SIZ1, HPY2 ligases do not contain the PINIT and SAP domains.

Single mutants of SUMO E3 ligases are not lethal and have contributed to elucidating the role of SUMOylation in plants. Loss-of-function mutants of SIZ1 and HYP2 cause a dwarf phenotype, the *athyp2-1* phenotype is not complemented by SIZ1 expression, and the double mutant is lethal. These results suggest that SIZ1 and HYP2 are not redundant and function independently. However, the combination of their activities is essential for a correct development of the plant (Ishida *et al*., 2012).

SUMO ligase E4 enzymes

Two more ligases have recently been described in *Arabidopsis*, PIAL1 and 2, which promote the formation of SUMO chains. Analysis of the mutants suggests that PIAL1 and 2 contribute to the regulation of salt stress and osmotic responses in addition to being involved in sulphate assimilation and sulphide metabolism. Their functions overlap, but do not show functional redundancy with SIZ1 ligase, which would be consistent with the model proposing that SIZ1 preferentially promotes SUMOylation of SUMO substrates, while PIALs would extend SUMO chains bound to substrates (Tomanov *et al.,* 2014).

SUMO proteases (ULPs)

The balance between stored free SUMO and the conjugate is regulated by specific SUMO proteases, also called ULPs. These enzymes have peptidase activity, which is required for maturation of SUMO, and isopeptidase activity for deconjugation of SUMO from the substrate (Yates *et al.*, 2016). SUMO proteases have an amino-terminal domain and a conserved carboxyl-terminal domain with protease activity (Li and Hochstrasser, 2003). The ULP N-terminal domain displays poor conservation and is considered to be determinant for the ULP biological activity, such as regulation of the subcellular localization.

SUMO proteases form the largest family of the various components of the conjugation machinery. Although in *Arabidopsis thaliana* only significant expression levels have been detected for 5 isoforms: EARLY IN SHORT DAYS 4 (ESD4), ESD4 LIKE SUMO PROTEASE (ELS1), OVERLY TOLERANT TO SALT 1 and 2 (OTS1, OTS2) and *ARABIDOPSIS* SUMO PROTEASE 1 (ASP1)

(Kurepa *et al.,* 2003; Murtas *et al.,* 2003; Colby, 2006; Augustine *et al*., 2009; Hermkes *et al.,* 2011; Kong *et al.*, 2017). All of them show peptidase or isopeptidase activity against AtSUMO1 and 2, while only ELS1 has peptidase activity against AtSUMO3 and none of them against AtSUMO5 (Chosed *et al.*, 2006; Colby, 2006). Endogenous peptidase activity could not be detected for AtSUMO3/5 in *Arabidopsis*, suggesting that AtSUMO3/5 must be matured at a low level or under specific circumstances (Lois, 2010).

Surprisingly, loss of function of SUMO proteases generates similar physiological effects to loss of function of E3 ligases, although they have different molecular effects. While the *siz1* mutant shows a reduction in conjugation, the *ots1/ots2* double mutant and the *esd4* mutant show a greater accumulation of SUMO conjugates. This fact suggests that the balance between SUMO conjugation and its deconjugation is strongly regulated, and a decrease or increase in SUMO conjugates can deregulate different cellular processes (Yates *et al.,* 2016).

4. Molecular implications of SUMO conjugation to proteins

The functional consequences of SUMOylation are unpredictable, but it is obvious that provides an additional surface for establishing interactions with other macromolecules. The following molecular consequences have been described:

1. **SUMOylation of the substrate can alter the ability to interact with other proteins**. For example, the modification by SUMO of lysine 14 of the ubiquitin conjugating enzyme E2-25K inhibits the interaction between the ubiquitin activating and conjugating enzymes, decreasing the ubiquitination capacity of the substrates (Pichler *et al*., 2005).
2. **Modification by SUMO can generate a new binding site** capable of recruiting other macromolecules through SUMO Interacting Motifs (SIMs) present in the interacting partner. SIMs interact non-covalently with SUMO and are characterized by a hydrophobic core flanked by acidic or phosphorylating amino acids (mostly serines, Ser) (Song *et al*., 2004). For example, SUMOylation of RanGAP promotes interaction with RanBP2. RanBP2 contains two SIM motifs at the carboxy terminus that promote binding to the SUMO-RanGAP complex and relocation of this complex to the Nuclear Pore Complex (NPC) (Werner *et al.,* 2012).
3. **SUMOylation can cause a conformational change of the target protein**. In cases that the SUMO substrate itself contains a SIM motif, an intramolecular interaction between SUMO and the SIM motif of the modified protein can occur, resulting in a conformational change of the protein (Kerscher *et al.,* 2006). This is the case of the

enzyme TDG (Thymine DNA Glycosylase), which binds to DNA to repair errors. SUMOylation of TDG results in a conformational change of this protein through the interaction of covalently bound SUMO and the SIM motif of TDG, allowing the release of TDG from DNA to initiate a new DNA repair cycle (Hardeland *et al.,* 2002).

4. **SUMOylation can affect the subcellular localization of the target protein**. RanGAP1, a protein associated with the nuclear pore complex, was the first SUMO substrate described (Matunis *et al.,* 1996). This study described how this modification directs RanGAP1 from the cytosol to the nuclear pore complex where it is required for nuclear import of proteins, activating the GTPase activity of the cytosolic/nuclear transporter protein Ran (Mahajan *et al.,* 1997); Mahajan *et al.,* 1998).
5. **Ubiquitination may depend on SUMOylation**. The formation of poly-SUMO chains can act as a binding domain of specialized E3 ubiquitin ligases known as STUBLs (SUMO-Targeted Ubiquitin Ligases) (Uzunova *et al.,* 2007; Xie *et al.,* 2007; Tatham *et al.,* 2008). STUBLs bind to poly-SUMO chains non-covalently through their multiple SUMO-interacting domains, allowing their dimerization, an essential feature for their activity and eventual polyubiquitination of both SUMO and substrate (Plechanová *et al.*, 2012; Xu *et al*., 2014). These polyubiquitinated SUMO targets can then be targeted for degradation via the ubiquitin-proteasome system (Uzunova *et al.,* 2007; Xie *et al*., 2007; Tatham *et al.,* 2008).

5. Approaches for identifying SUMO substrates

The identification of SUMOylated proteins is one of the main objectives of current research in the field of SUMOylation, since it provides the clues to elucidate the molecular mechanisms underlying the SUMO physiological roles proposed by studies using mutants of the different conjugation machinery components.

In plants, the initial approaches were targeted studies based on testing candidate genes known to regulate the physiological processes identified. The result was the identification of nine SUMO targets (Miura *et al*., 2005; Jin *et al.,* 2008; Miura *et al.,* 2009; Okada *et al.,* 2009; Cohen-Peer *et al*., 2010; Castaño-Miquel *et al.,* 2011; Park *et al*., 2011). Five conjugates were related to abiotic stress (ICE1, ABI5, MYB30, HSFA2, CAT3), three related to nitrogen metabolism (PHR1, NIA1; NIA2) and one related to development (FLD).

Considering that the expected SUMO proteome (subset of cell proteins modified by SUMO) will include thousands of proteins, the initial targeted approaches appear as an inefficient and

slow strategy. In the last decade, new approaches to identify SUMO conjugates emerged, such as proteomic studies in plant using a SUMO molecule fused to a peptide, that allows the purification of conjugates by affinity chromatography and subsequent analysis by mass spectrometry (Budhiraja *et al.,* 2009; Miller *et al.,* 2010; Park *et al*., 2011; Miller and Vierstra, 2011; López-Torrejón *et al.,* 2013; Rytz *et al*., 2018). Other groups opted for another approach based on the identification of protein-protein interactions with the components of the SUMOylation machinery (Garcia-Dominguez *et al.,* 2008; Nigam *et al.,* 2008; Elrouby *et al.,* 2013). The first identification of SUMO conjugates in plants using mass spectrometry was performed by *in vivo* expression of SUMO1, SUMO3 and SUMO5 tagged with a histidine-tag. Of the 14 identified SUMO conjugates, 5 were shown to be SUMOylated *in vitro* (Budhiraja *et al.,* 2009).

To optimize proteomic approaches, tagged SUMOs variants were expressed in *Arabidopsis* mutant plants (*sum1-1/sum2-1*) as genetic background to avoid competition and dilution with native SUMO Miller *et al.* (2010). This strategy resulted in the identification of up to 357 proteins conjugated to histidine-tagged SUMO. Many of the conjugates identified were specific to the stress conditions used, supporting the stress-specific modulation of the SUMO conjugate pool. On the other hand, the research group of Park and collaborators (2011) used a two-dimensional (2D) electrophoretic gel with the aim of screening for SUMO conjugates after performing thermal stress. In total, they identified 27 proteins involved in DNA and RNA metabolism and in different metabolic and signaling pathways.

Regarding the approach of identifying the proteins that interact with the SUMOylation machinery, the first to develop this strategy were Elrouby and Coupland (2010), who used a two-hybrid system where they identified 238 interactors of the SCE1 conjugator and the ESD4 protease. A similar screen using SIZ1 resulted in the identification of GTE3 and GTE5, which is a transcription factor involved in binding to acetylated histones (Garcia-Dominguez *et al.,* 2008).

The Gene Ontology evaluation of all SUMO conjugates in *Arabidopsis* relates SUMO to a wide range of molecular and biological activities (Castro et al. 2012). Many of the functional categories identified at the molecular level were similar to those evaluated in humans, suggesting the degree of conservation of these functions in eukaryotic organisms. Evaluation of *Arabidopsis* SUMOylome according to the regulated biological processes revealed that SUMO is implicated in a large number of activities including plant hormones, transport and signaling, development, seed dormancy, as well as a wide range of responses to different growth

conditions and stresses such as radiation, light, temperature, heat, cold and freezing, osmotic stress, drought, and responses to biotic stress (Elrouby, 2015).

Chapters 2 and 3 of the present PhD thesis are largely based on the identification of possible SUMO substrates, as well as the validation of selected candidates as *bona fide* SUMOylation targets. The validation of SUMO conjugation was determined by an assay based on the reconstitution of the SUMOylation machinery in *E. coli*. For this assay, two plasmids encoding the SUMOylation machinery are used. One plasmid encodes for the two subunits of the E1 heterodimer, *At*SAE1 and *At*SAE2, whereas a second one encodes for the E2 (*At*SCE1a) and one SUMO isoform (1, 2, 3 or 5) in their mature form. Using this type of analysis, SUMOylation of MYB30 and NAF genes were confirmed (Okada *et al.*, 2009).

6. Biological implications of SUMOylation in plants

SUMOylation has been shown to play a fundamental role in development, as well as being involved in the regulation of biotic, abiotic, and hormonal stress responses (**Figure I.4**). The binding of SUMO to target proteins leads to a change in substrate activity, generating or blocking interaction surfaces of the target protein. The identification of SUMO substrates and the study of gain and loss of function has been crucial to identify the biological effects of SUMO conjugation (Kurepa et al., 2003).

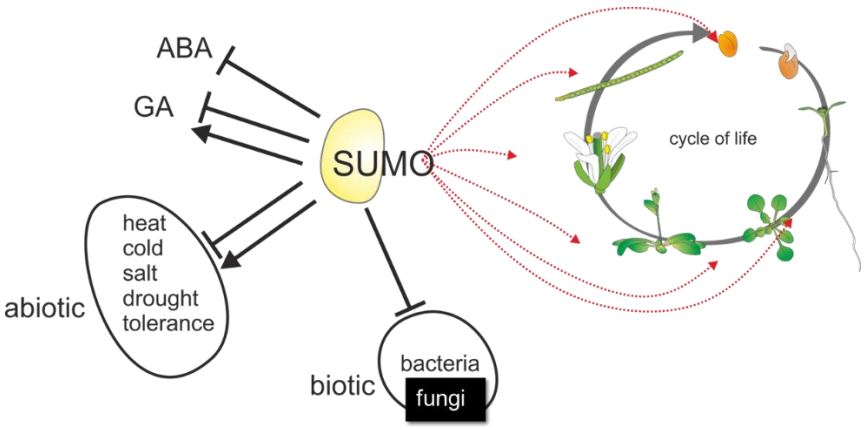


Figure I.4 – Biological roles of SUMOylation in plants. *SUMOylation is involved in the regulation of biotic, abiotic stress responses, and hormonal signaling, modulating all stages of plant development.*

6.1. The effect of SUMOylation on development

The *sae2*, *sce1* and *sumo1/2* mutants are lethal, the embryo does not develop correctly; providing an important link between SUMOylation and embryo development (Saracco *et al.,* 2007). Plants overexpressing a mutant version of SCE1 (E2) with the active site of Cys replaced by a Ser, showed reduced growth, early flowering, as well as hypersensitivity to abscisic acid (ABA) due to a reduction of SUMO conjugation. The regulation of the conjugation levels is very important for the correct development of the plant, as shown by the defects in the development and physiological responses that the mutants of the proteases present. For example, the *esd4* mutant has increased accumulation of SUMO conjugates and shows alterations including early flowering, dwarfism, and siliques and inflorescence damage (Reeves *et al*., 2002; Murtas *et al.,* 2003). Mutants of the OTS1 and OTS2 proteases do not show any apparent phenotype under normal growth conditions, but the overexpression of SUMO1 in the double mutant *ots1/ots2* produces a decrease in plant size (Conti *et al.,* 2008).

Contrary to what happens with mutants deficient in SUMO proteases, SUMO E3 ligase mutant plants show a reduction in the endogenous accumulation of conjugates (Catala *et al.*, 2007; Ishida *et al.,* 2009). It has been described that the E3 ligase *At*SIZ1 regulates the stability and development of the female gametophyte, necessary for the correct orientation of the pollen tube and subsequent fertilization, while the ligase *At*HYP2 is necessary for the correct development of the female and male gametophytes (Ling *et al.,* 2012; Liu *et al.,* 2014). On the other hand, *siz1* and *hyp2* have a dwarf phenotype, although it is due to different mechanisms. In the case of *siz1* mutants, this phenotype has been associated with the accumulation of SA (Lee *et al.,* 2006). In contrast, decreased cell proliferation in E3 ligase *At*HYP2 mutants is SA-independent (Ishida et al., 2011). Specifically, *siz1* mutant exhibits a dwarf phenotype with small leaves, early flowering, with defective female gametophytes, abnormal seed development, dormant seeds, and during photomorphogenesis exhibits short hypocotyls under different light or dark conditions, indicating that SIZ1 plays an important role in plant development (Catala *et al.,* 2007; Jin *et al*., 2008; Miura *et al.,* 2010; Ling *et al.,* 2012; Kim *et al.,* 2016; Lin *et al.,* 2016).

6.2. The effect of SUMOylation on abiotic stress

Organisms require rapid and reversible responses to environmental changes. This modulation of protein activity is possible through post-translational modifications. SUMOylation is an essential process in response to the different environmental stresses that organisms can suffer, as they induce a drastic accumulation of SUMO conjugates. In plants, this accumulation of conjugates has been observed in *Arabidopsis* in response to high temperatures, low temperatures, drought, salinity, sugars, exposure to heavy metals, incubation in hydrogen

peroxide, ethanol among others. This hyperaccumulation of SUMO conjugates is transient and SUMO reverts to its initial state after the stress is ended (Kurepa *et al.,* 2003; Miura *et al*., 2005; Kerscher *et al.*, 2006).

Arabidopsis seedlings exposed to 37 degrees show a rapid and dramatic increase in SUMO1 and 2 conjugates where almost all free SUMO is consumed (Kurepa, 2003). This modification is reversible and after a few hours the free SUMO is regenerated. SUMO1 overexpression reduces acquired thermotolerance, suggesting that SUMOylation reduces the capacity of plants to cope with lethal high temperatures following acclimation at sublethal high temperatures (Cohen-Peer *et al.,* 2010). Sensitivity to high temperature exposure has also been observed in *siz1-3* mutants, indicating that SIZ1 positively regulates basal thermotolerance (Yoo et al., 2006).

In addition to the role of SUMO against thermal stress, the importance of SIZ1 in the control of adaptation to low temperatures has also been described. SIZ1-dependent SUMOylation activates or stabilizes the ICE1 (Inducer of CBF Expression 1) protein, which controls the expression of the transcription factors CBF3/DREB1A (C-repeat Binding Factor 3/Dehydration Responsive Element Binding Factor 1A) in response to cold. SUMOylation of ICE1 stabilizes the protein by blocking its ubiquitination. In addition, the modification of ICE1 by SUMO negatively regulates MYB15, a repressor of CBF3/DREB1A, through its binding to MYB elements of the promoter. SIZ1 positively regulates the response to low temperatures (Miura et al., 2007).

It has been demonstrated that the *siz1* mutants are of special interest for the study of SUMO against different abiotic stresses, since they present an altered SUMOylation pattern that results in a general increase in its sensitivity against these types of stresses. The role of SIZ1 in the control of the response to phosphorus deficiency, water stress, in the maintenance of copper homeostasis and salt stress has been described (Miura *et al.*, 2005; Catalá *et al.,* 2007; Miura *et al.*, 2007; Chen *et al.,* 2011; Castro *et* al., 2012; Miura *et al.,* 2012).

Regarding the response to heavy metals, in response to copper, SIZ1-dependent SUMO conjugation increases in the first 30 minutes and decreases after 3 hours. In conditions of excess, the *siz1* mutant accumulated this micronutrient in the aerial organs, showing hypersensitivity to this metal. This phenotype could be partially explained if we take into account that the *siz1* mutant has induced expression of the metal transporters YSL1/3 (Yellow Stripe-Like 1 and 3) (Chen *et al.,* 2011).

6.3. The effect of SUMOylation on biotic stress

Plant damage caused by other living organisms such as bacteria, fungi, insects, nematodes, and viruses that compromises plant growth is known as biotic stress. The infection caused by

pathogens in crops result in significant agronomic losses worldwide, posing a threat to food security (Sharma *et al.,* 2021). Plants have developed several mechanisms to perceive the attack of pathogens and quickly develop specific response and defense programs. Plant resistance strategies against pathogens are based on a combination of morphological, biochemical, and molecular responses organized in multiple regulatory layers (Bari and Jones, 2009). Plant defense mechanisms are largely steered by PTMs to activate appropriate signaling pathways against the invading pathogen. Conversely, some infection strategies rely on the delivery of pathogen effectors that target host cell PTMs (Sharma *et al*., 2021).

Plants have different lines of defense against biotic agents. The first line is based on the molecular patterns associated with pathogens or microbes (PAMPs), which are the molecular determinants that trigger inducible immune responses (Cook *et al.,* 2015; Sharma *et al*., 2021). The second is called the hypersensitive response (HR) and is more specific than the previous one, since it directs the programmed cell death of infected plant cells, through the production of reactive oxygen species (ROS) (Jones and Dangl, 2006). Despite being useful signaling pathways, they can be suppressed and end up with infected plant cells. For this reason, the study of post-translational modification mechanisms such as phosphorylation, ubiquitination, SUMOylation, nitrosylation and glycosylation have been in the spotlight in recent years, as they have revealed their importance and involvement in plant defense responses to considerable pathogens (Stulemeijer and Joosten, 2008; Lois, 2010).

One of the purposes of pathogens when they infect the host is the proteolysis of host proteins. Several virulent factors with cysteine protease activity have been identified that possess peptidase and isopeptidase activity against SUMO, acting as SUMO-specific proteases. These virulent factors deregulate the balance of free and conjugated SUMO in the cell, compromising the host defense system (Hotson and Mudgett, 2004). Both SUMO conjugation and deconjugation have been described as one of the mechanisms involved in plant pathogen infection responses (Hotson and Mudgett, 2004; Mudgett, 2005). The role of the SUMOylation of 141 proteins involved in plant immunity was established by performing a Gene Ontology term analysis, obtaining an intersecting group between bacteria and fungi of 17 proteins, 3 between bacteria and viruses, and 1 protein between virus and fungal defense (Sharma *et al,* 2021) (**Figure I.5**).

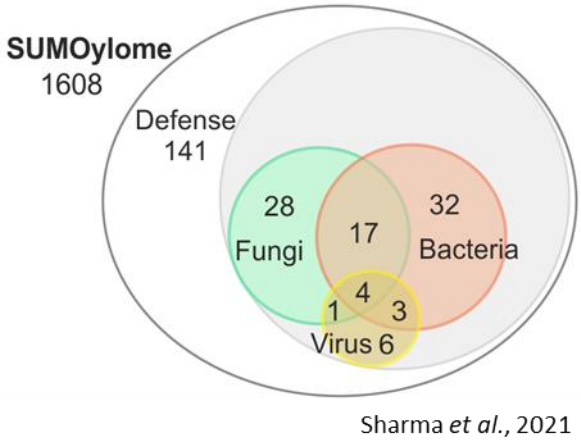


Figure I.5 – Venn diagram showing distribution of potential SUMO targets with a role in defense. *From a total of 141 candidates, 28 are specific to pathogenic fungi, 32 to bacteria and 6 to virus. The proteins involve in defense responses against multiple pathogens are indicated at the intersections.*

The first report on plant SUMOylation uncovered the protein-protein interactions between the tomato SUMO (T-SUMO) and the fungal effector ethylene-inducing xylanase. EIX is secreted by the fungus *Trichoderma viride* and elicits plant defense responses leading to programmed cell death. EIX was shown to interact with T-SUMO in yeast two-hybrid and *in vitro* pull-down experiments. The treatment with this fungal protein caused the reduction of T-SUMO mRNA levels in tomato leave, while the overexpression of T-SUMO attenuated induction of ethylene biosynthesis and cell death in transgenic tobacco plants (Hanania *et al.*, 1999).

Another of the first studies that related SUMOylation to biotic stress identified the interaction between the SUMO conjugating enzyme and RepAC1 (Replication Initiation Protein) of TGMV (Tomato Golden Mosaic Virus) and TYCLSV virus (Tomato Yellow Leaf Curl Sardine Virus) (Castillo *et al.,* 2004). Since then, different bacterial elicitors have been characterized with the ability to deconjugate SUMO substrates and manipulate the post-translational modification system (Park and Yun, 2013).

Another of the defense mechanisms of plants against infection by pathogens is the Systematic Acquired Response (SAR), which is preceded by the accumulation of Salicylic Acid (SA) that activates the SA signaling cascade involving the activation of pathogen response genes (PR genes). The *siz1-3* mutants present a greater accumulation of SA, therefore, the response to SAR is constitutively activated, causing a greater expression of PR genes in basal conditions. This cascade promoted by the accumulation of SA gives rise to greater resistance of these mutants against infection by the pathogen *Pseudomonas syringae* (PstDC3000) (Lee *et al.,* 2006). In addition, this accumulation of SA also results in stomatal closure, which reduces pathogen invasion and confers tolerance to water stress (Miura *et al.,* 2012).

Another evidence of the importance of SUMOylation against infection by pathogens is given by the existence of pathogen factors that interact with the SUMOylation machinery. Interestingly, several virulence factors with cysteine protease activity have been identified that have peptidase and isopeptidase activity against SUMO, acting as SUMO-specific proteases. These virulent factors deregulate the balance of free and conjugated SUMO in the cell, compromising the host's defense system (Hotson and Mudgett, 2004).

Besides, the SUMOylation of NPR1 has been described, an essential regulator in the basal and acquired resistance of plants, which confers immunity through a transcriptional cascade including transcriptional activators (TGA3) and repressors (WRKY70) of antimicrobial genes. SUMOylation of NPR1 by SUMO3 activates the expression of defense genes favoring the association of NPR1 from WRKY repressors to TGA transcriptional activators. In addition, SUMOylation of NPR1 also activates its own degradation allowing a transient induction of the immune response. Together with phosphorylation that inhibits NPR1 modification by SUMO, these PTMs enable dynamic and robust post-translational control to control the immune response (Saleh *et al.*, 2015).

In infection experiments of *Arabidopsis* by the necrotrophic fungi *Plectosphaerella cucumerina*, a depletion of free SUMO and SUMO conjugates were observed in the first 48 h after plant inoculation with fungal spores. It has been suggested that this depletion is the result of an unknown posttranscriptional mechanism that also affected the E1-large subunit SAE2 and the SCE1 conjugating enzymes turnover (Castaño-Miquel *et al.,* 2017). These are the first evidences for the existence of a pathogenic strategy targeting plant SUMOylation.

7. The effect of SUMOylation on hormonal responses

Hormones play an important role in the response to various stresses. ABA is a phytohormone that regulates numerous processes related to the correct development and adaptation of the plant to environmental changes. *At*SUMO1 and 2 overexpressing plants are insensitive to ABA, while plants with reduced *At*SCE1 expression show increased sensitivity to ABA, indicating that SUMOylation negatively regulates the response to ABA (Lois, 2003). The study of the effect of ABA in the mutants of the E3 ligase *At*SIZ1 led to the identification of the ABI5 transcription factor (ABA Insensitive 5) as a SUMO target dependent on SIZ1. Furthermore, SUMOylation of ABI5 at a single lysine prevents its own degradation via the proteasome, which is facilitated by AFP (ABI Five Binding Protein) (Lopez-Molina et al., 2003). Despite SUMO-modified ABI5 accumulates to a greater extent than the non-SUMOylated form, SUMOylation of the ABI5 factor negatively regulates ABA-mediated signaling in the cell (Miura et al., 2009).

It has also been observed that SUMOylation influences the accumulation of SA in the plant. Increased accumulation has been observed in SUMO1 and 2 overexpressing plants and in *siz1* and *esd4* mutants, suggesting that SUMO homeostasis is crucial for SA modulation in the cell (Lee *et al.,* 2006; Van den Burg *et al.,* 2010). Likewise, SUMO has been related to the regulation of signaling pathways of auxins, ethylene, brassinosteroids and cytokinins (Castro *et al.,* 2012).

Various authors highlight that the SUMO ligase E3 MMS21/HPY2 modulates auxin signaling and development processes (Ishida *et al.,* 2009; Zhang *et al.,* 2010; Okushima *et al*., 2014). The expression of MMS21 is regulated by the transcription factors PLETHORA1 (PLT1) and PLT2, which are induced by auxins. It has been hypothesized that PLT1 and PLT2 could act as auxin gradient translators and, which in turn, appear to be SUMO substrates (Ishida *et al.,* 2009).

Hormones play an important role in the response to various stresses, both abiotic and biotic, as well as in plant development. Therefore, understanding how SUMO regulates the response to the different hormones can help us understanding the molecular mechanisms that mediate the biological role of SUMO.

7.1 Ethylene

Plants are exposed to variable environmental conditions, to cope with them, plants produce phytohormones to react quickly and specifically to these changes. An important phytohormone in plants that responds to multiple stresses is ethylene (Dubois *et al*., 2018).

Ethylene is a gaseous phytohormone which is involved in a wide range of plant growth and development processes, including germination, leaf and flower regulation, senescence, cell elongation, fruit ripening, nodulation in symbiotic nitrogen fixation and defense against pathogens. Thanks to its gaseous form, ethylene can allow communication from plant to plant (Mattoo and Suttle 1991; Abeles *et al*., 1992; Goormachtig *et al.*, 2004; Glazebrook, 2005; Dubois *et al*., 2018). It is involved in response to environmental stresses such as nutritional stresses, drought, salinity, flooding, and oxidative stress, among others (Hattori *et al.*, 2009; Pan *et al.*, 2012; Iqbal *et al.*, 2013; Asgher *et al.*, 2014; Tao *et al.*, 2015). Almost all plant tissues can produce ethylene, although in most cases the amount of ethylene produced is very low. Its production can increase dramatically during various processes and can also be affected by various stimuli (Yang and Hoffman, 1984).

Ethylene was reported as a growth inhibitor (Abeles *et al*., 1992). It was first described by plant physiologist Dimitry Neljubow, who observed and abnormal growth of etiolated peas (*Pisum sativum*) when grown in illuminating gas that contained ethylene (Neljubow, 1901). This phenotype observed when plants grow under an ethylene atmosphere was called triple

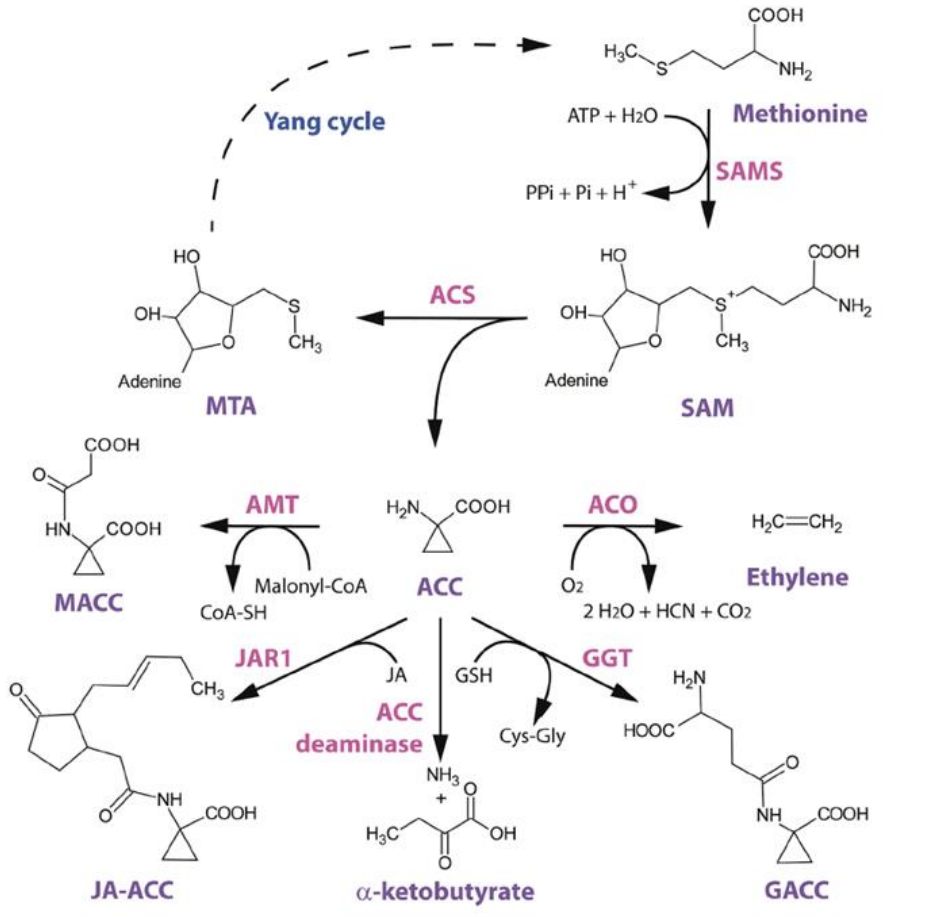
response. The triple response to ethylene is uniform and consists of root inhibition and hypocotyls elongation, radial swelling of the hypocotyl and root, and an exaggeration of the apical hook (Guzman and Ecker, 1990; Solano and Ecker, 1998; Merchante and Stepanova, 2017).

Constitutive ethylene signaling mutants are generally dwarfed with reduced cell growth, consistent with the growth reduction observed when plants are exposed to ethylene (Kieber *et al.*, 1993; Dubois *et al*., 2018). Consequently, ethylene-insensitive mutants are generally found to have larger rosettes with larger expanded leaves, as a result of enhanced cell growth compared to control plants (Hua *et al.*, 1995; Bleecker *et al.*, 1998; Dubois *et al*., 2018).

7.2. Ethylene biosynthesis

The biosynthesis of ethylene occurs through a metabolic pathway, where the general precursor is the amino acid methionine (**Figure I.6**). Methionine is converted to S-adenosylmethionine (SAM) by SAM synthetase using ATP. SAM is converted to 1-aminocyclopropane1-carboxylate (ACC), the immediate precursor of ethylene biosynthesis in higher plants, and 5'-methylthioadenosine (MTA) by ACC synthase (ACS). ACS is a member of the pyridoxal-5'-phosphate (PLP) dependent aminotransferases, which use PLP as a co-factor. The side product MTA is recycled back to methionine by the Yang cycle to prevent methionine depletion during high rates of ethylene production. ACS inhibitors, such as aminoethoxyvinylglycine (AVG) and aminooxyacetic acid (AOA), can decrease ACC biosynthesis. In a third step, ethylene is obtained from ACC by ACC-oxidase (ACO) through an oxygen-requiring reaction (Wang *et al*., 2002; Zhang and Wen, 2010; Van de Poel and Van Der Straeten, 2014; Song and Liu, 2015; Houben and Van de Poel, 2019).

The rate-limiting step for ethylene biosynthesis is mainly ACS, but recent studies suggest that ACO could be the rate-limiting enzyme under specific conditions as low oxygen concentration (Wang *et al*., 2002; Dorling and McManus, 2012).



Van de Poel and Van Der Straeten, 2014

Figure I.6 – Structural scheme of ethylene biosynthesis and 1-aminocyclopropane-1-carboxylicacid (ACC) conjugation/metabolism. *Methionine is converted into SAM by SAM synthetase (SAMS). Next, SAM is converted into methylthioadenosine (MTA) and 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC-synthase (ACS). MTA is recycled back to methionine by the Yang-cycle (dotted line indicates multiple enzymatic steps). ACC can be converted to ethylene by ACC-oxidase (ACO) in the presence of oxygen. ACC can also be converted to 1-malonyl-ACC (MACC) by the ACC-N-malonyl transferase (AMT) with the requirement of malonyl-Coenzyme-A. A second derivate of ACC is γ-glutamyl-ACC (GACC) which is formed by γ-glutamyl-transpeptidase (GGT) with the requirement of glutathione (GSH). Another novel derivate of ACC is jasmonyl-ACC (JA-ACC), which is formed by jasmonic acid resistance 1 (JAR1). ACC can also be metabolized by ACC deaminase into ammonium and α-ketobutyrate.*

The first successes in molecular cloning of the ACC synthase (ACS) (Sato and Theologis, 1989) and ACC oxidase (ACO) (Hamilton et al., 1991; Spanu et al., 1991) genes led to the demonstration that these enzymes belong to a multigene family and are regulated by a complex network of developmental and environmental signals responding to both internal and external stimuli (Wang *et al*., 2002). ACC promotes hypocotyl elongation under light conditions, in contrast to

the well characterized inhibition of longitudinal expansion in the dark (Smalle *et al*., 1997; Vandenbussche *et al.*, 2003).

7.3. Ethylene Responsive Factor (ERF)

Plants need to constantly adapt to climate changes by modifying gene expression patterns. In the modulation of gene expression, the presence of regulatory sequences and proteins capable of directing gene expression is necessary. The regulation of gene expression is one of the most important events in the control of development and responses to climate change. The master proteins in the regulation of gene expression are known as transcription factors.

Transcription factors are proteins that bind to DNA to control genes and stimulate or repress the transcriptional rate of their genes. Transcription factors have fundamental roles in almost all biological processes such as development, growth, and response to stress, and it is assumed that they have played a predominant role in the evolution of species (Tiessen *et al.* 2009). In the presence of ethylene, EIN3 and EIL1 induce the expression of numerous secondary transcription factors. One of the most relevant transcription factors are the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) family transcription factors. The ERF family belongs to the APETALA2 superfamily composed by 122 genes in *Arabidopsis* and are characterized by an APETALA2 (AP2)/Ethylene Responsive Element Binding Factor (EREB) domain, which consists of 40–70 conserved amino acids involved in DNA binding (Nakano *et al*., 2006, Dubois *et al.*, 2018; Xie *et al*., 2019). AP2/ERFs contain the four major subfamilies: APETALA2 (AP2), RELATED TO ABSCISIC ACID INSENSITIVE 3/VIVIPAROUS 1 (RAV), DEHYDRATION-RESPONSIVE ELEMENT BINDING proteins (DREBs) (subgroup A1–A6) and ETHYLENE RESPONSIVE FACTORS (ERFs). The ERF proteins own a highly conserved core AP2 DNA-binding domain called the ERF domain of about 58-59 amino acid sequences (Sakuma *et al*., 2002; Nakano *et al*., 2006; Xu *et al.,* 2008; Debbarma *et al*., 2019; Xie *et al*., 2019). In *Arabidopsis*, 65 ERFs have been identified and 31 have been studied at the phenotypic level. Of these, 22 show a growth phenotype when overexpressed or knocked down, providing multiple additional connections between ethylene and growth-regulating pathways (Dubois *et al.*, 2018).

Through directly binding to target gene promoters, AP2/ERFs can either activate or repress target gene expression. In addition to an N-terminal DNA binding domain, the C-terminal domain of AP2/ERFs mediates the transcriptional regulation of target gene in *Arabidopsis* and rice (Nakano et al., 2006).

These transcription factors are involved in diverse biological processes in plant, such as developmental processes, phytohormone signalling pathways and regulation of metabolic pathways, as well as post-translational control (Xie *et al*., 2019).

7.4. Biological roles of ethylene

Responses to abiotic stresses

ERFs regulate responses to numerous abiotic stresses such as cold, drought, heat, salt, and freezing. ERFs responses to multiple stimuli and regulation of different stresses allow them to form a more complex stress response network, some ERFs are rapidly and continuously induced, while others are regulated by prolonged stress, which indicates that they may have a mutual influence on each other's function (Dubois *et al.*, 2018; Xie *et al*., 2019).

ERF transcription factors are able to bind to dehydration responsive elements (DRE) and GCC box. ERF1 binds to DRE elements (A/GCCGAC) of several genes including early response to dehydration 7 (ERD7), responsive to desiccation 29B (RD29B) and RD20, conferring tolerance to various stresses including drought, heat, and salinity (Cheng *et al*., 2013; Husain *et al*., 2020).

The most intensively studied ERF in abiotic stress responses are the DREB proteins. Members of the DREB1/CBFs subfamily are rapidly induced in response to cold stress and, when expressed ectopically, improve tolerance to freezing. Metabolomic analyses show that many of the metabolites that accumulate during cold stress also accumulated in *Arabidopsis* plants ectopically expressing DREB1/CBF. The DREB2 subgroup has eight members in *Arabidopsis* and homologs in the genomes of many angiosperm species. Among the members of the DREB2 subfamily, DREB2A and DREB2B are induced by dehydration, high salinity and heat in an ABA-independent manner. The ectopic expression of a constitutively active form of DREB2A exhibits improved tolerance to drought, high salinity and heat stresses. By contrast, mutants in DREB2A are more sensitive to heat shock (Licausi *et al.*, 2013).

Ethylene helps to enhance ROS production under various abiotic stresses and its synthesis and signaling are mainly regulated by ERFs. The main reason behind the elevated level of ROS is the ERF1-mediated downregulation of superoxide dismutase (SOD) and peroxidase (POD), two ROS scavenger enzymes. The role of the interaction of ethylene and ROS in plants under metal stress is also reported (Husain *et al*., 2020).

Responses to biotic stresses

Pathogen recognition by the plant immune system is regulated by phytohormone signaling network. The main signaling branches are mediated by SA, JA, ethylene and phytoalexins, which interact between each other to provide specificity to defense responses (Tsuda *et al.,* 2009).

Nevertheless, the plant immune network is also influenced by other phytohormones such as auxins, cytokinins, ABA, GAs, and BRs (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

Ethylene biosynthesis is one of the immune responses of crops to pathogen and is associated with the induction of defense. In addition, ethylene biosynthesis in response to a pathogenic attack can promote disease development rather than alleviating it (Tzeng and De Vay, 1985; Abiri *et al*., 2017). Ethylene acts as modulator of interaction between plants with several enemies, activating or repressing determined branches of the defense network in combination with SA or JA-Ile (Groen *et al.,* 2013).

ERF transcription factors are fundamental molecules in the modulation of various plant development processes and play a crucial role in plant immunity. It has also been shown that various ERF transcriptional activators confer greater disease resistance when overexpressed and decrease resistance when disrupted (McGrath *et al.,* 2005; Bethke et al., 2009; Moffat *et al.,* 2012; Meng *et al.*, 2013; Dubois *et al*., 2013; Huang *et al*., 2016; Van der Broeck *et al.*, 2017; Dubois *et al*., 2018).

The AP2/ERF2 factors have a regulatory role in the expression of pathogenesis-related (PR) genes. ERF1 acts downstream of EIN3/EIL1 and is rapidly induced by both jasmonic acid and ethylene. ERF1 binds to GCC box in promoters of jasmonic- and ethylene-responsive plant defensin (PDF1.2) and basic chitinase (b-CHI). PR gene expression is rapidly induced conferring resistance to pathogens (Solano *et al*., 1998; Abiri *et al*., 2017). Several ERFs activate transcription of basic-type defense-related genes, PR genes, osmotin, chitinase and β-1,3-glucanase, although the set of target genes regulated by each ERF has not been completely elucidated. Probably, the most extensively characterized ERF are ERF1 and its homologues belonging to the ERF-IX group (Licausi *et al.*, 2013).

Ethylene can produce antagonistic effects on SA signaling by blocking its production (Chen *et al*., 2009), but ethylene and SA can also act synergistically to contribute to immunity against both necrotrophic and biotrophic pathogens (Tsuda *et al.*, 2009). The effect of ethylene on disease resistance depends on the pathosystem and the conditions, considering that many pathogens are also capable of producing ethylene, which makes interpretation of the results even more difficult (Van Loon *et al*., 2006). For example, strains of the bacterial pathogens *Pseudomonas syringae* pv. *glycena* and *Pseudomonas syringae* pv. *phaseolicola* have been shown to be able to produce ethylene *in planta*, and those unable to produce ethylene due to a mutation on the ACO gene are defective in their ability to grow in soybean plants (Weingart and Volksch, 1997; Weingart *et al.*, 2001).

In summary, all data points to a crucial SUMO in development of stress responses, including defense against pathogens. The mechanisms supporting a potential role of SUMO in the coordination of development-defense balance have not been identified. The present thesis aims to uncover this mechanism.

Objectives

The general objective of this thesis is to study the potential role of protein SUMOylation as a modulating mechanism of the interconnection between defense processes and plant development.

To achieve this objective, the following specific objectives have been developed:

Objective 1: Identification of the SUMOylome (proteins modified by SUMO) with a dual role in the regulation of plant development and defense processes by *in-silico* analysis.

Objective 2: Functional analysis of SUMOylation of the selected target proteins focusing on their ability to regulate development and defense processes in *Arabidopsis*.

Results

Chapter 1: Effect of altered SUMOylation on plant responses to auxin

One of the main objectives of this work was to study the interactions between SUMOylation and auxin signaling in the defense response in *Arabidopsis*. To achieve this aim, the first approach was to understand the effect of increasing or decreasing SUMOylation capacity of plants on plant responses to auxins. The conditions to be used for the evaluation of the phenotypic characteristics of the wild-type or SUMOylation- deficient lines in the presence or absence of auxins and auxin transport inhibitors were established.

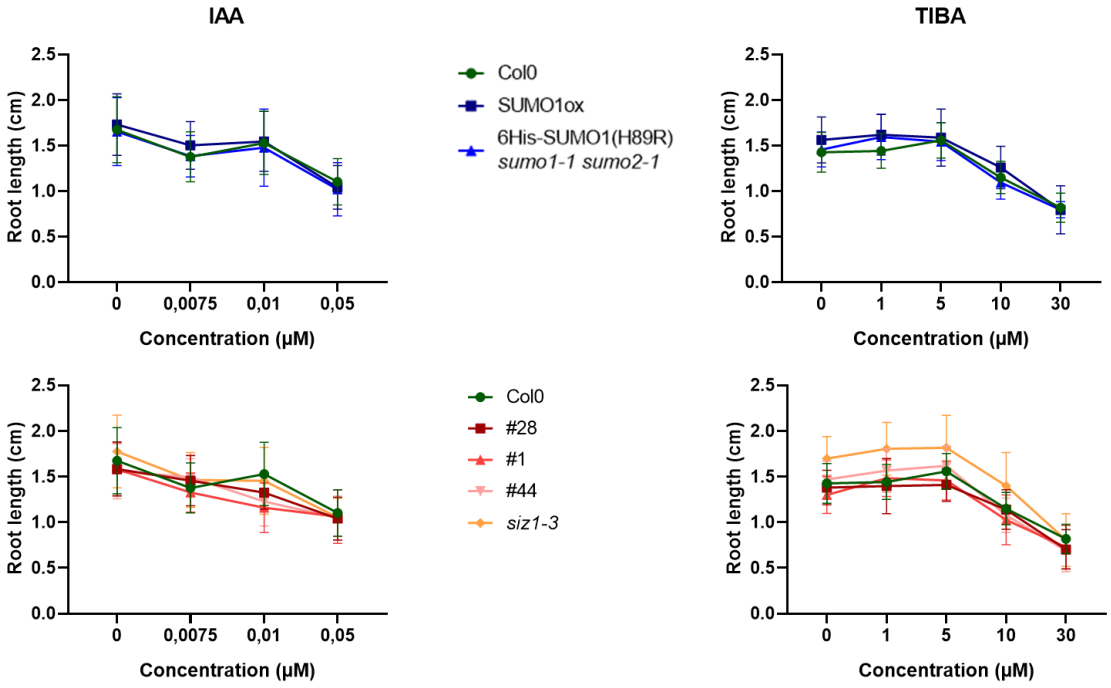


Figure 1.1 – Effect of altered SUMOylation in the plant responses to auxin. *Arabidopsis seeds were germinated on a MS with vitamins (MSv) medium for 3 days before seedlings were transferred to new MSv medium supplemented with IAA (0, 0.0075, 0.01 or 0.05 µM) or TIBA (0, 1, 5, 10 or 30 µM) for 4 additional days. At that point, total root length was measured for each growth condition from 3 biological replicates (30-60 plants per line and replicate). Growth conditions consisted of a photoperiod of 12 h light/12 h dark and constant temperature of 22 ºC. Plants with increased SUMOylation (SUMO1ox and 6His-SUMO1(H89R)) or decreased SUMOylation (lines #28, #1, #44 and siz1-3, from lower to higher reduction) were compared with Col0 plants.*

These assays were performed using transgenic *Arabidopsis* plants that express the E1 SAE2^{UFDCt} domain under the control of the CAMV 35S promoter, giving rise to different levels of accumulation of SUMO conjugates, inversely related to the expression levels of the SAE2^{UFDCt} domain (Castaño-Miquel *et al.,* 2017). A line exhibiting increased SUMOylation by His:SUMO1 expression under the control of the 35S promoter (Lois *et al.,* 2003) and a *sum1-1 sum2*-1 double

mutant, complemented with the His:SUMO1 H89R variant under the expression of the endogenous promoter of SUMO1 was also included (Miller *et al.,* 2010).

With the results obtained at the different concentrations (**Figure 1.1**), both auxin and auxin transport inhibitor, no significant differences were observed in the root phenotype in plants with altered levels of SUMOylation compared to the control. These results don't support the hypothesis that SUMOylation influences the response to auxins, at least in the lines studied.

Chapter 2: Identification of SUMO targets

Previous studies have shown that protein SUMOylation is necessary for the resistance of *Arabidopsis* to infection by the necrotrophic fungi *Botrytis cinerea* and *Plectosphaerella cucumerina* (Castaño-Miquel *et al.,* 2017). In addition, they demonstrated that protein SUMOylation is depleted in response to infection, suggesting that the SUMOylation machinery might be a target of fungal pathogenicity. These studies used *Arabidopsis* transgenic lines in which protein SUMOylation is inhibited, by means of a novel strategy based on the *in vivo* disruption of the interaction of the E1-E2 SUMO enzymes, through the expression of the E1 domain responsible for E2 recruitment (SAE2UFDCt). These transgenic lines show growth defects, are smaller, have fewer leaves and a lower seed production than wild type, and the extent of these phenotype defects correlate with the degree of SUMOylation inhibition in a dose-dependent manner (Castaño-Miquel *et al.,* 2017). This study clearly demonstrates that protein SUMOylation plays an important role in plant growth and development, as well as in the defensive response; and it is the starting point of the doctoral thesis proposed here, whose general objective is to study the role of SUMOylation at the crosstalk between the defense response and the development processes of plants.

2.1. *In-silico* selection of SUMO candidates

For the purpose of identifying possible SUMO candidates with a role in development and defense, the first approach was to make a compilation of possible SUMO substrates identified in bibliography. First, a database was created using the FileMaker Pro Advanced v17 program which contains relevant information about the substrates, such as the biological function, whether they have been experimentally validated as SUMO substrate and related publications, among others. The **Table 2.1** summarizes the sources of the SUMO substrates added to the database.

In first place, data on SUMO substrates included in the SUMO Gene Network (SGN) V1 database was collected. SGN consists of a collection of genes which are experimentally linked to plant SUMOylation pathway (Castro *et al.,* 2016). Starting from this database, 512 possible SUMO substrates where annotated.

150 proteins from Augustine and Vierstra (2018) were introduced. This publication describes the SUMOylation system in plants, as well as a list of SUMO targets from articles published from 2003 to 2018 involved in biological process such as reproductive development, response to biotic and abiotic stress and transcriptional silencing genes, among others.

Table 2.1 – Sources used for identification of SUMO substrate candidates by data mining. *The table shows the reference, the description and the number of substrates found.*

Reference	*Description*	*Nº of substrates retrieved*
SUMO Gene Network	Online database	512
Augustine and Vierstra, 2018	SUMO targets (2003-2018)	150
Nukarinen et al., 2017	Proteome and phosphoproteome of plants of Col0 and mutants in PIAL1 and PIAL2	54
Rytz et al., 2018	SUMO conjugates from plants grown under normal and heat stress conditions were purified by affinity chromatography and identified by mass spectrometry	51

54 possible targets found by Nukarinen *et al.* in 2017 was also added to the database. In this article they analyzed the proteome and phosphoproteome of extracts from the complete aerial part of plants obtained in early flowering stages, both from Col0 and from mutants of the PIAL1 and PIAL2 ligases, involved in the formation of SUMO chains. The authors select those proteins significantly accumulated in *pial1pial2* mutants and whose peptides have a higher probability of possessing a phosphorylation site. From the proteome analysis, the proteins involved in SUMOylation [SAE2 (SUMO-activating enzyme 1), SCE1 (SUMO-conjugating enzyme 1), SUMO1 and SUMO2 (small ubiquitin-related modifiers 1 and 2)] were detected and quantified, observing similar amounts between ligase mutants and wild type but increased in mutants lacking SIZ1. Proteins showing changes in concentration, along with phosphopeptides showing distinct phosphorylation patterns between wild-type and mutants, were subjected to a Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), showing that SUMOylation influences phosphorylation. Finally, SUMOylation motifs of 54 proteins (represented by 99 phosphopeptides in the dataset) with a significantly changed phosphorylation state were analyzed.

Additionally, in Rytz *et al*. (2018) SUMOylome was identified in SUMO E3 ligase *siz1* and *mms21* mutants expressing the His:SUMO1H89R variant, designed to facilitate the identification of the SUMO acceptor lysine during mass spectrometry analysis. In this work, SUMO substrates present in plants grown at normal temperatures or exposed to heat stress were purified by affinity chromatography and identified by mass spectrometry. Among the candidate proteins, candidates were selected based on: not being present in wild-type plants samples [6His-SUMO1(H89R) *sumo1-1 sumo2-1*, ecotype Col 0] and identified in which at least two peptides spectral matches (PSM). From this data, those SUMO1 substrates considered abundant based

on their detection in at least 3 of the 5 replicates of the heat stressed wild type and *siz1-2* mutant samples are selected, evaluating whether they have a significant change in SUMOylation. With the results published in this study, it was decided to add to the database 51 proteins described as SUMO targets involved in the response to abiotic stress.

A

Biological process	Reference	Upload	Expected	Fold Enrichment
regulation of defense response (GO:0031347)	249	25	6	4.04
response to bacterium (GO:0009617)	493	30	12	2.45
response to other organism (GO:0051707)	988	51	24	2.08
response to external biotic stimulus (GO:0043207)	988	51	24	2.08
response to biotic stimulus (GO:0009607)	989	51	24	2.07
negative regulation of post-embryonic development (GO:0048581)	111	15	2	5.43
seedling development (GO:0090351)	115	13	2	4.54
negative regulation of developmental process (GO:0051093)	144	16	3	4.47
regulation of flower development (GO:0009909)	180	20	4	4.47
regulation of post-embryonic development (GO:0048580)	387	42	9	4.36
regulation of multicellular organismal development (GO:2000026)	535	46	13	3.46
regulation of shoot system development (GO:0048831)	263	21	6	3.21
reproductive shoot system development (GO:0090567)	405	29	10	2.88
regulation of developmental process (GO:0050793)	720	51	17	2.85
flower development (GO:0009908)	389	27	9	2.79
reproductive structure development (GO:0048608)	1219	83	30	2.74
reproductive system development (GO:0061458)	1221	83	30	2.73
post-embryonic development (GO:0009791)	1477	98	36	2.67
tissue development (GO:0009888)	572	37	14	2.60
shoot system development (GO:0048367)	743	47	18	2.54
embryo development ending in seed dormancy (GO:0009793)	560	34	13	2.44
embryo development (GO:0009790)	576	34	14	2.37
system development (GO:0048731)	1830	107	45	2.35
seed development (GO:0048316)	726	42	18	2.33
fruit development (GO:0010154)	757	43	18	2.28
multicellular organism development (GO:0007275)	2613	134	64	2.06
anatomical structure development (GO:0048856)	2965	144	73	1.95
developmental process (GO:0032502)	3147	147	78	1.88

B

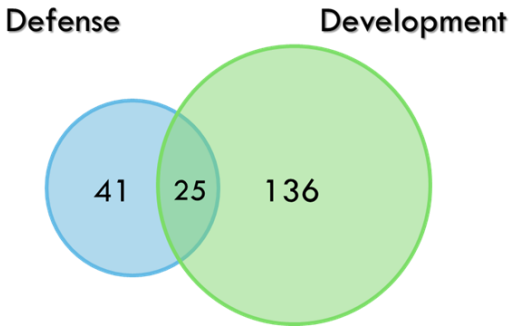


Figure 2.1 – Classification of the 644 SUMO substrate candidates according to their relationship with defense or development. (A) *Analysis of the biological processes using Gene Ontology Enrichment. Defense-related proteins are shown in orange and development-related proteins in green.* ***(B)*** *Venn diagram showing the number of proteins involved in defense, the number of proteins involved in development, and the number of proteins involved in both processes.*

In total, 644 potential unique SUMO substrates were identified and added into the database. As we are interested in defense and development candidates, all SUMO targets were introduced into the Gene Ontology Enrichment for Plants available on the TAIR website

[https://www.*arabidopsis*.org/tools/go_term_enrichment.jsp]. Using the biological category as criteria, those related to development or with defense were selected (**Figure 2.1 A**).

Table 2.2 – List of the 25 identified proteins predicted to have a role in both development and defense with their correspondent description.

ID	*Gene*	*Description*
AT1G32640	**MYC2, JIN1, ZBF1, JAI1**	Transcription factor
AT5G47220	**ERF2**	Ethylene-responsive transcription factor
AT4G17490	**ATERF6, ERF-6-6, ERF6**	Ethylene-responsive transcription factor
AT5G61600	**ERF104**	Ethylene-responsive transcription factor
AT3G28910	**MYB30**	Transcription factor
AT5G22330	**RIN1, ATTIP49A**	RuvB-like protein
AT2G35980	**ATNHL10, NHL10, YLS9**	NDR1/HIN1-like protein
AT3G01500	**ATBCA1, ATSABP3, CA1, SABP3**	Beta carbonic anhydrase 1, chloroplastic
AT1G05010	**ACO4, EAT1, EFE**	Aminocyclopropane-1-carboxylate oxidase
AT1G43850	**SEU, SEUSS**	Transcriptional corepressor
AT2G32700	**LUH, MUM1**	Transcriptional corepressor
AT4G32551	**LEUNIG, LUG, RON2**	Transcriptional corepressor
AT2G18790	**HY3, OOP1, PHYB**	Phytochrome B
AT1G25540	**PFT1, GLH1, MED25**	Mediator of RNA Polymerase II Transcription Subunit
AT5G60410	**SIZ1**	E3 SUMO-Protein Ligase SIZ1
AT4G38130	**HD1, HDA1, HDA19, RPD3A**	Histone deacetylase
AT1G77300	**ASHH2, CCR1, EFS, LAZ2, SDG8**	Histone-lysine N-methyltransferase
AT4G20260	**ATPCAP1, MDP25, PCAP1**	Plasma membrane-associated cation-binding protein
AT5G02500	**HSP70, HSC70-1**	Probable mediator or RNA polymerase II transcription subunit
AT5G08790	**ANACO81, ATAF2**	Putative transcriptional activators with NAC domain
AT3G10490	**ANACO51, ANACO52, NAC52, NAC052, SGS1**	NAC domain containing protein
AT5G13010	**EMB3011, CUV, PINP1, PRP16**	Pre-mRNA-splicing factor ATP-dependent RNA helicase
AT3G09840	**ATCDC48, ATCDC48A, CDC48, CDC48A**	Cell division control protein
AT3G12140	**ATEML1, EML1**	Protein EMSY-LIKE
AT4G02640	**BZO2H1, ATBZIP10**	Basic leucine zipper

Taking the GO list classification, two list were created, one list with unique candidates related to development and another list with unique candidates related to defense. Both lists were merged into one list containing development and/or defense related candidates. Finally, the proteins that appeared more than once were analyzed, understanding that these proteins

had a role in both development and in defense (**Figure 2.1 B**). In this way, we obtained a total of 25 possible SUMO targets with a function in development and in defense (**Table 2.2**).

To reduce the candidates to evaluate, an analysis based on the response of these genes to different treatments was performed, studying the effect against fungi infection and fungal elicitors as chitin (**Figure 2.2 A**). This analysis was complemented with an interaction network to see the protein connections (**Figure 2.2 B**).

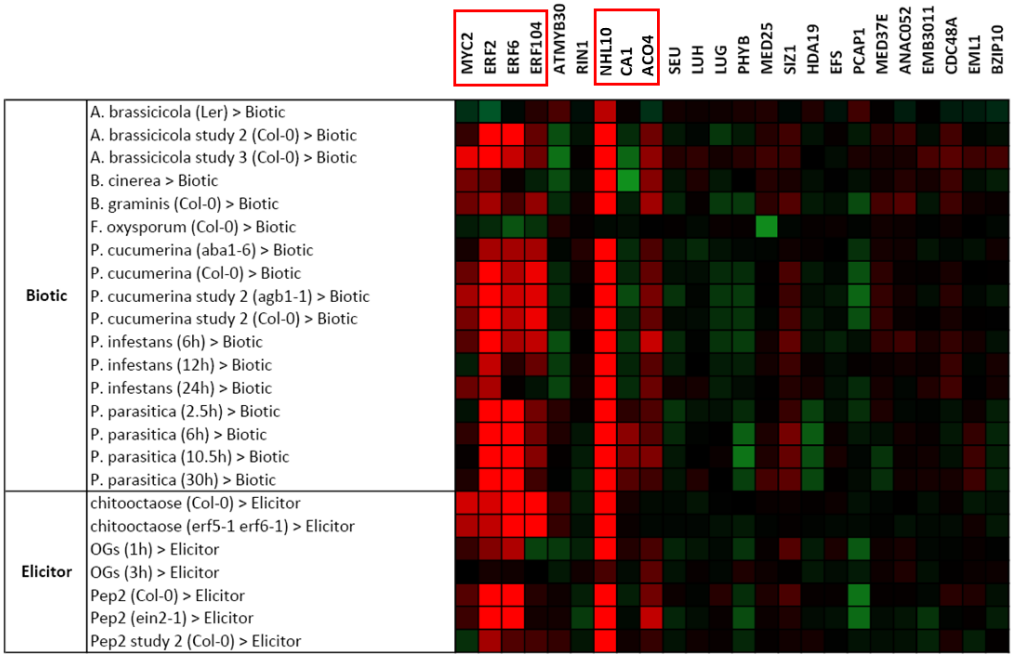
7 of those proteins were selected to be evaluate as bona fide SUMO substrates. ERF1 and ICE2 were also included based on the relevant role in development and defense and that they were validated as SUMO substrates. EIN3, an important transcription factor in the ethylene signaling pathway and involved in defense and development, was confirmed previously as SUMO substrate in this research group (Schapire and Lois, in preparation) (**Table 2.3**).

Table 2.3 – Complete list of the proteins selected for evaluation as bona fide SUMO substrates with their correspondent description.

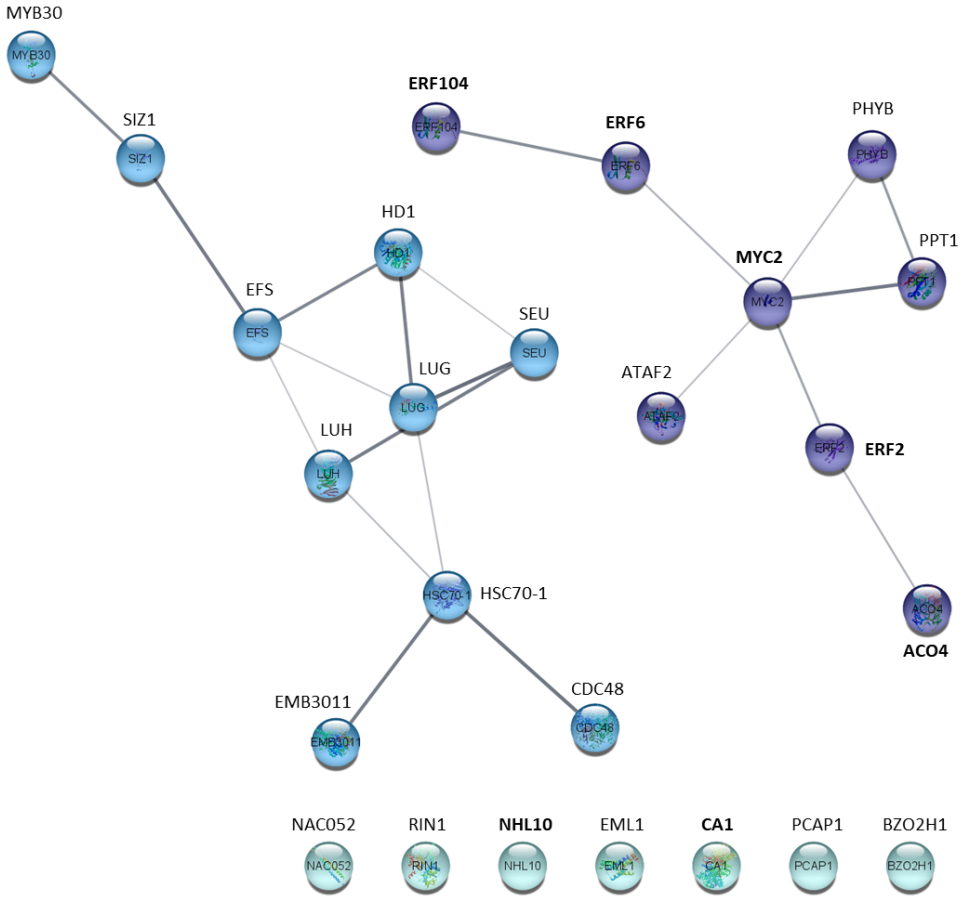
ID	*Gene*	*Description*
AT1G32640	**MYC2**	Transcription factor MYC2
AT5G47220	**ERF2**	Ethylene-responsive transcription factor 2
AT4G17490	**ERF6**	Ethylene-responsive transcription factor 6
AT5G61600	**ERF104**	Ethylene-responsive transcription factor 104
AT2G35980	**NHL10**	NDR1/HIN1-like protein 10
AT3G01500	**CA1**	Beta carbonic anhydrase 1, chloroplastic
AT1G05010	**ACO4**	Aminocyclopropane-1-carboxylate oxidase 4
AT3G20770	**EIN3**	Ethylene Insensitive 3
AT3G23240	**ERF1**	Ethylene-responsive transcription factor 1
AT1G12860	**ICE2**	Transcription Factor SCREAM 2

Figure 2.2 – Expression and network analysis of genes listed in Table 2.2. (A) *Genevestigator Clustering Analysis. Genes were clustered based upon their expression in response to different treatments (rows). The color scale represents log2 ratio of fold change. The red box represents the cluster of genes upregulated under infection with fungi and under treatment with fungal elicitors.* ***(B)*** *Interaction Network representing the connection between the 25 proteins. This network consists of 25 nodes and 21 interactions. STRING was used for the network creation and Cytoscape for the visualization. 7 selected proteins for the SUMO substrate validation are highlighted in bold.*

A


MYC2
ERF2
ERF6
ERF104
ATMYB30
RIN1
NHL10
CA1
ACO4
SEU
LUH
LUG
PHYB
MED25
SIZ1
HDA19
EFS
PCAP1
MED37E
ANAC052
EMB3011
CDC48A
EML1
BZIP10
Biotic
A. brassicicola (Ler) > Biotic
A. brassicicola study 2 (Col-0) > Biotic
A. brassicicola study 3 (Col-0) > Biotic
B. cinerea > Biotic
B. graminis (Col-0) > Biotic
F. oxysporum (Col-0) > Biotic
P. cucumerina (aba1-6) > Biotic
P. cucumerina (Col-0) > Biotic
P. cucumerina study 2 (agb1-1) > Biotic
P. cucumerina study 2 (Col-0) > Biotic
P. infestans (6h) > Biotic
P. infestans (12h) > Biotic
P. infestans (24h) > Biotic
P. parasitica (2.5h) > Biotic
P. parasitica (6h) > Biotic
P. parasitica (10.5h) > Biotic
P. parasitica (30h) > Biotic
Elicitor
chitooctaose (Col-0) > Elicitor
chitooctaose (erf5-1 erf6-1) > Elicitor
OGs (1h) > Elicitor
OGs (3h) > Elicitor
Pep2 (Col-0) > Elicitor
Pep2 (ein2-1) > Elicitor
Pep2 study 2 (Col-0) > Elicitor

B


MYB30
SIZ1
ERF104
ERF6
PHYB
HD1
PPT1
MYC2
EFS
SEU
LUG
ATAF2
LUH
ERF2
HSC70-1
ACO4
EMB3011
CDC48
NAC052
RIN1
NHL10
EML1
CA1
PCAP1
BZO2H1

2.2. Validation of candidates

Based on these results, the conjugation of the candidates has been validated by an assay based on the reconstitution of the SUMOylation machinery in *E. coli* (Okada *et al*, 2009) (**Figure 2.3 A**). To carry out this assay, two types of plasmids carrying the SUMOylation machinery have been used: the first one brings the two subunits of the heterodimer E1, AtSAE1 and AtSAE2 and the second one brings E2 (AtSCE1a) and one AtSUMO isoform (1, 2, 3 or 5). In our experimental design, the AtSUMO1 isoform, which presents a higher conjugation rate, has been used. In this system, the mature form of AtSUMO1 is expressed, which is modified to expose the C-terminus Gly-Gly sequence, necessary for covalent binding to the target protein. As a negative control, AtSUMO1 with the C-terminus mutated to Ala-Ala has been used. AtSAE1 and AtSCE1 are fused to an S-tag at their C-terminus, whereas AtSAE2 and AtSUMO1 are fused to a His-tag at their N-terminus to allow detection by Western-blot (**Figure 2.3 B**) (Okada *et al., 2009)*.

On the other hand, the SUMO substrate candidate protein has been cloned into the pET28 vector, allowing fusion to a His-tag and a T7-tag at the N-terminus. Using the putative substrate and the AtSUMO1 isoform, the SUMOylation reaction occurs in *E. coli* and SUMOylation of the substrate is subsequently analyzed by immunodetection by Western-blot (**Figure 2.3 B** and **C**).

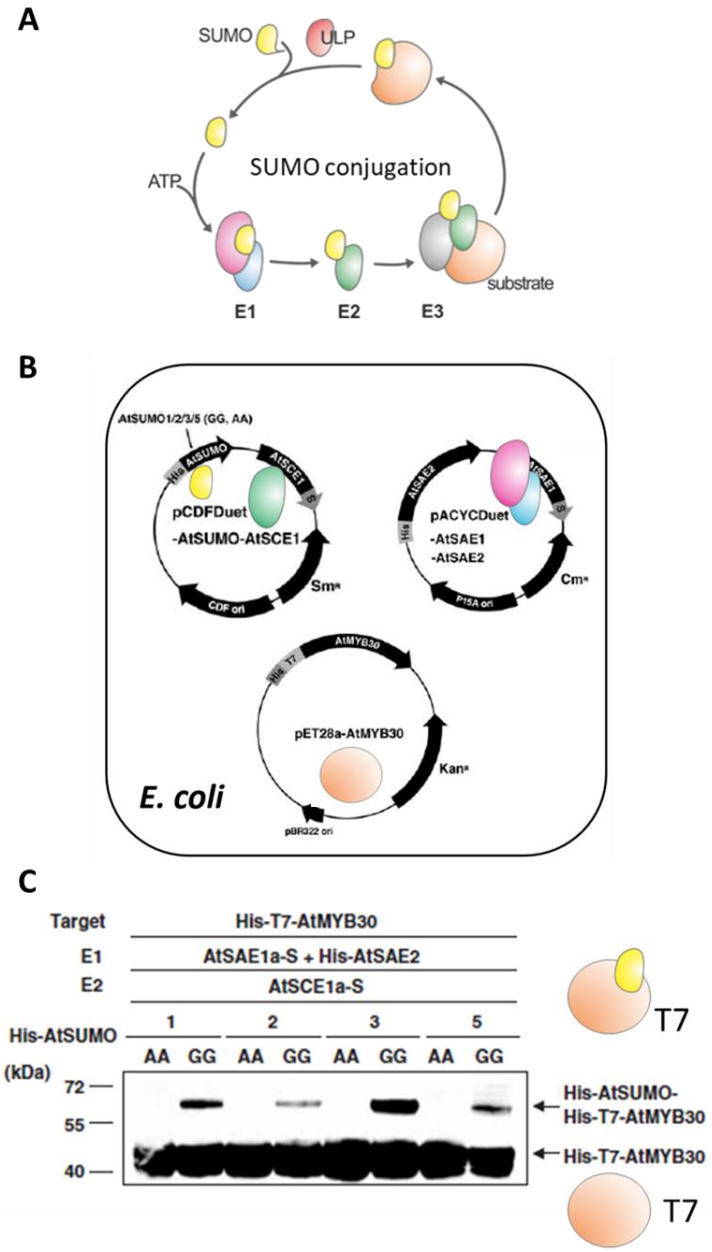


Figure 2.3 – Principles of E. coli SUMOylation assay. (A) *SUMO conjugation/deconjugation cycle.* ***(B)*** *Schematic representation of the plasmid used for the reconstitution of the SUMO machinery in E. coli. The plasmid used are pCDFDuet-ATSUMO-AtSCE1, pACYCDuet-AtSAE1-AtSAE2 and pET28a-AtMYB30 (used as positive control). In Arabidopsis, the SUMO conjugation is composed of the SUMO isoforms SUMO1 (At4g26840), SUMO2 (At5g55160), SUMO3 (At5g55170), and SUMO5 (At2g32765), the of AtSUMO mature forms and AtSAE2 were His-tagged at their N-termini. AtSCE1 and AtSAE1 were S-tagged at their C-termini. The target protein was His- and T7-tagged at the N-terminus. As negative control, the Gly-Gly C-terminal motif is modified to Ala-Ala.* ***(C)*** *the SUMOylation of the target substrate is confirmed by Western Blot analysis using anti-T7 antibody. (Adapted from Okada et al., 2009).*

By performing this assay and using the substrates described in **Table 2.3**, the SUMOylation of the candidates ERF2, ERF6, ERF104 and ACO4 was confirmed (**Figure 2.4**). While we didn't confirm the SUMOylation of the ERF1, ICE2 and CA1 candidates. In the case of NHL10 and MYC2 we were unable to obtain conclusive results.

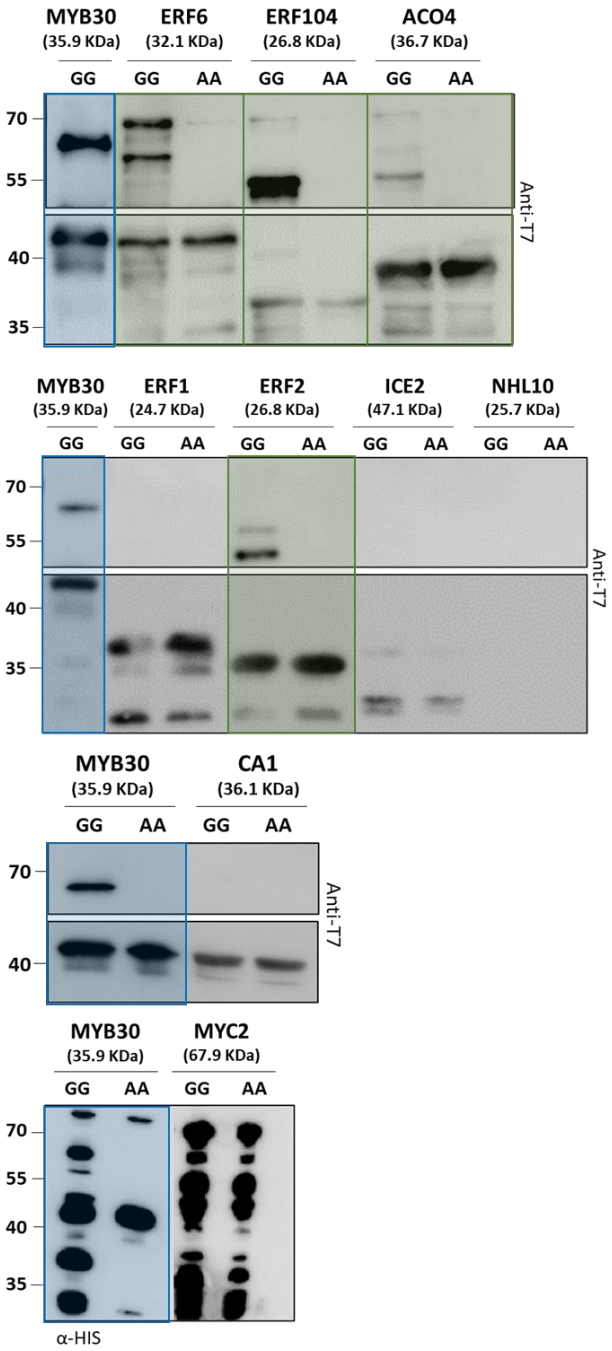


Figure 2.4 – Substrate validation in E. coli SUMOylation assay. *The E.coli strain containing a reconstituted SUMOylation assay (Okada et al., 2009) was transformed with one candidate of interest and induction of recombinant protein production was performed. Total protein extracts from bacteria were resolved by SDS-PAGE and substrate SUMOylation was monitored by western blot. Those substrates modified by active SUMO (GG), ERF2, ERF6, ERF104 and ACO4, are highlighted in green. As a negative control, in a non-conjugable form of SUMO (AA) was used. In the case of NHL10 and MYC2 the results were non-conclusive. MYB30, in blue, was used as control.*

Table 2.4 – List of the confirmed SUMO candidates. *The description and the expected molecular weight are shown. EIN3 is also included since it has been previously confirmed as a SUMO substrate by our group (Schapire and Lois, in preparation).*

Name	*Protein*	*kDa*
ERF2	Ethylene-responsive transcription factor 2	26.8
ERF6	Ethylene-responsive transcription factor 6	32.1
ERF104	Ethylene-responsive transcription factor 104	26.8
ACO4	Aminocyclopropane-1-carboxylate oxidase 4	36.7
EIN3	Ethylene Insensitive 3	71.4

Confirmed SUMO targets from the **Table 2.4**. are involved in the ethylene signaling pathway, which is of special interest since ethylene is a hormone known to play a role in both development and in defense (Song and Liu, 2015; Jordá *et al.,* 2016; Houben and Van de Poel, 2019). **Figure 2.5** represents a schematic illustration of the ethylene pathway and the position of these genes. ACO4, which catalyzes the conversion of ACC to ethylene, is located in the cytosol while the transcription factors EIN3 and ERFs are in the nucleus.

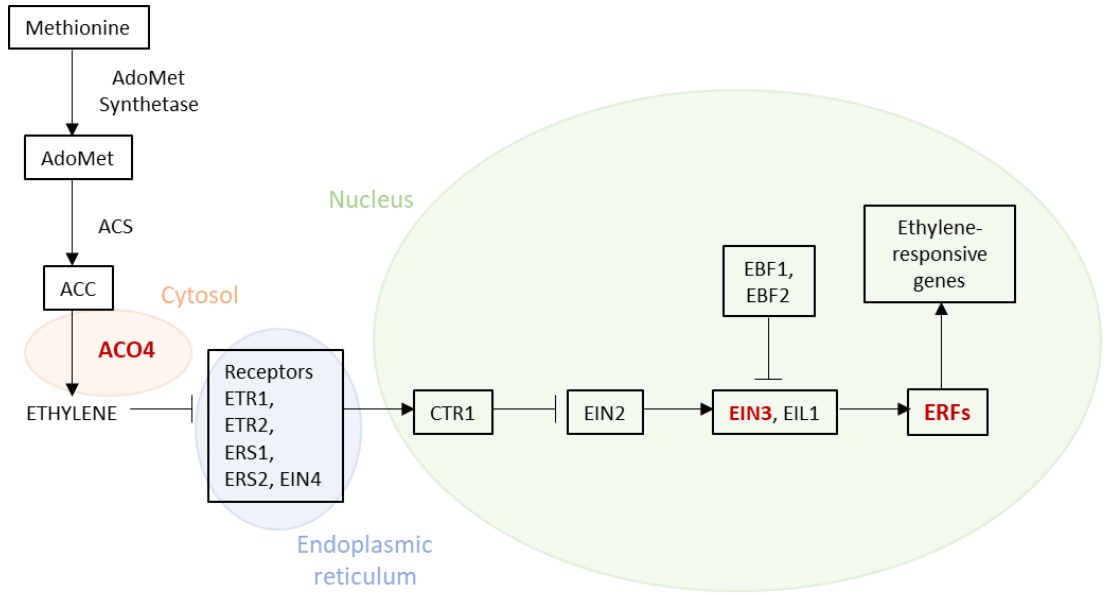


Figure 2.5 – Simplified scheme of the confirmed SUMO candidates positioned in the biosynthesis and signaling pathway of ethylene, including their cellular localization. *Proteins confirmed as SUMO substrates are indicated in red; ACO4 is responsible for catalyzing the conversion of ACC to ethylene, while EIN3, ERF2, ERF6 and ERF104 are transcription factors responsible for regulating the expression of genes related to the defense response. (Adapted from Song and Liu, 2015).*

Chapter 3: Generation of the SUMOylation deficient variants

Once we have confirmed the SUMOylation of some of the candidates, to analyze the effect of the SUMOylation of the validated proteins, the next step is to obtain the SUMOylation-deficient variants to be compared against the native version of the protein.

SUMOylation is a reversible post-translational modifier, which is covalently conjugated to a lysine residue in a substrate protein. Identification of SUMO acceptor sites is essential for mutagenesis studies aimed at understanding the molecular consequences of SUMOylation on target protein function.

3.1. Identification of the SUMO-acceptor lysine(s)

SUMOylation motif is formed by a hydrophobic residue (Ψ), a lysine (K), any amino acid (x) and an acidic amino acid (E/D) (Okada *et al*., 2009; Elrouby and Coupland, 2010) (**Figure 3.1 A**). The identification of these SUMOylation sites and their lysines is essential for carrying out the directed mutagenesis assays. Once potential SUMOylation lysines are identified, they are replaced by arginine, another amino acid with positive charged side chains that show similar physicochemical properties to lysine but is not SUMOylable (**Figure 3.1 B**).

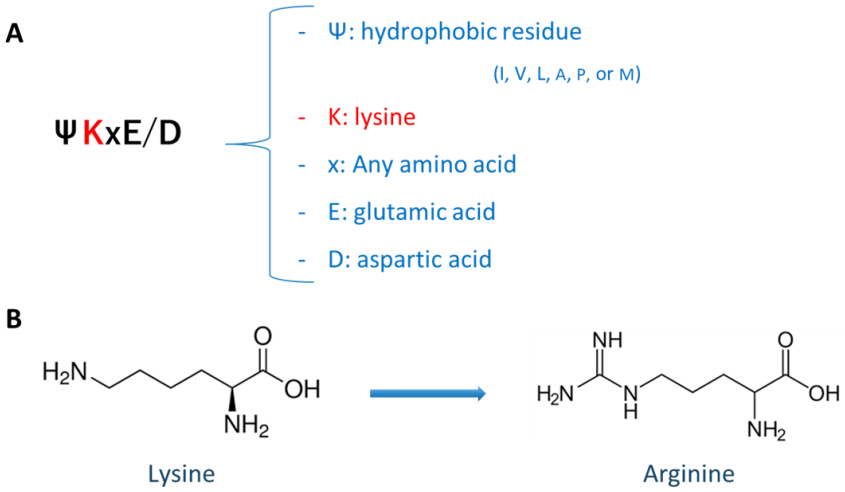


Figure 3.1 – Properties of the consensus SUMOylation motif. (A) *The core consensus motif ΨKxE/D is formed by a hydrophobic residue (I, V, L, A, P or M), the targeted lysine (K, in red), any amino acid (x) and a glutamic (E) or aspartic acid (D).* ***(B)*** *Lysine and arginine play similar functions because of being basic amino acids with higher pKa values (constant used to describe the acidity of a molecule). Yet, they present different chemical properties which affect their capacity to interact with other amino acids and biomolecules (Li et al., 2013). Taking advantage of this, we mutated the lysine from the consensus motif to arginine to obtain the non-SUMOylable variant without destabilize the physicochemical properties of the protein.*

3.1.1. ERF2

The lysines K87 and K228 were found in two ERF2 SUMOylation motifs. K87 is located in a canonical SUMO motif, which is highly conserved across land plants, while K228, which also belongs to a canonical SUMO motif, is less conserved (**Figure 3.2 A**). When the role of these lysine residues as SUMO acceptor sites were analyzed by Western-blot, it was observed that each single mutation removes one band, whereas the double mutant completely abolishes SUMOylation (**Figure 3.2 B**). The SUMOylation of ERF2 is carried out by two lysines, K87 and K228.

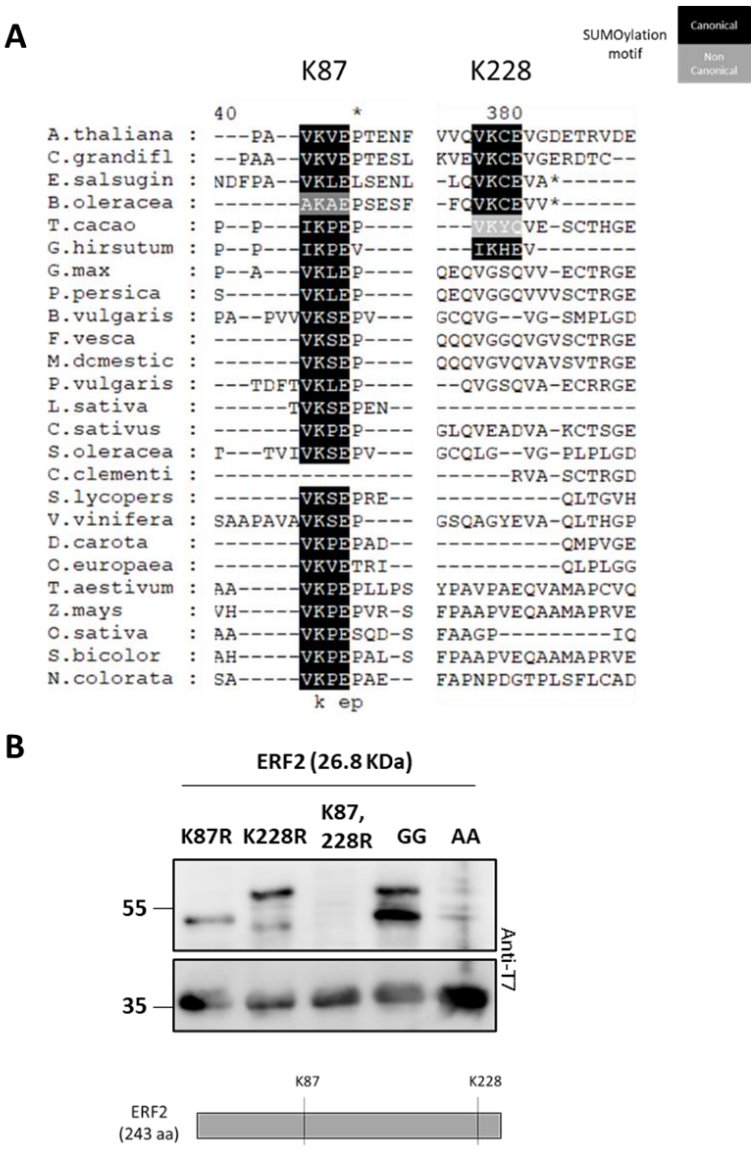


Figure 3.2 – Analysis of the ERF2 SUMO-acceptor lysine residues. (A) *Sequence conservation of the SUMOylation motif from ERF2 orthologs from different higher plants (taxid: 3193). Black or gray shading represent the canonical or non-canonical SUMOylation, respectively. The SUMOylation motif corresponding to the lysine K87 displays the highest conservation along the different species.* ***(B)*** *Candidate lysine residues were validated in the E. coli SUMOylation assay. SUMOylation of native ERF2 and the mutant variants K87R and K228R were analyzed by Western Blot using anti-T7 antibody.*

3.1.2. ERF6

The lysines K53, K63, K72, K121 and K231 were found in five ERF6 SUMOylation motifs. K231 is the most conserved lysine throughout land plants. K72 is also located in a canonical SUMO motif but it's not conserved. Among non-canonical lysine residues, K53 is highly conserved, and it's located in an inverted SUMO motif. The lysine K63 and K121 are non-canonical and no conserved lysine residues (**Figure 3.3 A**). When these lysine residues were analyzed individually, the bands corresponding to the SUMOylation were reduced but not completely removed. The same occurred with the quadruple mutant K63, 72, 121, 231R. The K53 was added in last place obtaining the quintuple mutant where the SUMOylation is completely abolished (**Figure 3.3 B**). The SUMOylation of ERF6 is carried out by five lysine residues, K53, K63, K72, K121 and K231.

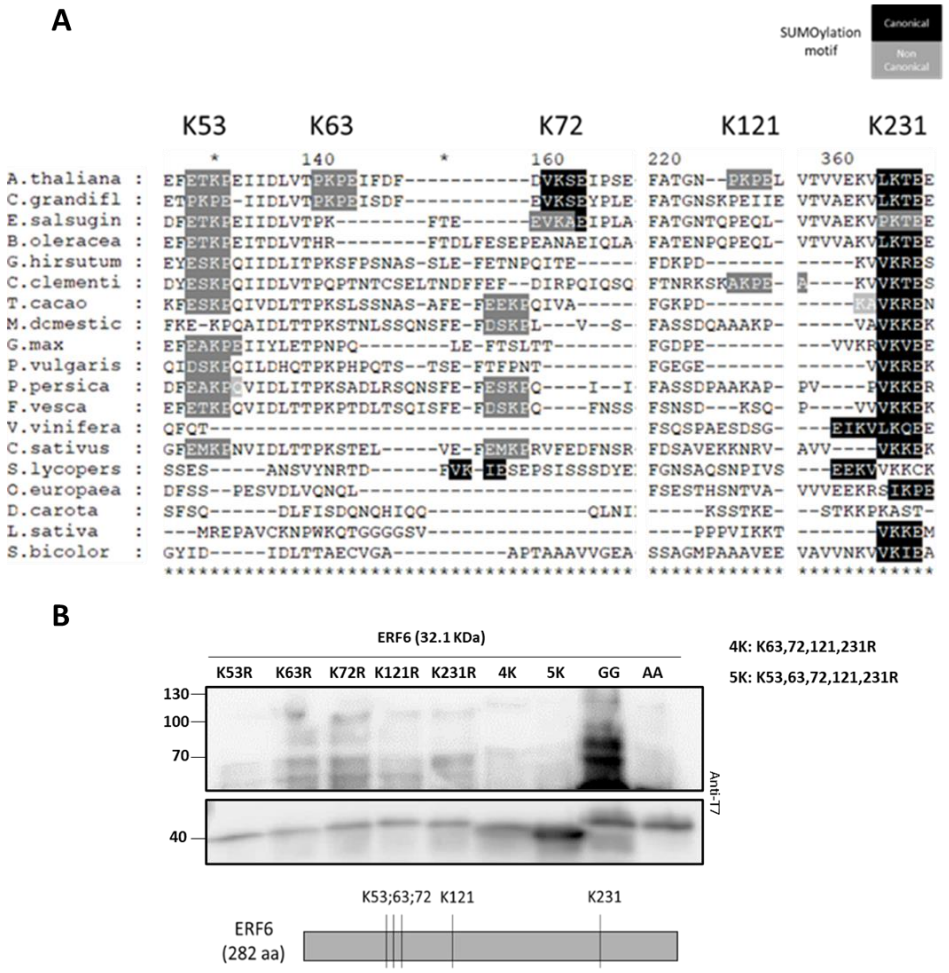


Figure 3.3 – Analysis of the ERF6 SUMOylation lysines. (A) *Sequence comparison of SUMOylation motif from ERF6 orthologs in different higher plants (taxid: 3193). The canonical SUMOylation motif is presented in black and the non-canonical is presented in grey. The SUMOylation motif corresponding to the lysine K231 is more conserved along the different species and the lysines K63 and K121 are less conserved.* ***(B)*** *Results of the SUMOylation of ERF6 and its correspondent lysine mutations K53R, K63R, K72R, K121R and K131R by Western Blot using anti-T7 antibody.*

3.1.3. ERF104

The lysine K81, K178 and K183 were found in three ERF104 SUMOylation motifs. K183 is the most conserved lysine throughout plant lands. Inside the non-canonical lysine residues, K81 and K178 are less conserved. K178 belongs to an inverted SUMO motif (**Figure 3.4 A**). The single mutation K183R impaired SUMOylation but not completely. Additional mutations of K81 and K178 contributes to reduce ERF104 SUMOylation to greater degree than single K183 (**Figure 3.4 B**). No more potential SUMOylation motifs were found in this protein.

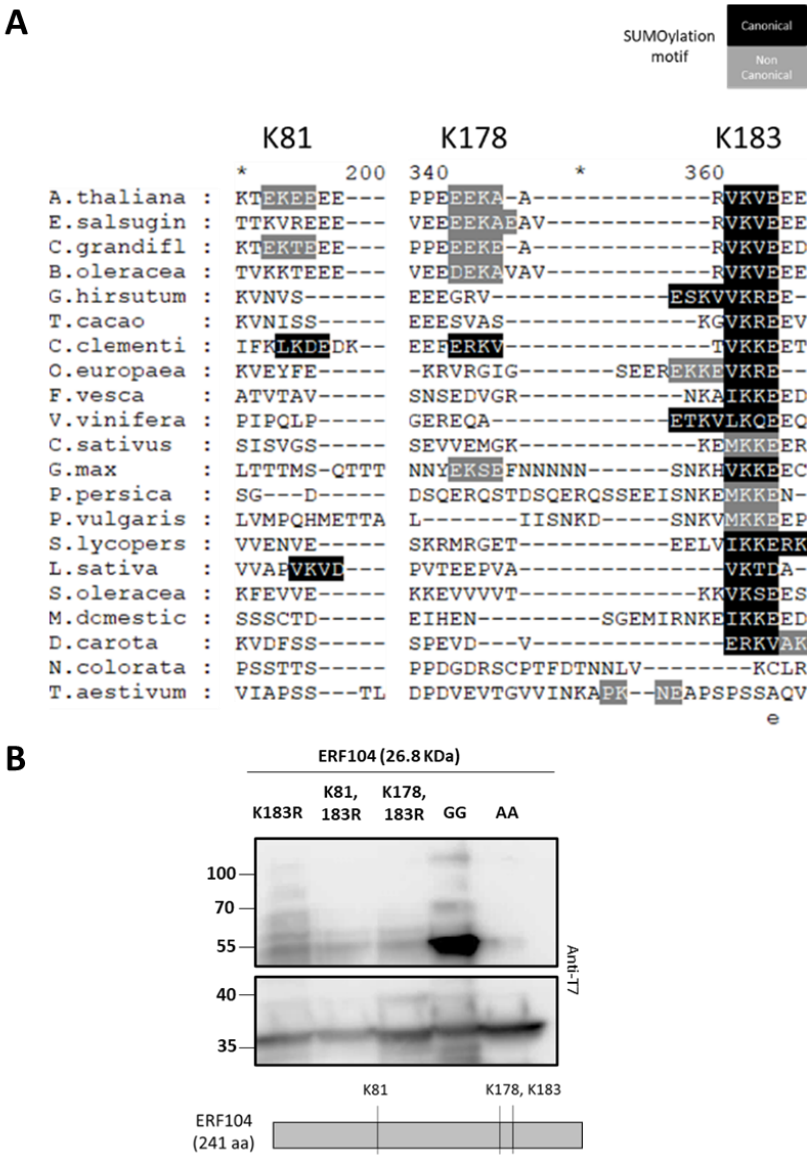


Figure 3.4 – Analysis of the ERF104 SUMOylation lysines. (A) Sequence comparison of SUMOylation motif from ERF104 orthologs in different higher plants (taxid: 3193). *The canonical SUMOylation motif is presented in black and the non-canonical is presented in grey. The SUMOylation motif corresponding to the lysine K183 is more conserved along the different species.* ***(B)*** *Results of the SUMOylation of ERF104 and its correspondent lysine mutations K183R, K81,183R and K178,183R by Western Blot using anti-T7 antibody.*

3.1.4. ACO4

In first place, the lysines K191 and K295 were found in two ACO4 SUMOylation motifs. K191 belongs to an inverted canonical SUMO motif, whereas K295 is in a non-canonical SUMO motif. Both are highly conserved in land plants (**Figure 3.5 A**). When these lysine residues were analyzed, we did not observe even a significant reduction SUMOylation (**Figure 3.5 B**). As we were unable to identify the lysine for SUMOylation in ACO4, new candidate lysine residues were searched to evaluate.

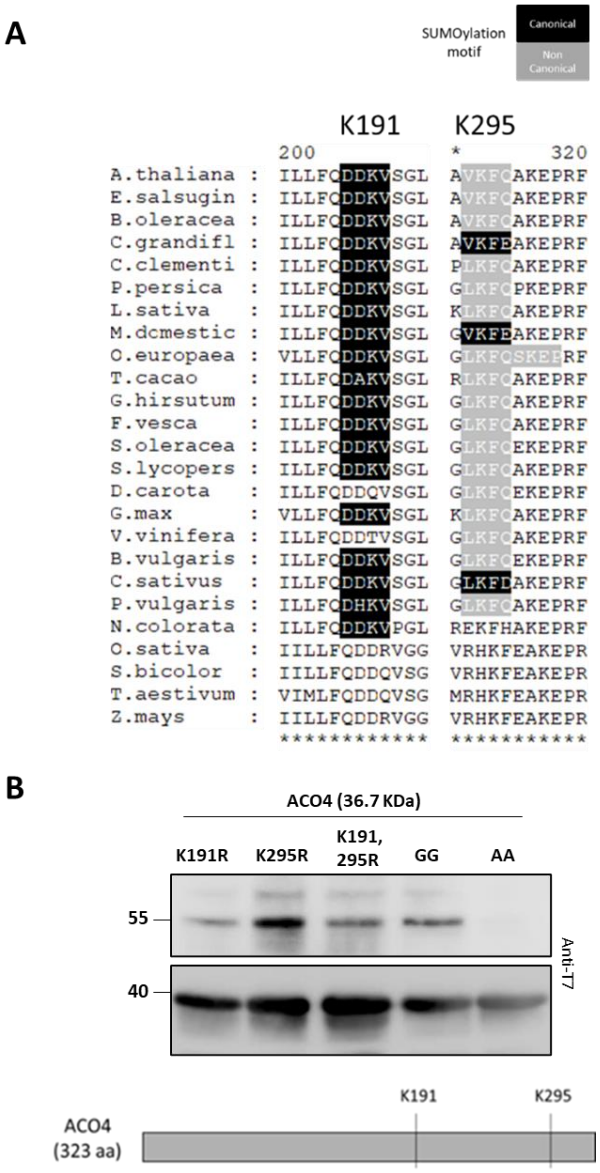


Figure 3.5 – First part of the analysis of the ACO4 SUMOylation lysines. (A) Sequence comparison of SUMOylation motif from ACO4 orthologs in different higher plants (taxid: 3193). *The canonical SUMOylation motif is presented in black and the non-canonical is presented in grey. The SUMOylation motif corresponding to the lysines K191 and K295 are high conserved along the different species.* ***(B)*** *Analysis of the SUMOylation of ACO4 and its correspondent lysine mutations K191R and K295R by Western Blot using anti-T7 antibody.*

In this second attempt, lysine K11, K23, K120, K151 and K289 were found in five ACO4 SUMOylation motifs. All these lysine residues belong to a non-canonical SUMO motif, K23 and K120 are in an inverted SUMO motif. All of them are conserved in land plants, specially K289 (**Figure 3.6 A**). Despite the extension of the analysis, where seven candidates were analyzed, no SUMO acceptor lysine was identified (**Figure 3.6 B**).

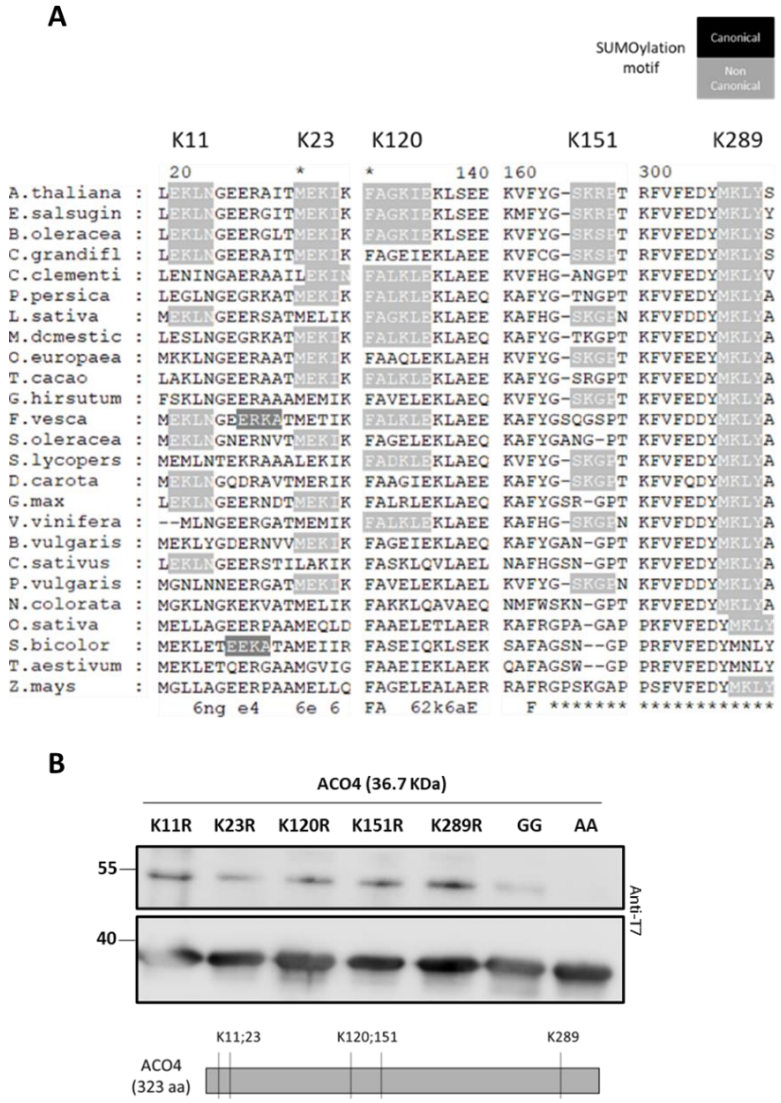


Figure 3.6 – Additional analysis of the ACO4 SUMO-acceptor lysine residues. (A) *Sequence comparison of SUMOylation motif from ACO4 orthologs in different higher plants (taxid: 3193). The canonical SUMOylation motif is presented in black and the non-canonical is presented in grey. The SUMOylation motif corresponding to the lysine K289 is more conserved along the different species.* ***(B)*** *Analysis of the SUMOylation of ACO4 and its correspondent lysine mutations K11R, K23R, K120R, K151R and K289R by Western Blot using anti-T7 antibody.*

Figure 3.7 – Localization of the analyzed lysines K11, K23, K120, K151, K191, K289 and K295 in the structure of ACO4. *The protein structure (PDB 1W9Y) was obtained from SWISS-MODEL webpage (https://swissmodel.expasy.org/) and visualize by PyMol v.2.3.4.*

ACO4 is a homo-tetramer consistent of four chains, each one with its sequence (**Figure 3.7**). In each chain we can find an iron molecule surrounded by a lysine, an arginine, and a glutamic acid. In addition to the SUMOylation consensus site, SUMO acceptor lysine residues are usually located within loop motifs in the protein tertiary structure. The identified lysine K11, K129, K151, K191 and K295 are located inside a loop, which support their potential role as SUMO acceptor sites. Despite of the fact that the position of these lysine residues was interesting, we couldn't

identify any lysine for ACO4 SUMOylation, highlighting the multiple challenges involving the validation of SUMO substrates.

3.2. Summary of the analysis of the SUMO candidates

The **Table 3.1** shows the results of all the performed analysis with the selected candidate SUMO substrates. ERF2, ERF6, ERF104 and ACO4 were confirmed as SUMO targets. We confirmed SUMOylation of ERF2 K87 and K228 lysine residues; ERF6 K53, K63, K72, K121 and K231 lysine residues and ERF104 K183 lysine. However, despite testing various lysines for the ACO4 protein, some of them apparently located in a conserved SUMOylation motif and in exposed areas from the protein structure (**Figures 3.5 A, 3.6 A** and **3.7**), complete elimination of SUMOylation wasn't achieved.

Table 3.1 – Results of the analysis of candidate SUMOylation substrates including the analyzed lysine residues as SUMO acceptor sites. *The canonical SUMOylation motifs are represented in black and the non-canonical in grey. The most conserved lysine residues across higher plants (taxid: 3193) orthologs are displayed in larger font size, and the less conserved lysine residues are displayed in smaller font size. The identified SUMO-acceptor lysine residues are highlighted in green, while the lysine residues non-competent as SUMO-acceptor site are in red.*

SUMOylation motif: Canonical / Non Canonical

	SUMOylation	I/L/V K x E/D		A/P/M K x E/D			Others					
ERF2	Yes	K87	K228									
ERF6	Yes	K72	K231	K53	K63	K121						
ERF104	Yes	K183		K81	K178							
ACO4	Yes	K191					K11	K23	K120	K151	K289	K295
CA1	No											
ERF1	No											
ICE2	No											
MYC2	Inconclusive											
NHL10	Inconclusive											

3.3. Evaluation in transient expression in *Nicotiana benthamiana*

To use a transversal approach to validate the identified SUMO substrates, transient expression experiments in *Nicotiana benthamiana* were performed. For the development of this experiment, the native variants of the studied proteins were agroinfiltrated together with the conjugable or the non-conjugable of the SUMO variant. The empty vector pBA002 was used as

negative control. As we couldn't observe the expression of ERF2, ERF6 and ERF104 proteins (**Figure 3.8 A**), it was decided to infiltrate with the proteasome inhibitor MG132 50 µM (**Figure 3.8 B**) or with the ethylene precursor ACC 10 µM (**Figure 3.8 C**) 3 days after the agroinfiltration. However, only ACO4 had detectable expression levels.

Despite giving up this approach due to the lack of ERF protein expression, the samples expressing ACO4 allowed us to collaborate with the company Agrisera to validate a new batch of anti-ACO4 antibodies (https://www.agrisera.com/en/artiklar/aco4-1-aminocyclopropane-1-carboxylate-oxidase-4.html) (**Figure 3.8 D**).

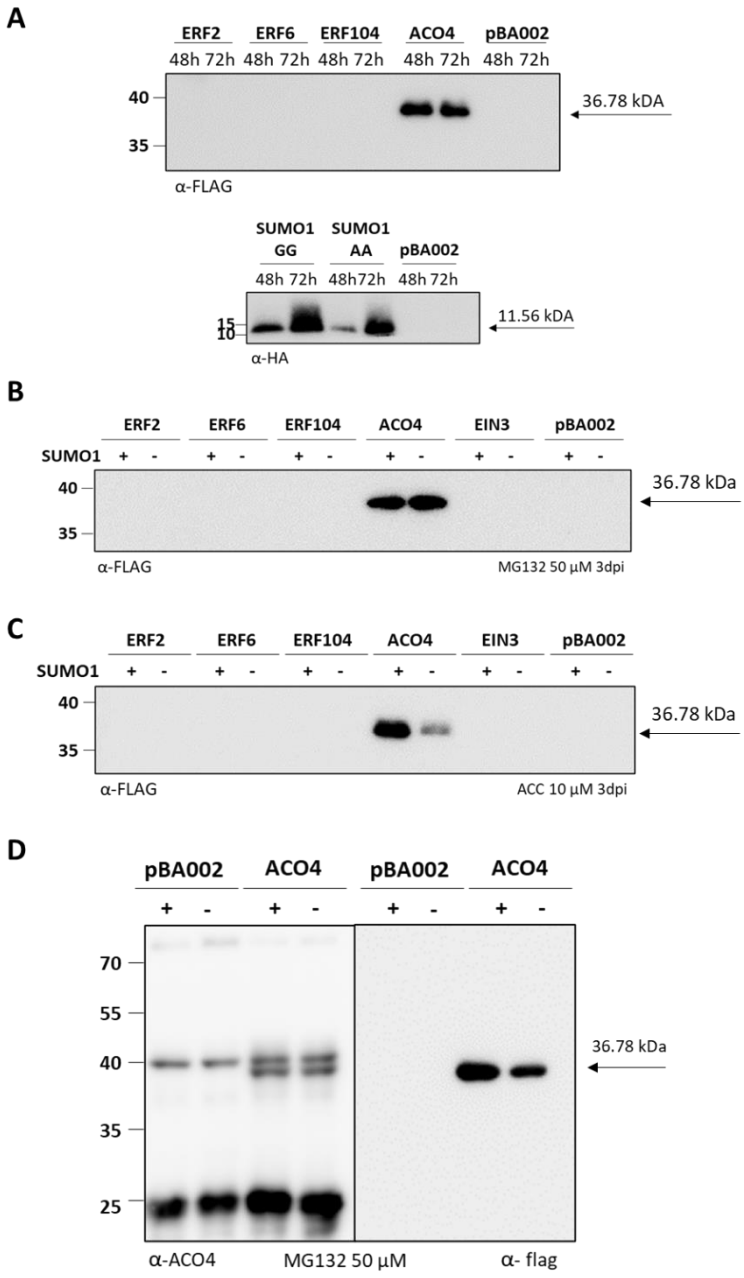


Figure 3.8 – Validation of SUMO-conjugates in Nicotiana benthamiana transient expression experiments. **(A)** N. benthamiana was infiltrated with ERF2, ERF6, ERF104, and conjugable SUMO1 (+) or non-conjugable SUMO1 (-). The empty vector pBA002 was used as negative control for each SUMO conjugate candidate. Samples were collected at 48 and 72 hours after agroinfiltration. As ERF2, ERF6 and ERF104 expression was not detected, new agroinfiltration experiments were performed and, after 3 days, MG132 50 µM **(B)** or ACC 10 µM **(C)** was additionally infiltrated. Samples were collected at 6 hours post treatment infiltration. The empty vector pBA002 was used as negative control. **(D)** In collaboration with Agrisera Company, anti-ACO4 antibodies were tested, which allowed us to confirm the identity of the signal detected with the anti-flag antibodies. The empty vector pBA002 was used as negative control.

Chapter 4: Characterization of *Arabidopsis* transgenic plants expressing native and SUMOylation-deficient variants of the selected candidates

4.1. Generation of *Arabidopsis* transgenic plants expressing native and SUMOylation-deficient variants of the selected candidates

In order to analyze the effect of the SUMOylation on the development and defense of the confirmed proteins, we developed two genetic strategies. In the first place, a complementation assay with the native and the non-SUMOylable variants of the proteins. This complementation assay was carried out with T-DNA insertion mutants of *erf5/erf6, erf104* and *aco4* from the public collections. The last one only with the SUMOylable variant because we were unable to identify the SUMOylable lysine.

Some authors didn't observe differences in the susceptibility/resistance phenotype of *erf6* single mutants in an infection with the necrotrophic fungi *Botrytis cinerea*, but did observe susceptibility to the pathogen in double mutants *erf5/erf6* (Moffat *et al.,* 2012). Based on these results, we decided to perform genetic complementation studies with the double mutant and not with the single mutant.

In parallel, functional tests were carried out in Col0 background with the native protein of the inconclusive proteins (MYC2 and NHL10), with ACO4 and with the native and SUMO-deficient variant of ERF2, ERF6 and ERF104.

Finally, we selected two independent lines from the native variants and another two from the non-SUMOylable variants based on their expression levels and with the aim of having the same expression levels in each case to be compared (**Figure 3.9**).

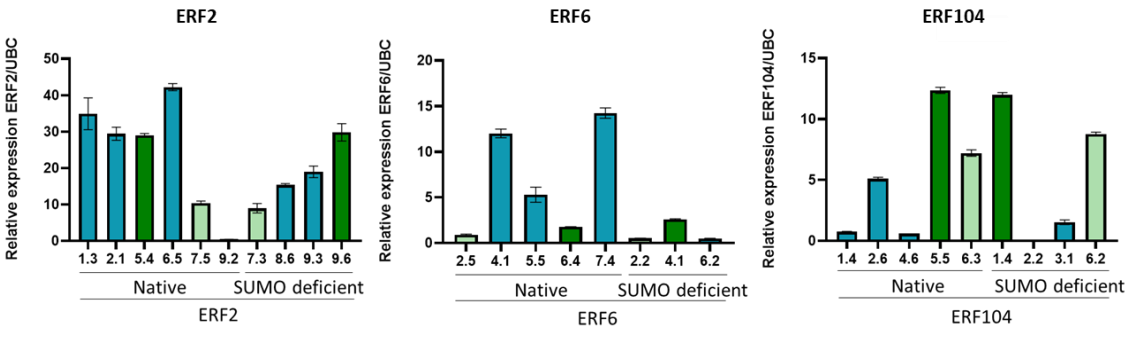


Figure 3.9 – Analysis of the expression levels of Arabidopsis transgenic lines expressing ERF2, ERF6 or ERF104 under the 35S promoter. *The bars in green represent the transgenic lines selected to further characterization. Light green and dark green indicate low and high expressing transgenic lines, respectively.*

4.1.1. Gene regulation

To compare the effect of SUMOylation we selected four lines for each gene depending on their expression level. In this way, we can compare, for example, ERF2 with lower level of expression (●) with its corresponding ERF2 non-SUMOylable variant (●). The same occurs with the one with higher expression level (●). The comparison of lines with the same expression levels of native or SUMOylation-deficient variants allow us to discriminate between effects resulting from differences in the expression levels or SUMOylation status of the protein under study.

In the first place, we decided to analyze how SUMOylation of selected proteins can affect the regulation of other genes. Since the proteins under study are transcriptional regulators of genes related to the response to ethylene, we analyzed the effect of the SUMOylation on the regulation of genes belonging to this pathway. Based on bibliography, STRING and Genevestigator analysis, 40 genes related to ethylene pathway and interacting in some way with ERF2, ERF6 and ERF104 were selected to perform the transcriptional analysis (**Table 4.1**).

Table 4.1 – List of selected genes whose expression levels were analyzed by qRT-PCR. The efficiency of the primers was determined and shown in the table.

AGI number	Name	Gene annotation	Efficiency
At5g25760	UBC	Ubiquitin	1,988
At1g18570	MYB51	High indolic glucosinolate 1	2,000
At1g19670	CLH1	Coronatine-induced protein 1	1,984
At1g22070	TGA3	Transcription factor TGA3	2,000
At1G27730	STZ	Related to Cys2/His2-type zinc-finger proteins found in higher plants	1,846
At1g32640	MYC2	Transcription factor MYC2	1,938
At1g51660	MKK4	Mitogen-activated protein kinase kinase 4	2,000
At1g73500	MKK9	Mitogen-activated protein kinase kinase 9	1,872
At1g75040	PR5	Pathogenesis-related protein 5	1,970
At1g80840	WRKY40	WRKY transcription factor 40	1,944
At2g27050	EIL1	ETHYLENE INSENSITIVE 3-like 1 protein	2,000
At2g31230	ERF15	Ethylene-responsive transcription factor 15	1,930
At2g38470	WRKY33	WRKY transcription factor 33	1,907
At2g40140	CZF1/SZF2	SALT-INDUCIBLE ZINC FINGER 2	2,000
At2g43790	MPK6	MITOGEN-ACTIVATED PROTEIN KINASE 6	2,000
At2g46400	WRKY46	WRKY DNA-BINDING PROTEIN 46	1,923
At3g04720	PR4	Pathogenesis-related 4	1,996
At3g12500	PR3	Pathogenesis-related 3	1,956
At3g15210	ERF4	Ethylene-responsive transcription factor 4	1,961
At3g20770	EIN3	Ethylene insensitive 3 family protein	2,000
At3g44260	CAF1a	CCR4- ASSOCIATED FACTOR 1A	2,000
At3g45640	MPK3	MITOGEN-ACTIVATED PROTEIN KINASE 3	2,000
At3g55980	SZF1	SALT-INDUCIBLE ZINC FINGER 1	2,000
At4g11280	ACS6	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6	2,000
At4g24570	DIC2	DICARBOXYLATE CARRIER 2	2,000
At4g33720	CAPE3	CAP Pathogenesis-related protein	1,970
At4g36920	AP2	Integrase-type DNA-binding superfamily protein	2,000
At5g47230	ERF5	ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 5	1,939
At5g51190	ERF105	ETHYLENE RESPONSE FACTOR 105	1,954
At1g72920	TN9	Disease resistance protein	2,000
At3g04210	TN13	Disease resistance protein	2,000
At2g18980	PRX16	Peroxidase	2,000
At2g26020	PDF1.2b	Plant defensin	2,000
At3g23240	ERF1	Ethylene-responsive transcription factor 1B	1,877
At5g36910	THI2.2	Thionin-2.2	1,998
At5g47220	ERF2	ERF2	2,000
At4g17490	ERF6	ERF6	2,000
At5g61600	ERF104	ERF104	1,922

Figure 4.1 aim to represent a more complete description of the ethylene signaling pathway by annotating all the genes found in bibliography.

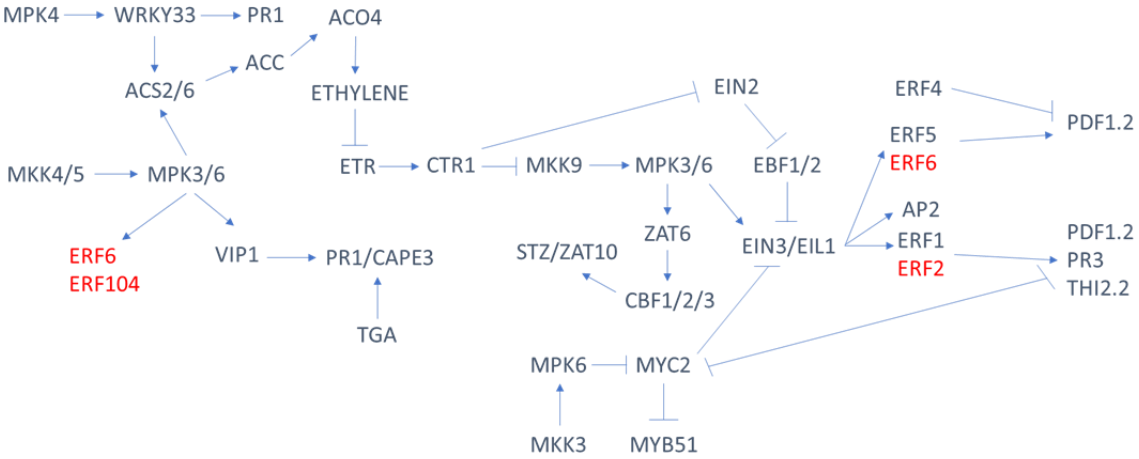


Figure 4.1 – Scheme of the biosynthesis and signaling pathway of ethylene. *Our genes of interest, ERF2, ERF6 and ERF104, in red, are transcription factors responsible for regulating the expression of genes related to defense. (Adapted from Bethke et al., 2009; Son et al., 2011; Santino et al.; 2013; Merchante et al., 2015; Song and Liu, 2015; Xie et al., 2019 and KEGG pathway analysis using DAVID (https://david.ncifcrf.gov/)).*

Through a KEGG pathway analysis, we could observe that those genes belong to the MAPK and ethylene signaling pathways and are involved in plant-pathogen interaction, as well as in the signal transduction of plant hormones (**Figure 4.2 A**).

According to biological processes (**Figure 4.2 B**), most genes are part of biotic response processes to bacterium, wounding and fungus and abiotic stresses like hypoxia and cold. They also participate in processes related to intracellular signal transduction, phosphorylation signaling cascades and secondary metabolite biosynthetic process, among others.

By means of the transcription analysis of the genes presented in **Table 4.1**, 17 genes with different expression levels have been identified (**Figure 4.3**). ERF2 presents mainly an activating role, ERF6 has an activating and a repressor role depending on the target gene, whereas ERF104 is characterized by showing a repressor role of the genes analyzed. SUMOylation deficient variants have compromised the activating or repressing activity of the transcription factor analyzed.

By analyzing their KEEG pathways and the biological processes (**Figure 4.4**), we also found that those genes belong to the signal transduction of MAPK and plant hormones and participate in biotic and abiotic response processes, such as fungal infection and cold stress responses, similar to those shown in **Figure 4.2 B**.

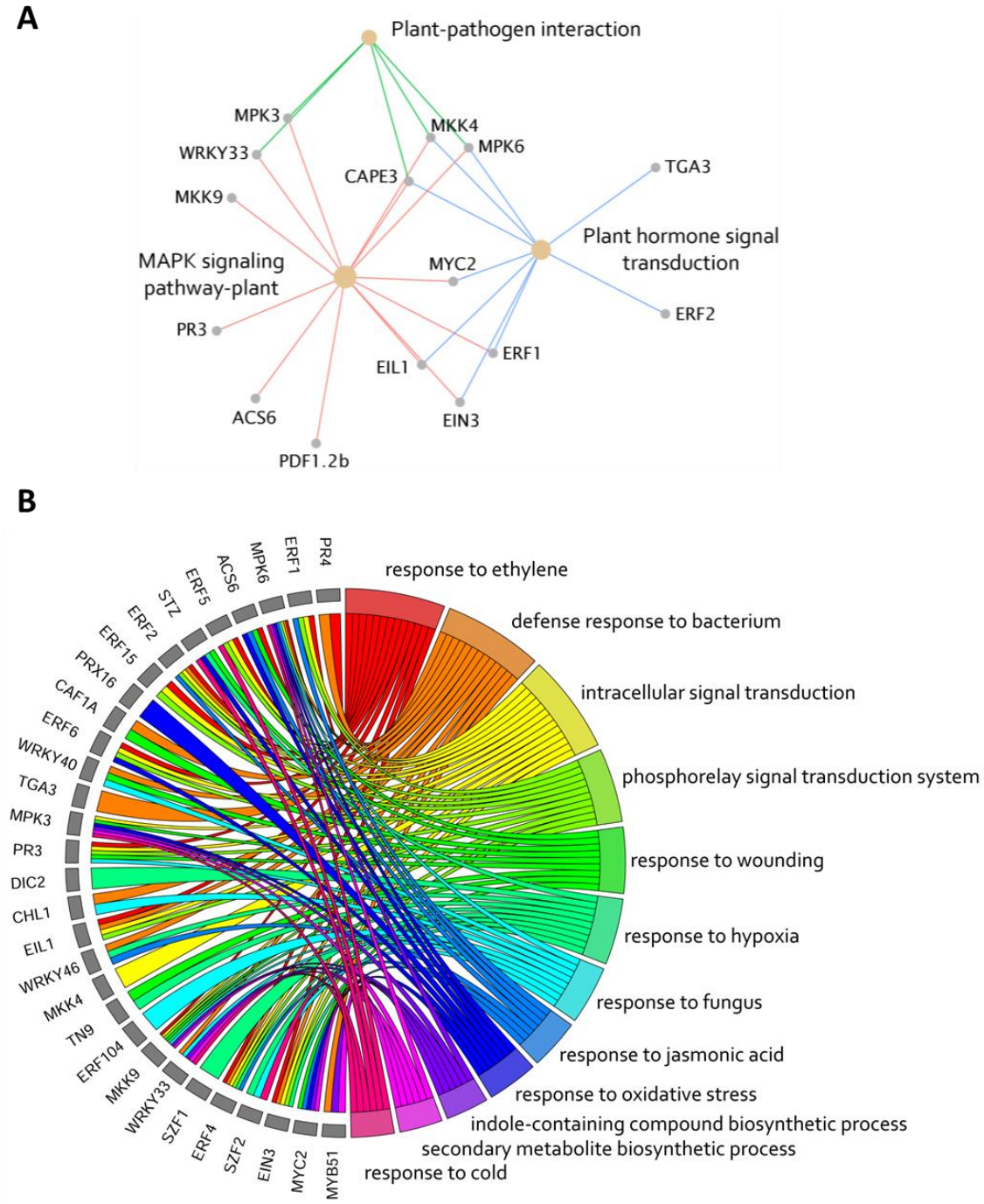


Figure 4.2 – Analysis of the 40 genes selected to evaluate the transcriptomics. (A) *Representation of the enriched KEGG pathway in which those genes are implicated. Most of them belong to the MAPK signaling pathway in plants and are involved in Plant hormone signal transduction and plant-pathogen interaction.* ***(B)*** *Circos Plot representation of those genes linked to their corresponding Biological Process. Only the Biological Processes with 6 or more genes are represented. It highlights the existence of Biological Processes corresponding to biotic such us abiotic stresses.*

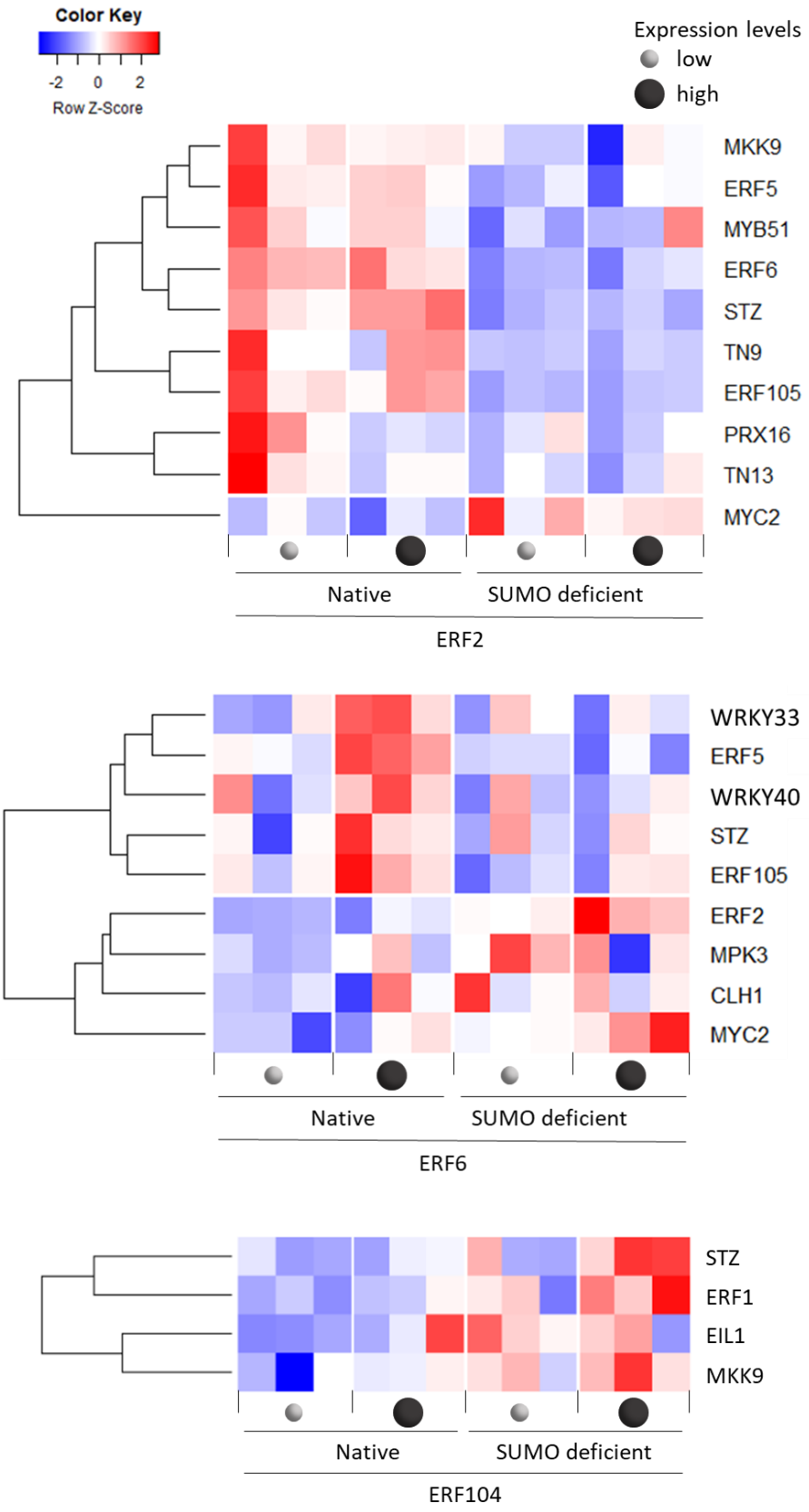


Figure 4.3 – Differential expression levels with respect to col0 of genes regulated by ERF2, ERF6 and ERF104, their native and SUMOylation-deficient variants. *Heat map representing three biological replicates for each transgenic line clustered based on expression values. Red and blue shadings represent higher and lower relative expression levels, respectively.*

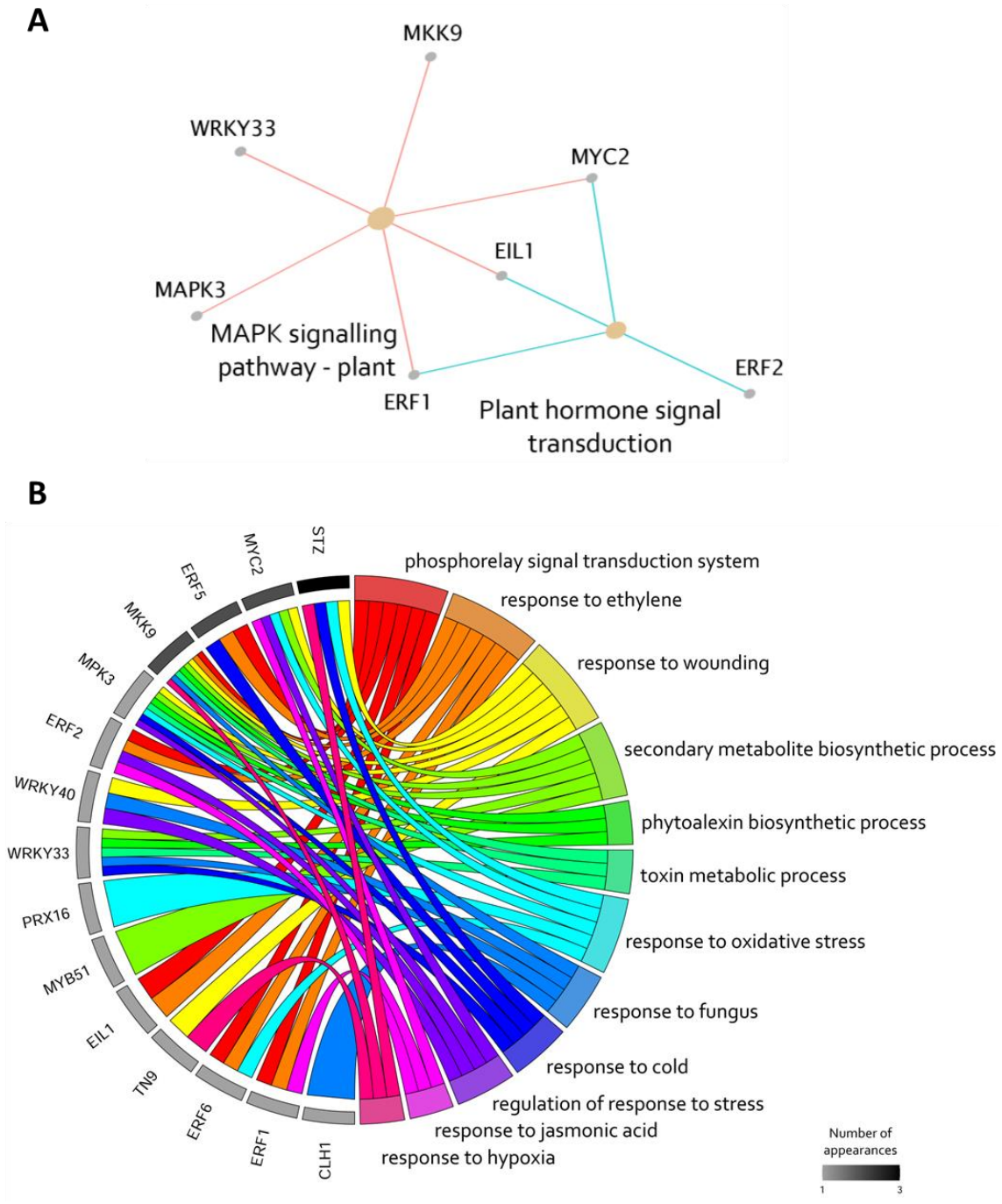


Figure 4.4 – Analysis of differentially expressed genes depending on the SUMOylation status of ERF2, ERF6 and ERF104 represented in Figure 4.3. (A) *Representation of the enriched KEGG pathway in which those genes are implicated. Most of them belong to the MAPK signaling pathway in plants and are involved in Plant hormone signal transduction.* ***(B)*** *Circos Plot representation of those genes linked to their corresponding Biological Process. Only the Biological Processes with 3 or more genes are represented. The scale corresponds to the appearance of the correspondent gene in one, two or three of the heatmaps of the Figure 4.3, which represents the regulation by one, two or three of our genes of interest. It highlights the existence of Biological Processes corresponding to biotic such us abiotic stresses.*

4.1.2. Plant development

Once it was confirmed that SUMOylation plays a role in regulation of different genes, the generated lines were phenotypically characterized to study the effect of the SUMOylation on development.

Lines with altered levels of SUMOylation generated previously in this laboratory have shown growth defects, being smaller with fewer leaves and lower seed production compared to Col0 depending on the level of SUMOylation, indicating that SUMOylation plays a role in development (Castaño-Miquel et al., 2017). Based on these results, we wanted to analyze whether SUMOylation affects to the development of our plants under normal conditions by evaluating different growth aspects (**Figure 4.5**).

When rosette area was analyzed in adult plants, neither ERF2, ERF6 nor ERF104 presented significant differences between SUMOylable and non-SUMOylable variants. Analyzing the length of the main branch, ERF2 and ERF6 SUMO deficient variants show a reduced main branch length, on the contrary to ERF104 which shows a longer main branch. Nevertheless, differences observed neither ERF6 nor ERF104 are not significant. In the case of the flowering analysis, SUMOylation of ERF2 and ERF6 seems to affect the beginning of the flowering by advancing and delaying it respectively, but only the differences in ERF6 according to SUMOylation were significant.

In parallel, the root phenotype of seedlings of ERF2, ERF6 and ERF104 was measured in 0.5 MS plates, but no significant differences were found (**Figure 4.6 A**). On the contrary, there were significant differences in the number of lateral roots (**Figures 4.6 B, C** and **D**). SUMOylation of ERF2 positively affects lateral roots development, unlike ERF6 where it has a negative effect. In the case of ERF104, it seems that the non-SUMOylable variants have a higher number of lateral roots, but this is not statistically significant.

SUMOylation of ERF2, ERF6 and ERF104 doesn't seem to significantly affect the phenotype in adult plants but does affect root development in seedlings, resulting in an altered number of lateral roots.

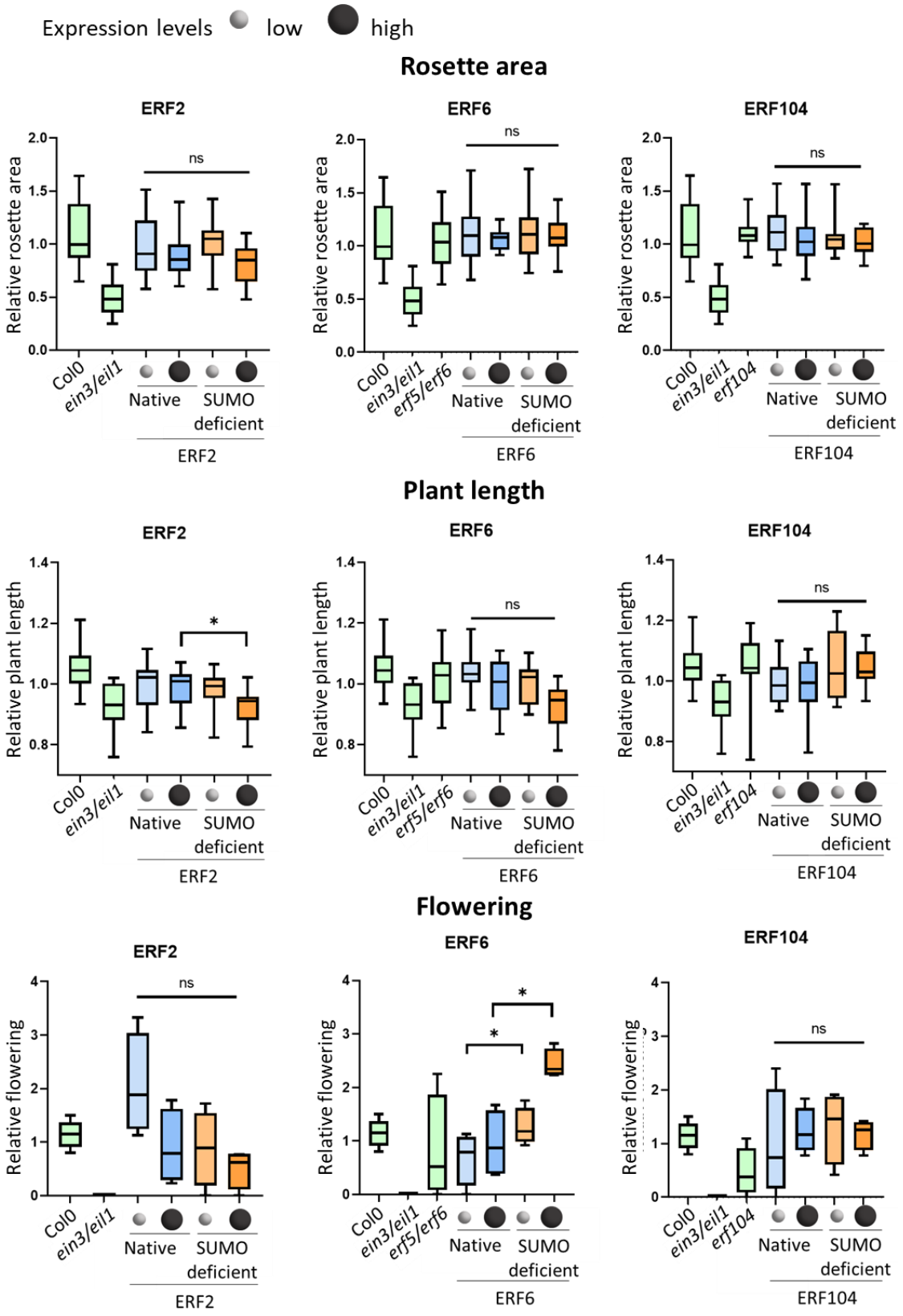
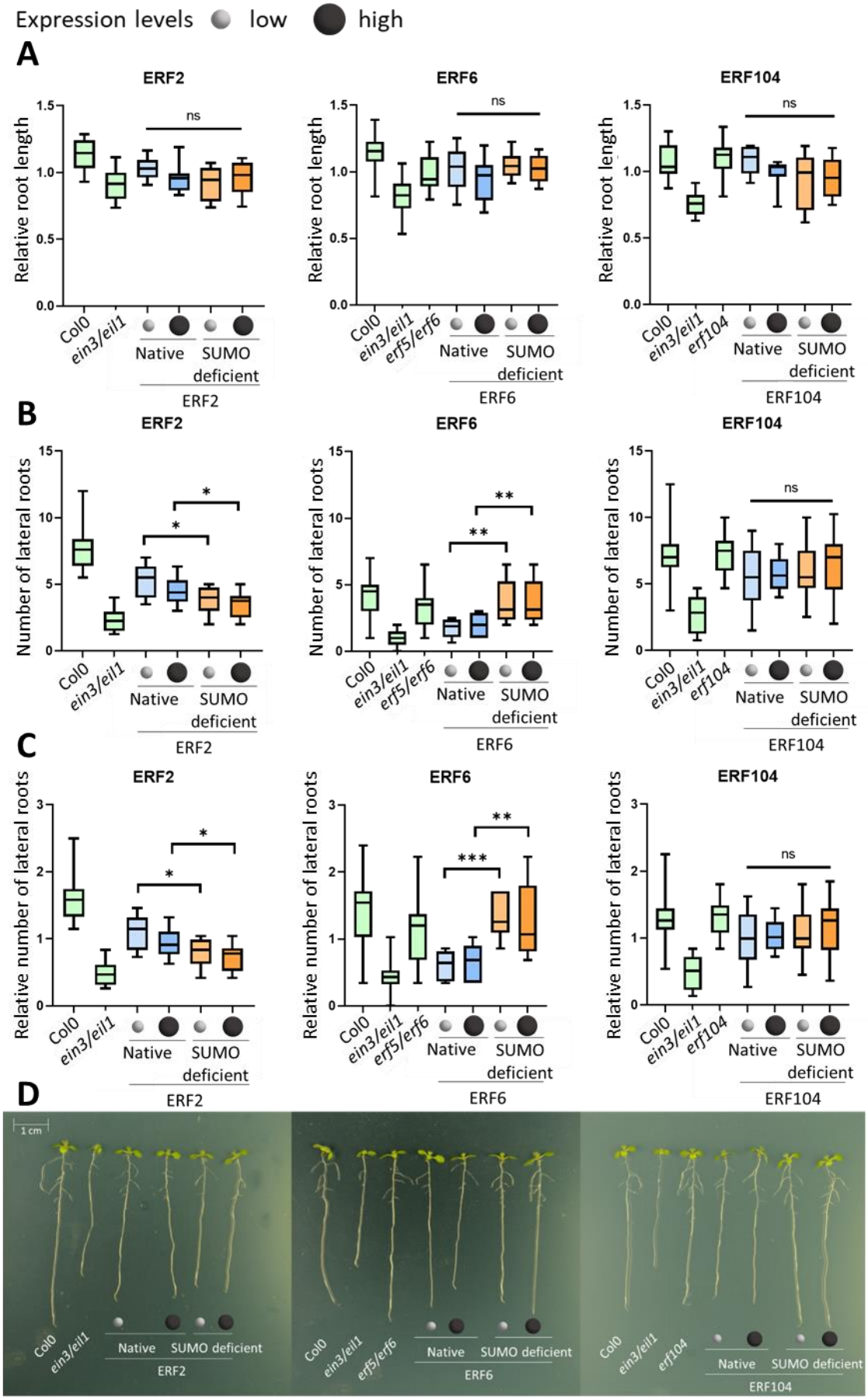


Figure 4.5 –Analysis of plant size and flowering of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The results correspond to the relative measure per tray analyzed in 9 biological replicates (5 plants per line and replicate) from 21 days old plants in Long Day conditions. In case of plant length, from 8 weeks old plant in Long Day conditions. Asterisk * corresponds to significant difference at p.value<0.05.*

Figure 4.6 – Analysis of root development of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The results correspond to the relative root length **(A)** and to the total number **(B)** or relative number **(C)** of roots per plate analyzed in 4 biological replicates from 8 days old seedlings in 12-12 22 ºC conditions. Asterisk *,** and *** correspond to significant difference at p.value<0.05, p.value<0.01 and p.value<0.001 respectively. **(D)** Photograph from representative roots. Plants per line: ERF2 (20, 20, 14, 15, 15, 13), ERF6 (25, 21, 19, 13, 12, 14, 12), ERF104 (17, 16, 19, 15, 12, 14, 14).*


Expression levels low high
A
ERF2
ERF6
ERF104
Relative root length
ns
Col0
ein3/eil1
erf5/erf6
erf104
Native
SUMO deficient
B
Number of lateral roots
*
**
C
Relative number of lateral roots
***
D
1 cm

4.1.3. Effect of the SUMOylation on other physiological processes

4.1.1.1. Analysis of tolerance to heavy metals

Exposure to heavy metals affects ethylene biosynthesis in plants, causing the generation of ROS and the induction of oxidative stress (Keunen *et al.,* 2016). ERFs such as ERF1, ERF2 and ERF5 have been overexpressed under exposure to cadmium and copper (Herbette *et al.*, 2006; Weber *et al.*, 2006; Keunen *et al.* 2016), activating the MPK3 and MPK6 pathway, precursors in ethylene biosynthesis (Liu *et al.*, 2010). Therefore, we wanted to study whether SUMOylation had any role in the regulation of heavy metal toxicity, carrying out a screening using various heavy metals available in the laboratory. Finally, $CuSO_4$ 30 µM, $CdCl_2$ 15 µM and H_3BO_4 750 µM were selected.

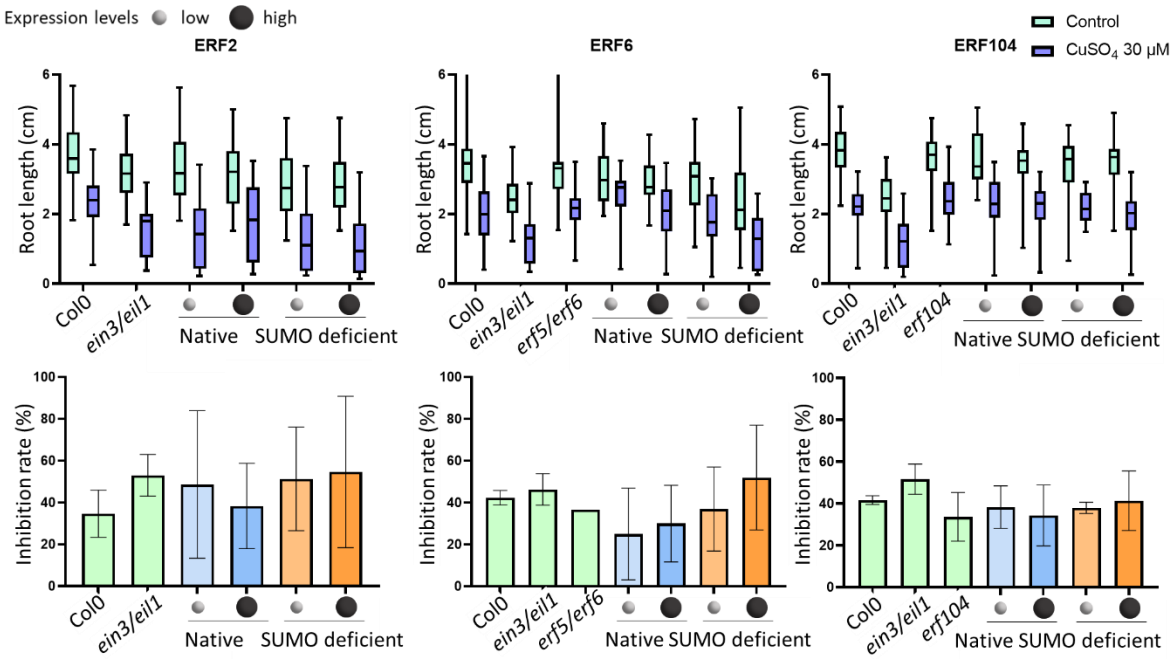


Figure 4.7 – Sensitivity of copper-induced root growth inhibition of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *Percentage of inhibition root under $CuSO_4$ 30 µM treatment. The results correspond to roots length and percent of inhibition rate in 3 biological replicates from 8 days old seedlings in 12-12 22 ºC conditions. No significant differences were observed. Plants per line: ERF2 (22, 19, 23, 24, 25, 25), ERF6 (24, 25, 24, 16, 27, 30, 20), ERF104 (14, 26, 20, 22, 25, 15, 18).*

With the addition of copper to the medium, we can observe more susceptibility with the non-SUMOylable variants of ERF2 and ERF6, but this susceptibility is not significant (**Figure 4.7**).

With the addition of cadmium to the medium, we can observe more susceptibility with the ERF2 non-SUMOylable variants and more resistance with the ERF104 SUMOylable variants, but none of them are significant (**Figure 4.8**).

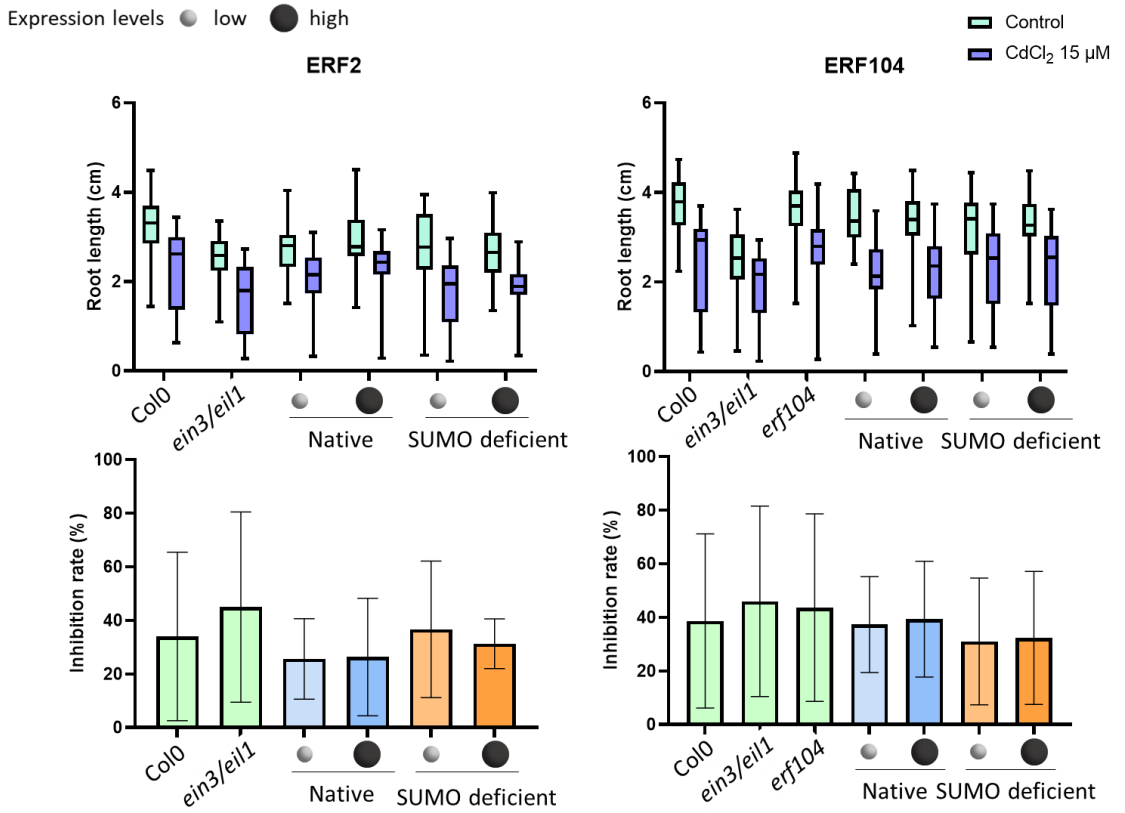


Figure 4.8 – *Sensitivity of cadmium-induced root growth inhibition of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter.* *Percentage of inhibition root under $CdCl_2$ 15 µM treatment. The results correspond to roots length and percent of inhibition rate in 3 biological replicates from 8 days old seedlings in 12-12 22 ºC conditions. No significant differences were observed. Plants per line: ERF2 (23, 18, 24, 32, 23, 34), ERF104 (23, 25, 24, 15, 20, 17, 21).*

Finally, with the addition of boron to the medium, we can observe that the susceptibility/resistance phenotype to boron depends on the ERF2 expression levels, but none of the results are significant (**Figure 4.9**).

Despite the fact that heavy metals can alter the ethylene signaling pathway, we couldn't see any significant difference in susceptibility/resistance phenotype when comparing the native and the SUMO-deficient variants. Perhaps the experimental conditions don't allow discriminating between the SUMOylated and non-SUMOylated activity of the ERFs studied, or the high number of ERFs and genes that regulate the ethylene signaling pathway are masking the toxicity effect.

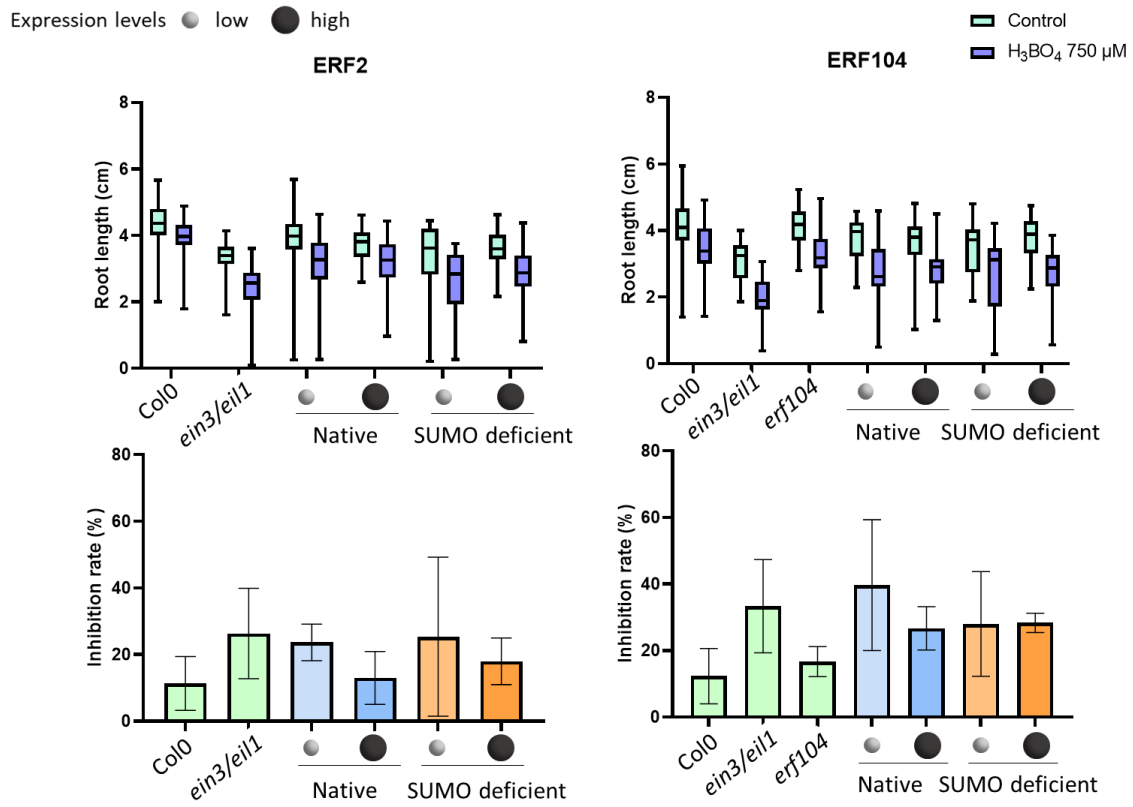
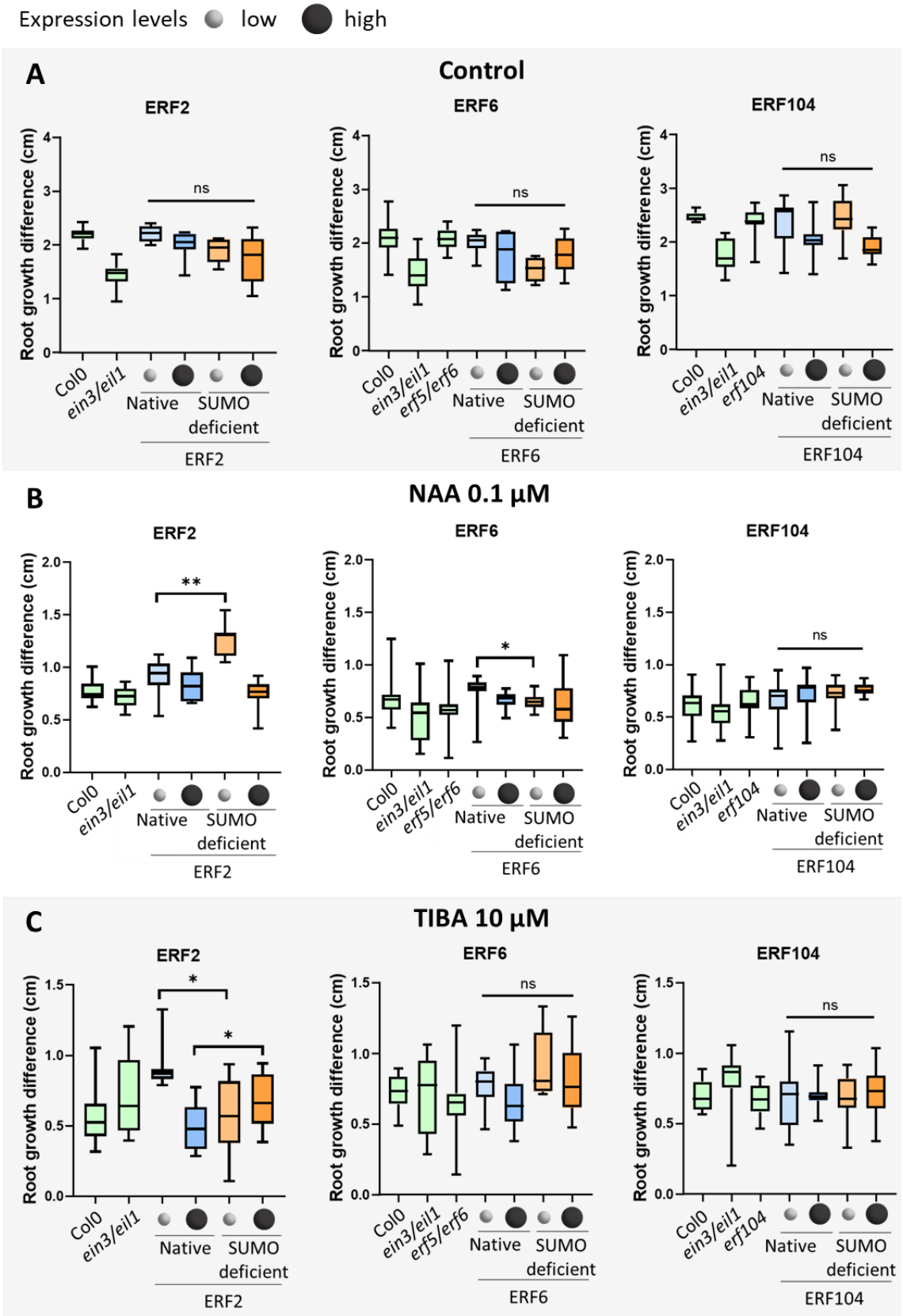


Figure 4.9 – Sensitivity of boron-induced root growth inhibition of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *Percentage of inhibition root under H_3BO_4 750 µM treatment. The results correspond to roots length and percent of inhibition rate in 3 biological replicates from 8 days old seedlings in 12-12 22 ºC conditions. No significant differences were observed. Plants per line: ERF2 (17, 18, 13, 13, 12, 12), ERF104 (16, 10, 16, 11, 10, 10, 12).*

4.1.1.2. Responses to hormone treatment

As our genes of interest belong to the ethylene signaling pathway and this hormone is linked to the pathway of other hormones as jasmonic acid, besides that we saw that the SUMOylation can interfere with root development, giving rise to a greater or lesser number of lateral roots, we considered analyzing this effect in response to different hormones (**Figure 4.10**).


Expression levels low high
A
Control
ERF2
ERF6
ERF104
Root growth difference (cm)
ns
Col0
ein3/eil1
erf5/erf6
erf104
Native
SUMO deficient
B
NAA 0.1 µM
**
*
C
TIBA 10 µM

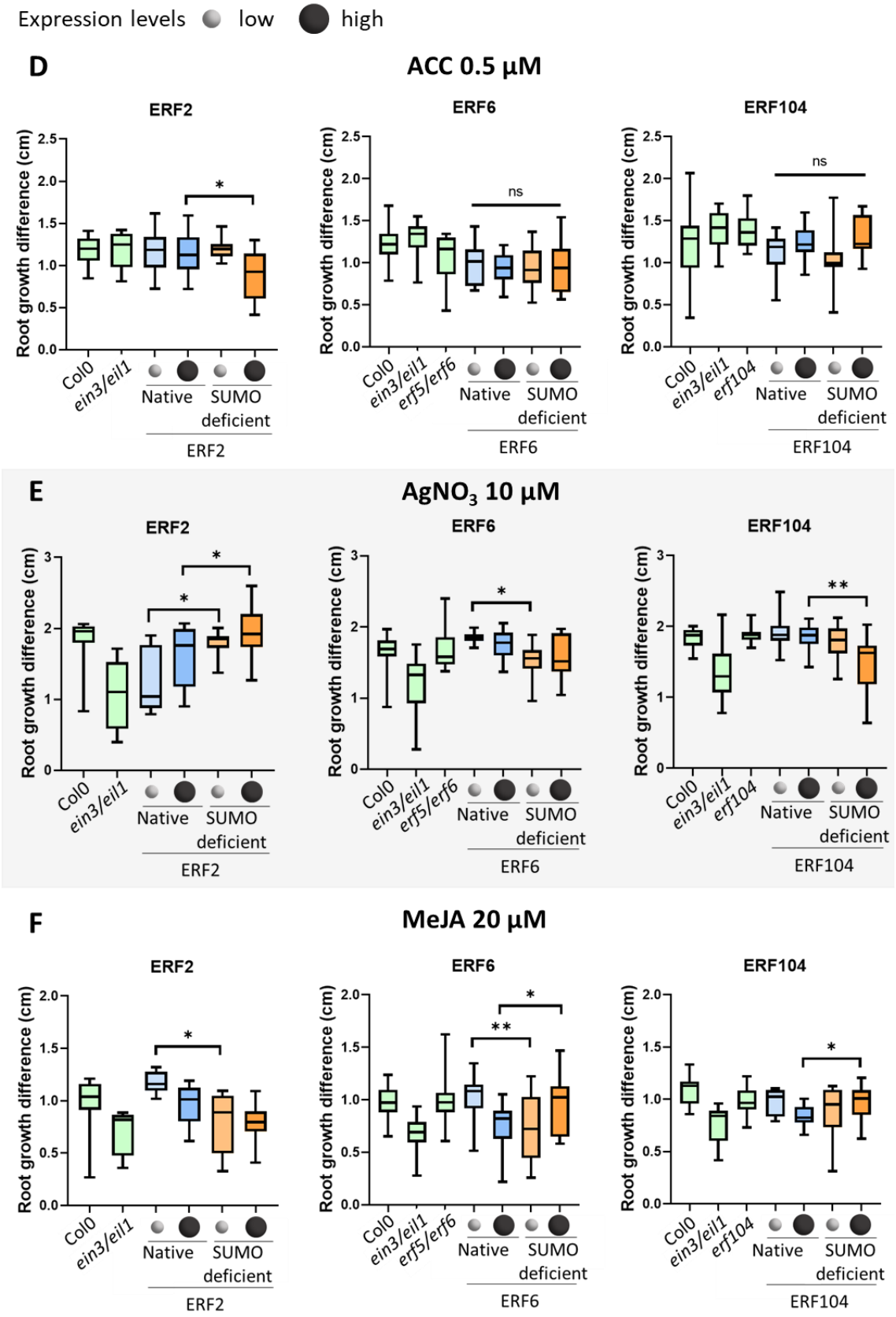

Expression levels low high
D
ACC 0.5 µM
ERF2
ERF6
ERF104
Root growth difference (cm)
Col0
ein3/eil1
erf5/erf6
erf104
Native
SUMO deficient
ns
E
AgNO3 10 µM
F
MeJA 20 µM

Figure 4.10 – Sensitivity of hormone-induced root growth inhibition of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The seedlings were growth for 4 days in a 0.5 MS with vitamins and transferred to a new plate with NAA, TIBA, ACC, $AgNO_3$ or MeJA, or without as control, for 3 days. The results correspond to the difference between the root measures from day 0 and 3 days of treatment in 12-12 22 ºC conditions. Asterisk * and ** correspond to significant difference at p.value<0.05 and p.value<0.01 respectively. Number of plants analyzed per line ordered as in the x-axis: ERF2 (A: 20, 20, 11, 12, 13, 12, B: 19, 13, 10, 12, 12, 13, C: 18, 18, 13, 13, 14, 14, D: 19, 14, 13, 13, 10, 13, E: 19, 17, 12, 11, 12, 12, F: 18, 15, 12, 13, 12, 12), ERF6 (A: 14, 12, 22, 14, 10, 10, 12, B: 16, 12, 19, 13, 15, 13, 10, C: 13, 8, 20, 13, 11, 10, 11, D: 12, 10, 16, 12, 15, 14, 15, E: 13, 13, 17, 13, 14, 15, 10, F: 14, 14, 20, 16, 10, 13, 11), ERF104 (A: 13, 11, 15, 16, 14, 13, 13, B: 14, 8, 14, 15, 13, 14, 13, C: 13, 11, 16, 15, 13, 12, 13, D: 14, 13, 14, 15, 14, 14, 13, E: 16, 16, 15, 14, 10, 10, 11, F: 17, 12, 15, 13, 14, 14, 11).*

With auxin treatment, the addition of NAA to the medium significantly increased root growth in ERF2 non-SUMOylable variants and significantly slowed root growth in ERF6 non-SUMOylable variants. We didn't observe any difference in ERF104. With the addition of the auxin transport inhibitor, TIBA, we saw that the expression levels of ERF2 and ERF6 could interfere in the inhibition of growth root.

With the treatment of the ethylene precursor, ACC, we only observed a significant reduction in root growth in ERF2 non-SUMOylation variants, the opposite occurring with de addition of the ethylene inhibitor, $AgNO_3$, where the variants deficient in SUMOylation of ERF2 showed less growth, while the same variants of ERF6 and ERF104 showed an increase in root growth.

With the addition of methyl jasmonate to the medium, we observed a reduction in root growth in non-SUMOylable variants of ERF2, while the response to this hormone seems to depend on the level of expression in ERF6 and ERF104.

The results obtained reflect the complexity of the system under study, where multiple layers of regulation and factors converge at a physiological response. There are cases where the effect of expression levels may mask the effect of the regulation by SUMO. However, it is well demonstrated that SUMOylation of ERF2 and ERF6 has an important role in the developmental processes analyzed, while SUMOylation of ERF104 seems not to be relevant for the analyzed parameters.

4.1.1.3. Analysis of senescence development

As ethylene is a hormone that induces senescence in plants (Hai-Chun Jing *et al.,* 2005; Iqbal *et al.,* 2017) and our genes of interest belong to the ethylene pathway, we decided to check whether the SUMOylation can alter the beginning of the senescence.

Figure 4.11 shows the Fv/Fm analysis of the ERF2, ERF6 and ERF104 lines at 3, 4 and 5 days of light/dark conditions. Under dark conditions (**Figure 4.11 B**), we were unable to see differences between the SUMOylable and non-SUMOylable variants, but under normal conditions, in both ERF2, ERF6 and ERF104, SUMO-deficient variants possess significantly higher photosynthetic capacity compared to SUMOylable variants.

Although darkness affects the photosynthetic capacity of plants, light inhibition equally affects if the variants are SUMOylable or non-SUMOylable. However, with the control plants corresponding to ERF2 and ERF6, we observed that SUMOylation reduces the photosynthetic capacity of the plants, decreasing the Fv/Fm ratio under normal conditions.

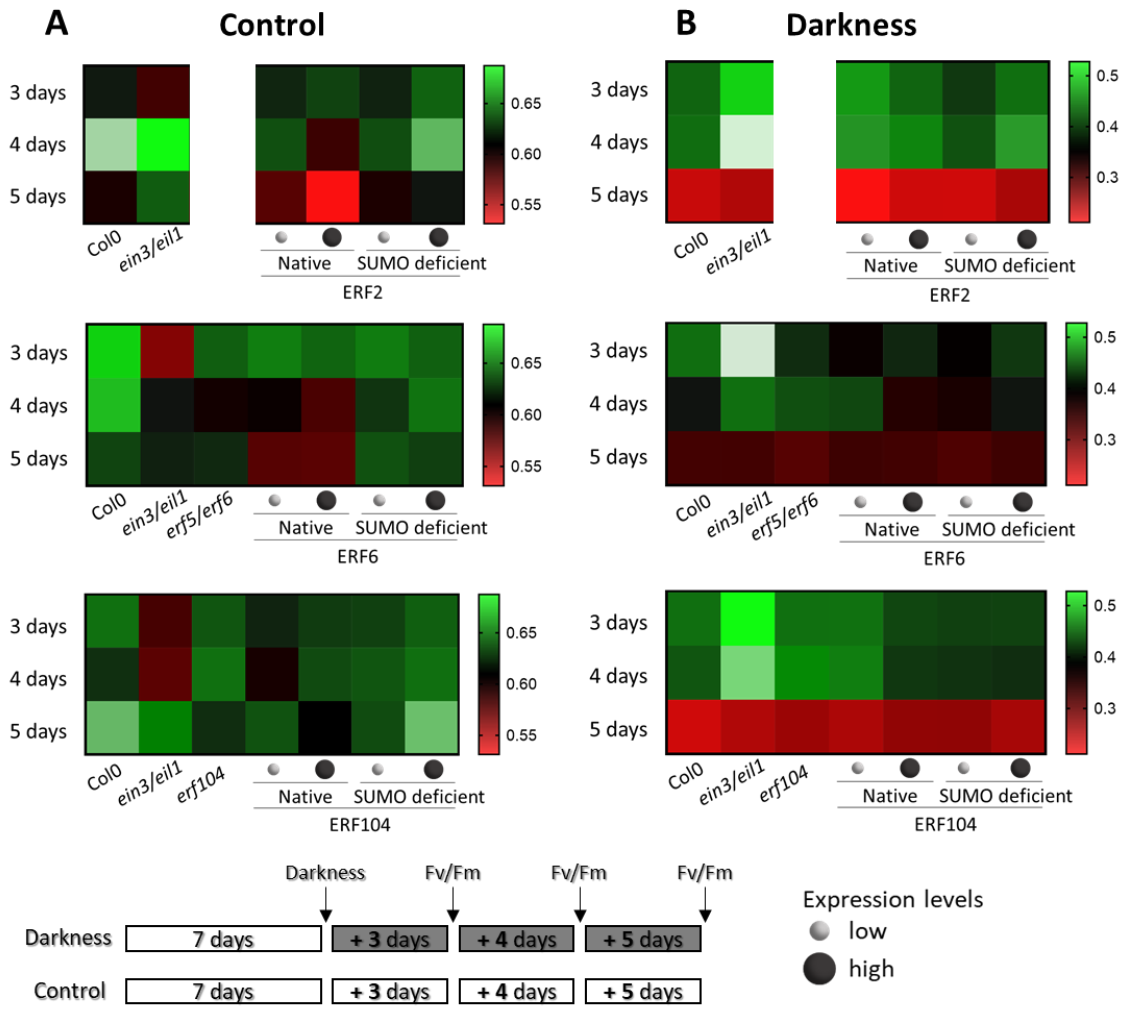


Figure 4.11 – Analysis of the senescence of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The heatmaps show the Fv/Fm ratio corresponding to photosynthetic capacity. Plants were grown under 12-12 photoperiod and 22 ºC for 7 days and transferred to dark or kept in light for + 3, 4 or 5 additional days. 3 biological replicates (10 plants per line and replicate).*

4.1.1.4. Analysis of tolerance to cold treatment

Among the biological processes in which the genes that showed differences in expression levels are involved, is cold stress. To analyze whether the SUMOylation of ERF2, ERF6 and ERF104 can regulate resistance to cold stress, two assays were performed, one with an acclimation period and one without acclimation.

4.1.1.5. With acclimation

With the experiment carried out with acclimation, after the recovering time of 7 days, all the plants were alive showing a red color in their leaves representing different levels of anthocyanin accumulation. Therefore, the accumulation of anthocyanins of each line was measured, observing a lower accumulation in SUMOylation deficient variants (**Figure 4.12**).

By performing a cold stress experiment with acclimation, we couldn't analyze the survival rate of the plants, but we can directly study the effect of this stress through the accumulation of anthocyanins, where SUMOylation seems to play an accumulative role.

4.1.1.6. Without acclimation

With the experiment carried out without acclimation, we could observe that most of the plants were affected, which allowed us to analyze the survival rate of the plants. In this case, we didn't observe accumulation of anthocyanins.

The lines that overexpress ERF6 showed a greater resistance to these conditions, obtaining a survival rate of 100%; this gene seems to play an important role in cold resistance. On the contrary, the ERF2 lines were affected at these temperatures, but the recovery of some plants was observed, while in the ERF104 lines the percentage of mortality was 100% (**Figure 4.13**). However, we were unable to find differences between the native or SUMO-deficient variants.

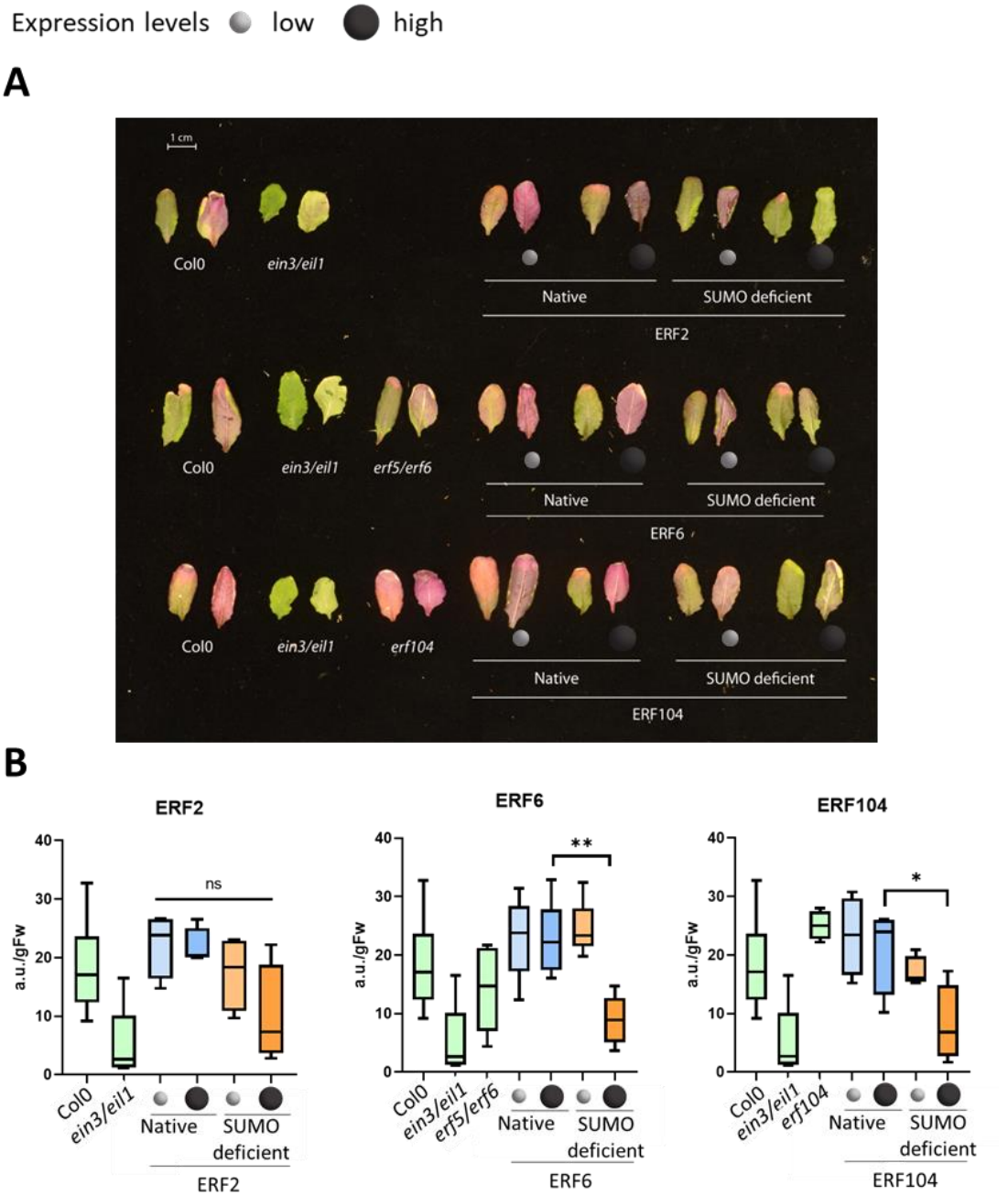
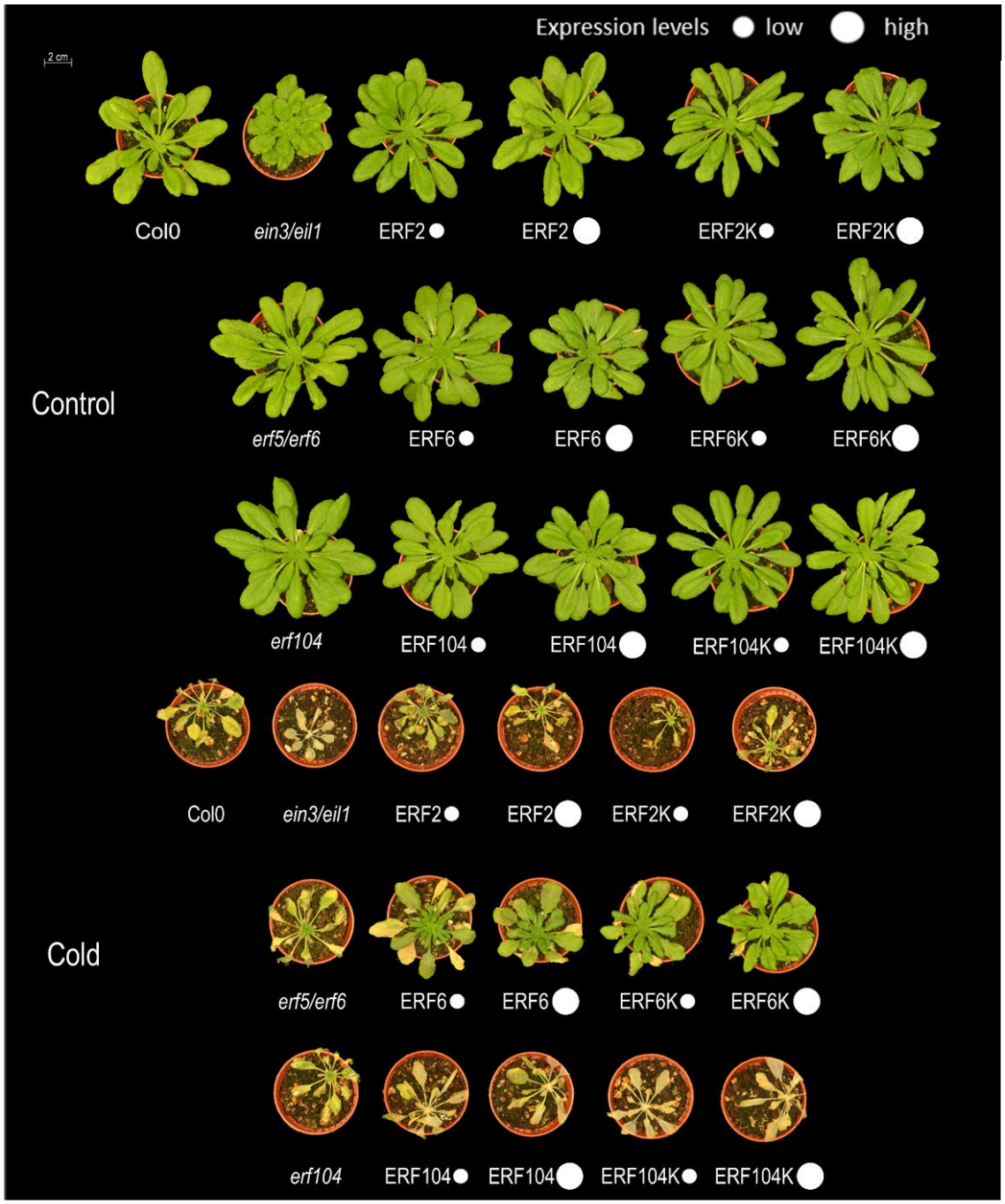


Figure 4.12 – Accumulation of levels of anthocyanins of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The plants were growth in Long Day conditions for 21 days following of a temperature acclimatization of 7 days at 4 ºC, after that, the temperature was reduced until -8 ºC keeping it for 1 hour. A new acclimatization of 12 hours at 4 ºC was performed before return the plants to normal Long Day conditions.* ***(A)*** *Representation of the front and back of a leaf. The anthocyanins accumulation is higher on the back part of the leaf.* ***(B)*** *Graphic representation of the accumulation of anthocyanins measured after 7 days of recovering analyzed in 1 biological replicates (5 plants per line and replicate).*


Expression levels low high
2 cm
Col0
ein3/eil1
ERF2
ERF2
ERF2K
ERF2K
Control
erf5/erf6
ERF6
ERF6
ERF6K
ERF6K
erf104
ERF104
ERF104
ERF104K
ERF104K
Col0
ein3/eil1
ERF2
ERF2
ERF2K
ERF2K
Cold
erf5/erf6
ERF6
ERF6
ERF6K
ERF6K
erf104
ERF104
ERF104
ERF104K
ERF104K

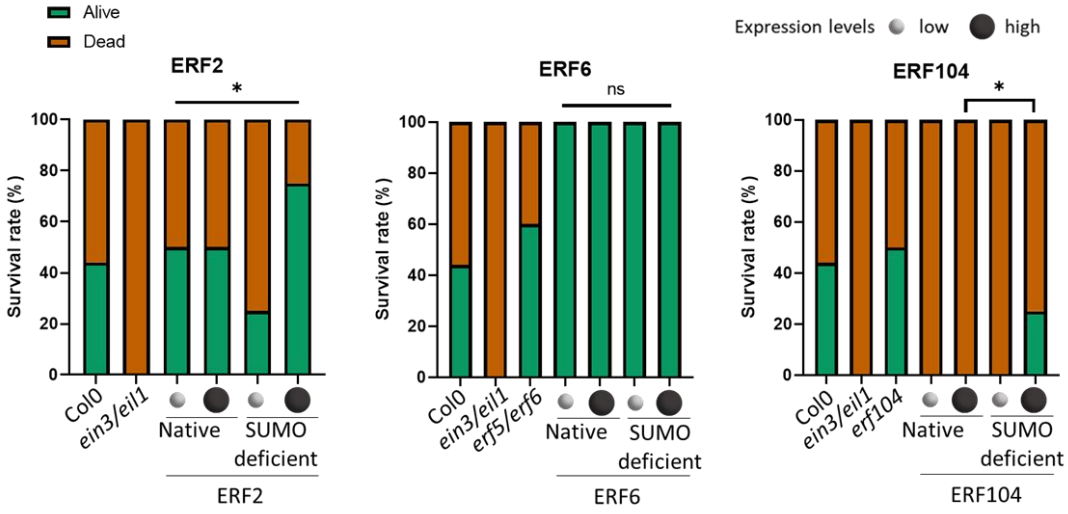

Alive
Dead
Expression levels low high
ERF2
ERF6
ERF104
Survival rate (%)
100
80
60
40
20
0
*
ns
*
Col0
ein3/eil1
erf5/erf6
erf104
Native
SUMO deficient
ERF2
ERF6
ERF104

Figure 4.13 – Analysis of cold-resistance phenotype without acclimatization of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104 under the 35S promoter. *The plants were growth in Short Day conditions for 5 weeks following of a sudden drop in temperature until -3 ºC for 4 days. After that, the plants return to normal Short Day conditions for its recovering for 7 days. The results correspond to the rate of survival and death analyzed in 1 biological replicates (5 plants per line and replicate). Asterisk * corresponds to significant difference at p.value<0.05.*

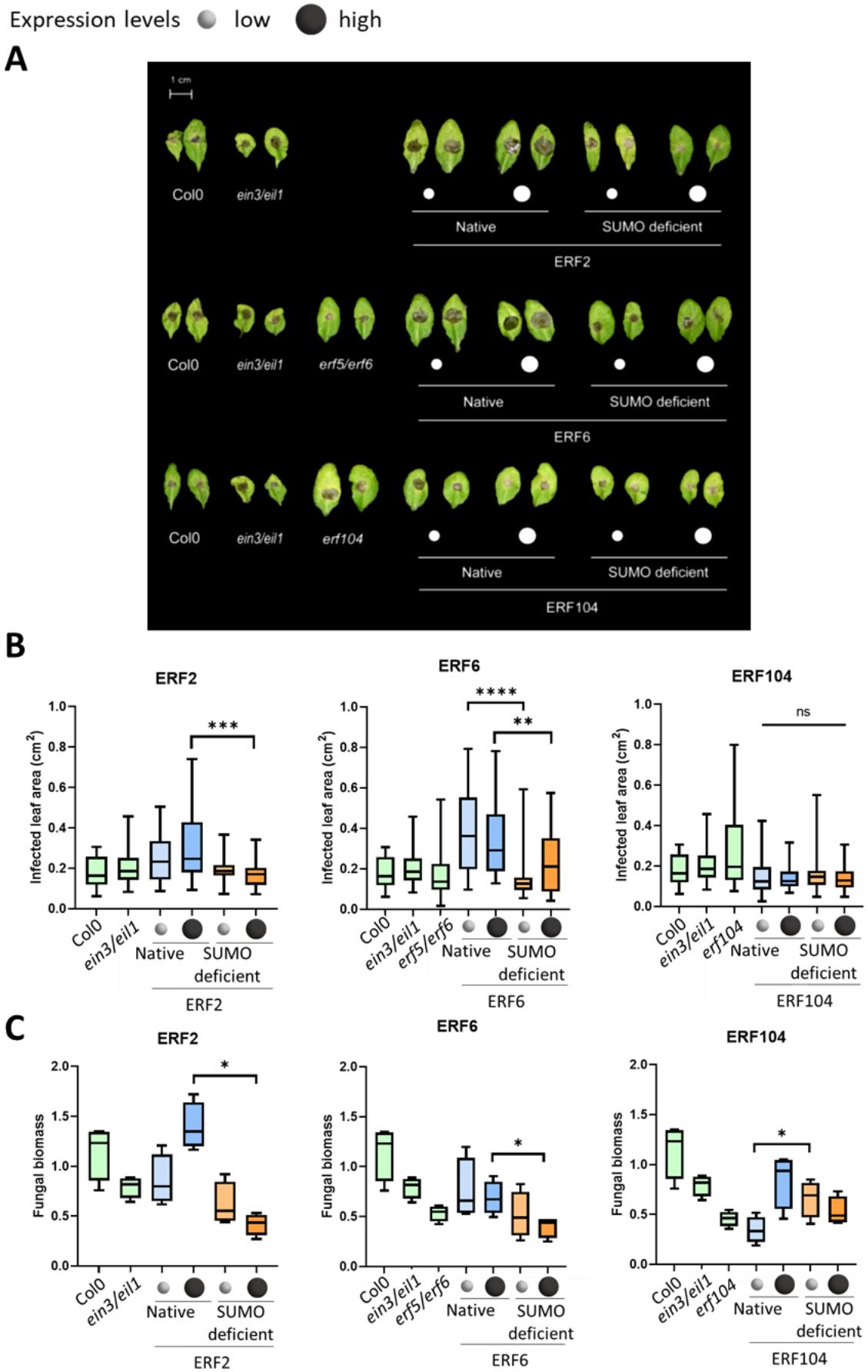
4.1.1.7. Defense against Botrytis infection

Another biological process in which genes that showed differences in expression levels are involved is the response to fungus. Various studies have shown that post-translational modification mechanisms such as phosphorylation, ubiquitination, nitrosylation and glycosylation, as well as SUMOylation are involved in the defense response of plants against pathogens (Stulemeijer and Joosten, 2010; Lois, 2010; Castaño-Miquel *et al*., 2017; Campos *et al*., 2019).

Lines with altered levels of SUMOylation generated in this laboratory have shown greater susceptibility to the infection by the necrotrophic fungus *Botrytis cinerea,* demonstrating that the SUMOylation is necessary for fungal resistance (Castaño-Miquel *et al.,* 2017). Based on these results, we decided to infect the ERF2, ERF6 and ERF104 lines in order to analyze whether the SUMOylation could affect the susceptibility of these plants to infection.

The non-SUMOylable variants of ERF2 and ERF6 were significantly more resistant to infection with *B. cinerea,* showing a greater lesion area and amount of fungal biomass in infected leaves. In contrast, SUMOylation of ERF104 doesn't appear to to affect the susceptibility-resistance phenotype (**Figure 4.14**).

Figure 4.14 – Analysis of the susceptibility-resistance phenotype of Arabidopsis transgenic lines expressing SUMOylable and non-SUMOylable variants of ERF2, ERF6 or ERF104, under the 35S promoter, infected with Botrytis cinerea. *The plants were growth in Short Day Conditions for 4 weeks before being infected with 10 µL of 3.95x10^6 spores/ml of Botrytis cinerea. The results correspond to 3 biological replicates (3 leaves and 10 plants per line).* ***(A)*** *Photograph from representative leaves. The infected leaf area* ***(B)*** *and fungal biomass* ***(C)*** *were measured 3 days post infection. Asterisk **, *** and **** correspond to significant difference at p.value<0.01, p.value<0.001 and p.value<0.0001 respectively.*


Expression levels low high
A
1 cm
Col0
ein3/eil1
Native
SUMO deficient
ERF2
Col0
ein3/eil1
erf5/erf6
Native
SUMO deficient
ERF6
Col0
ein3/eil1
erf104
Native
SUMO deficient
ERF104
B
ERF2
ERF6
ERF104
Infected leaf area (cm2)
***
****
**
ns
Col0
ein3/eil1
erf5/erf6
erf104
Native
SUMO deficient
C
Fungal biomass
*

Discussion

Auxins play a major role in regulating plant growth and development and, together with other plant hormones, are responsible for balancing plant growth and defense. Defense against infection by pathogens is frequently mediated by an interaction of different hormones (Kazan and Manners, 2009; Fu *et al.,* 2015). Auxins stimulate cell division and cell elongation under normal conditions, favoring tumor formation after specific bacterial infection. Furthermore, many plant pathogens can produce indole acetic acid (IAA) through their interaction with plants (Kazan and Manners, 2009; Ludwig-Müller, 2015). Auxins have a positive regulatory role in resistance to necrotrophic fungi. The application of the auxin transport inhibitor (TIBA) before an infection is capable of favoring the resistance or susceptibility of the plant depending on the pathogen, possibly related with the effect of this hormone on SA and JA signaling pathways (Llorente *et al*., 2008; Kazan and Manners, 2009; Nafisi *et al*., 2015).

One of the first objectives of this project was to identify a potential role of SUMOylation in auxin signaling and defense response, for which an analysis of different auxin concentrations and auxins transport inhibitors in lines with altered levels of SUMOylation were performed. However, the results obtained did not support a major role of SUMO in the regulation of auxin signaling. This prompted us to reevaluate the initial hypothesis.

Post-translational modifications (PTMs) of proteins can lead to changes in protein interaction with other molecules, subcellular localization, and even protein activity and stability. A single protein can be modified by multiple PTMs at the same time or at different times in the cycle, so PTMs act as key players in determining protein function (Nukarinen *et al.*, 2017; Sharma *et al.,* 2021).

Plant defense mechanism are largely directed by PTMs to activate the appropriate signaling pathways against the pathogen. On the contrary, some infection strategies rely on the delivery of pathogen effectors that target host cell's PTMs. SUMO is an essential PTM belonging to the Ubl family of modifiers. In plants, SUMO regulates multiple biological processes, ranging from development to defense against environmental challenges (Castro et al., 2012; Benlloch and Lois, 2018; Rosa and Abreu, 2019; Sharma *et al*., 2021).

SUMOylation is mediated by the heterodimeric SUMO-activating enzyme (subunits SAE1 and SAE2 in *Arabidopsis*), by the SUMO-conjugating enzyme (SCE1 in *Arabidopsis*) and by SUMO E3 and E4 ligases (SIZ1, HPY2, PIAL1 and PIAL2 in *Arabidopsis*) (Nukarinen *et al.*, 2017; Augustine and Vierstra, 2018). The small Ubiquitin-like MOdifer is covalently attached to a K (lysine) residue located in the consensus sequence ΨkxE/D, where Ψ is a hydrophobic amino acid, on the substrate. As a reversible PTM, specific cysteine proteases remove SUMO from the substrate

(Johnson, 2004; Jürgen Dohmen and Dohmen, 2004; Augustine and Vierstra, 2018; Sharma *et al*., 2021). This multicomplex system provide several opportunities for pathogen intervention. The identification of SUMO substrates is one of the main objectives of current research in the field of SUMOylation. It provides the necessary knowledge to molecularly relate the function of SUMO and the different physiological alterations observed in plants carrying mutations in different components of the conjugation machinery.

Quantitative proteomic studies examining the dynamics of SUMO conjugation with heat, ethanol and ROS treatment revealed that these stresses generally increase the accumulation of SUMO conjugates rather than modify a new target population. Most of the genes regulated by heat stress are located in the nucleus and have functions related to chromatin modification and transcription, along with an enrichment of proteins involved in RNA metabolism, suggesting that RNA processing is especially critical for defense against SUMO-influenced stress (Miller *et al*., 2013; Augustine and Vierstra, 2018; Rytz *et al*., 2018).

In the current study, 644 potential SUMO substrates were found from which 25 SUMO targets with a role in both development and defense were identified. Gene Ontology (GO) is a widely resource knowledge base on gene functions. The ontology covers three distinct aspects of gene function: molecular function (the activity of a gene product at the molecular level), cellular component (the location of gene product activity related to biological structures) and biological processes (a larger biological program in which the molecular function of a gene is used) (The Gene Ontology Consortium, 2019). This resource helped us to classify all the possible substrates based on their biological function (**Figure 2.1**), noting that targets are more involved in development than in defense. This may be due to the different stages of development that SUMO targets can be widely distributed throughout the plant's life cycle.

In order to reduce the candidates to be evaluated, a total of 9 candidates for SUMOylation evaluation were finally selected based on their relationship with defense against different types of pathogens and responses to fungal elicitors (**Figure 2.2**).

MYC2 is a basic helix-loop-helix transcription factor that functions as both a positive and negative regulator of light and multiple hormones signaling pathways, emerging as a master regulator of most aspects of the jasmonate and abscisic acid signaling pathways. MYC2 participates in JA-regulated plant development, such as lateral and adventitious root formation or flowering time. It also has a negative role as regulator of salicylic acid-mediated defense against bacterial pathogens (Kazan and Manners, 2013; Gautam *et al*., 2021).

ERF1, ERF2, ERF6 (ERF103) and ERF104 are Ethylene response transcription factors (ERFs). ERFs are members of the AP2/ERF superfamily, one of the largest families of plant transcription factors. The members of the ERF family can be divided into 12 groups based on the amino acid alignments of the AP2/ERF domains. ERF proteins can bind to the GCC box (AGCCGCC), a short cis-acting element found in the promoters of many jasmonic acid (JA)/ethylene (Et)-inducible and pathogenesis-related (PR) genes and can positively or negatively regulate transcription (Moffat *et al.,* 2012). Several ERF transcriptional activators confer increased disease resistance when overexpressed and compromised resistance when disrupted. Overexpression of the transcriptional activators, ERF1 and ERF2, upregulated transcription levels of defense genes, such as PDF1.2 and b-CHI (McGrath *et al.,* 2005, Moffat *et al.,* 2012, Meng *et al.,* 2013) and increased resistance to the necrotrophic pathogen *Fusarium oxysporum* (McGrath *et al.,* 2005). ERF proteins also play a role in a variety of developmental processes such as cell expansion, leaf petiole development and some mediate the cytokinin response (Moffat *et al*., 2012).

Recently, several ERF transcription factors have been shown to be the substrates of pathogen-responsive MAPKs. ERF104 was identified as a substrate for MPK6 that plays an important role in plant resistance to bacterial pathogens (Bethke *et al.,* 2009). In Meng *et al.,* (2013), ERF6 is also identified as a substrate of both MPK3 and MPK6 in *Arabidopsis*. The two Ser-Pro sites clustered in the C-terminal region of ERF6 are phosphorylated by MPK3/MPK6 *in vitro* and in vivo. Gain-of-function expression of a phospho-mimicking ERF6 mutant and loss-of-function expression of an ERF6-EAR chimeric repressor demonstrate that ERF6 plays important roles downstream of MPK3/MPK6 in regulating plant defense in response to fungal pathogen.

NHL10, or also known as YLS9, is a senescence-related NDR1/HIN1-like protein. NHL10 transcripts accumulate in senescent rosette leaves and at lower levels in roots, making this gene a useful molecular marker for leaf senescence in *Arabidopsis*. Its transcription is also upregulated during leaf yellowing and hypersensitive response caused by *Cucumber mosaic virus* infection and whose products are localized in the chloroplasts (Zheng *et al*., 2004), as well as treatment with *Bacillus subtilis* (Elsharkawy *et al*., 2021).

ACO4, or also known as EFE, is an Aminocyclopropane-1-carboxylate oxidase 4 responsible for catalyzing the conversion of ACC to ethylene. This gene is predominantly expressed in *Arabidopsis* seedlings shoots and its overexpression promotes ethylene synthesis compared to wild type, resulting in reduced seedling growth, thus ACO4 is necessary for proper growth and development of *Arabidopsis* seedlings (Moon *et al*., 2021). In this article they also observed that brassinosteroids inhibit ACO4 expression.

ICE2 is an INDUCER or CBF EXPRESSION2 transcription factor which encodes MYC-type bHLH (basic helix-loop-helix) transcription factors. This gene confers cold stress tolerance by inducing the CBF/DREB1 regulon and regulates stomatal formation and flowering time. Overexpression induced meristem freezing tolerance, resulting in an activation of CBF1 and CBF3 genes and ABA biosynthesis by induction of NCED3. ICE2 may be involved in JA-dependent defense pathway. Its overexpression in transgenic *Nicotiana benthamiana* has been shown to cause high antimicrobial activity in homogenized tobacco leaves (Kurbidaeva *et al*., 2014).

The confirmed SUMO substrates ERF2, ERF6, ERF104, ACO4 and EIN3 are involved in the ethylene signaling pathway. Ethylene biosynthesis affects multiple processes such as germination, senescence, fruit ripening, as well as responses to various stresses such as flooding or high salt. Once this gaseous hormone is biosynthesized, ethylene diffuses through the plant and interacts with ethylene receptors to trigger ethylene responses (Binder, 2020). Overexpression of genes related to this hormone is reported to improve tolerance to drought, salt and freezing (Xu *et al.,* 2008).

SUMOylation is an essential process, knockout mutants affect early steps of the SUMO conjugation pathway, such as the E1-activating or the E2-conjugating enzymes. Despite the embryonic lethality of mutants of essential components of the SUMOylation machinery, the use of knockout mutants has been limited to the study of specific ligase-dependent functions, such as SIZ1 or MMS21. Mutants in SUMO conjugation have made a significant contribution to functional analysis of SUMOylation (Nukarinen *et al.*, 2017). Recent studies have used other mutants that have altered SUMO conjugation and have improved our understanding of SUMO's role in plant development. The *sumo1/sumo2* knock-down mutant is partially sterile and shows phenotypes of dwarfism, early flowering, inflorescence disruption and advanced senescence (Lois *et al*., 2003). The *siz1* and *mms21* mutants show dramatic pleiotropic growth defects (Ishida *et al*., 2009; Miura *et al*, 2013). The effect of SUMOylation on the phenotype of different lines with altered levels of SUMOylation was analyzed in Castaño-Miquel et al., 2017, observing a direct relationship between size and seed production of the plant with SUMOylation.

One approach to study the effect of SUMOylation on a target protein involves the complementation of mutant lines of the corresponding gene and compare the native and the non-SUMOylation variant. We developed non-SUMOylation variants of ERF2, ERF6 and ERF104 by lysine-directed mutagenesis of the SUMO motif. However, we were unable to identify the corresponding lysine in ACO4. We attempted to analyze the variants using *Nicotiana benthamiana* through a transient expression assay. With this experiment we could not detect

protein expression of ERF2, ERF6 and ERF104. In the case of ACO4, which was expressed, we couldn't identify differences in SUMOylation and non-SUMOylation of ACO4. *N. benthamiana* system is very simple but it may not be the best strategy to express these ethylene related proteins.

ERF genes may have redundant functions, which could compromise the functional analysis of single mutants. Double or triple mutants may be necessary to study the role of each gene in the activation of plant defense responses (Lorenzo *et al.,* 2003). ERF1 and ERF2 participate in the regulation of JA, just as ERF5 and ERF6 play redundant role in defense (McGrath *et al*., 2005; Moffat *et al.,* 2012). As *erf5* and *erf6* single mutants didn't show phenotypic effects (Moffat *et al*., 2012), it was decided to use *erf5/erf6* double mutant for the complementation assays.

Finally, the T-DNA insertion mutant for ERF2 wasn't available, which led us to transform Col0 plants instead of mutant plants. For experimental consistency, we finally developed the gain-of-function of ERF2, ERF6 and ERF104 along with their respective SUMOylation-deficient versions.

Plants have developed several mechanisms to cope with different biotic and abiotic stresses. One important step in the control of stress responses seems to be the transcriptional activation or repression of genes (Chen *et al.,* 2002). Transcription factors are proteins located upstream of the target genes capable of binding to DNA-regulatory sequences that act as enhancers or silencers and modulate gene transcription rate. Transcription factors are enriched in the plant SUMOylome, where the SUMOylation modification is usually associated with reduced transcription. Although the molecular consequences depend on the specific target proteins, often these consequences result in altered subcellular localization, activity, or stability, allowing or preventing protein-to-protein interactions. One of the main consequences of the SUMOylation of transcription factor is the altered levels of expression associated with its chromatin binding sites, controlling access to the target sites, and regulating the ability to bind DNA (Rosonina *et al.*, 2017).

ERFs play important roles in many diverse developmental processes and stress responses in plants. Overexpression improves disease resistance and tolerances to drought, salt and freezing in transgenic plants (Xu *et al.,* 2008; Xie *et al.*, 2019). Within the genes regulated by ERF2, ERF6 and ERF104, in SUMOylable and non-SUMOylable variants, there are some genes that belong to these processes. STZ is regulated positively by SUMOylation of ERF2 and ERF6 and negatively in ERF104. It acts as a transcriptional repressor to increase stress tolerance. Its expression is strongly induced by dehydration, high-salt and cold stresses and its overexpression shows a delay in growth and tolerance to drought stress (Sakamoto *et al.*, 2004). Along the genes within

the regulation of response to stress we can find MYC2, negatively regulated by SUMOylation of both ERF2 and ERF6. MYC2 regulates most aspects of Jasmonate (JA) signaling pathway and plant development, is a positive regulator of defense against and wound, as well as oxidative stress and negative regulator of defense against pathogens (Kazan and Manners, 2013). MPK3 is negatively regulated by SUMOylation of ERF6, this gene improves salt tolerance negatively regulating the protein stability of ARR1, ARR10 and ARR11 promoting their degradation in response to salt stress. Furthermore, the *mpk3* single mutant shows increased tolerance, while its activation attenuates freezing tolerance (Li *et al.,* 2017; Yan *et al*., 2021). ERF2 is also within this group of genes, it is downregulated by SUMOylation of ERF6. ERF2-silenced tomato plants were susceptible to infection with *S. lycopersici*, decreasing the hypersensitive response and ROS production (Yang *et al.*, 2021). ERF5, positively regulated by SUMOylation of ERF2 and ERF6, whose expression is induced by abiotic stresses such as salinity, drought, flooding, wounding, and low temperatures, and its overexpression in tomato results in high tolerance to drought and salt stress (Pan *et al.,* 2012). The last gene belonging to the stress response regulation group is WRKY40, which is upregulated by SUMOylation of ERF6. WRKY40 is a transcriptional repressor in plant cells that belongs to a transcription factors family involved in plants responses to abscisic acid (ABA), both biotic and abiotic stress (Chen *et al.,* 2010).

In general, all genes regulated by ERF2, ERF6 and ERF104 are involved in both abiotic and biotic stress, which is very useful to analyze the effect of the SUMOylation on these ERFs under these conditions.

Previously, the effect of SUMOylation on the phenotype of different lines with altered levels of SUMOylation was previously analyzed, observing a direct relationship between the size and seed production of the plant with SUMOylation (Castaño-Miquel et al., 2017). In the present work, SUMOylation could play a role in the ERFs lines analyzed but there is no significant difference in rosette area, plant length or root length. However, differences were observed in the onset of flowering and number of lateral roots.

A delay in flowering is observed in non-SUMOylable ERF2 plants, while advanced flowering is observed in ERF104 non-SUMOylable plants, although those results weren't significant. In contrast, significant results are observed in ERF6 plants, in which the corresponding SUMO-deficient variants show earlier flowering compared to native variants. Loss of SUMOylation is reported to cause early flowering, in which different SUMO components of the SUMOylation machinery are removed, such as ESD4, ULP1a/ELS1, SIZ1 and OTS1/OTS2 (Reeves *et al*., 2002;

Murtas *et al.,* 2003; Catala *et al.,* 2007; Jin *et al*., 2008; Miura *et al.,* 2010; Park *et al*., 2011; Ling *et al.,* 2012; Xu and Yang, 2013; Kim *et al.,* 2016; Lin *et al.,* 2016).

SUMOylation has been reported to promote lateral root formation. SUMOylation-deficient mutants of *sumo1/2* and *siz1-2* in *Arabidopsis* and apple, respectively, had developed fewer lateral roots than the correspondent wild-type, regardless of the growth conditions (Hammoudi *et al.,* 2021; Zhang *et al*., 2021). With the lines analyzed, we can observe the three possible scenarios: if we suppress the SUMOylation of the protein, we observe fewer lateral roots in ERF2 lines and more lateral roots in ERF6 lines. On the contrary, we didn't obtain a significant difference with ERF104 lines. The suppression of SUMOylation is consistent with the results for ERF2 lines but not for ERF6 lines in which the SUMOylation reduces the number of lateral roots. It could depend on the regulation of the different genes and how SUMOylation can directly or indirectly affect the formation of lateral roots. Among the genes whose expression is modified by SUMOylation of ERF6 we can see that MYC2 is repressed with SUMOylation. The expression of this gene positively affects the number of lateral roots (Gangappa *et al*., 2010), such that the higher the expression of MYC2 in non-SUMOylable variants of ERF6, the more lateral roots.

In all cases, overexpression or ERF2, ERF6 and ERF104 results in fewer lateral roots compared to Col0, regardless of SUMOylation. It corresponds to the possible negative effect of ethylene on the development of lateral roots (Houben *et al.,* 2022).

We have also seen that SUMOylation affects senescence under normal conditions, noting that non-SUMOylable lines show a higher Fv/Fm ratio compared to native variants. This result corresponds to the hypothesis that SUMOylation influences senescence, but with an effect contrary to that observed in Xu and Yang (2013) where the *sumo1/sumo2* double mutant presents early senescence.

To further unravel the effect of SUMOylation on these transcription factors analyzed, AP2/ERF family transcription factors were found to be key regulators of many abiotic stresses. Several AP2/ERF mutants have been identified for altered responses to abiotic stress and sensitivity to metals or hormones (Kurepa *et al.,* 2003; Miura *et al*., 2005; Kerscher *et al.*, 2006; Xie *et al*., 2019).

Although exposure to heavy metals affects ethylene biosynthesis, the treatment with different heavy metals to ERF2, ERF6 and ERF104 lines didn't show significant results. Ethylene biosynthesis increases in response to treatment with Cd, Cu, Fe, Zn. In the case of Cd and Cu, this increase is due to an upregulation of ACC synthase transcription and enhanced activity

(DalCorso *et al*., 2010; Abozeid *et al*., 2017). The addition of Boron also inhibits the elongation of root cells via ethylene/auxin/ROS-dependent pathway (Camacho-Cristóbal *et al*., 2015).

Copper ions can elicit defense responses along the ethylene signaling pathway. $CuSO_4$ treatment rapidly increases ethylene production and increases the expression of several defense-related genes, as well as ethylene biosynthesis genes (Peñarrubia *et al*., 2015; Zhang *et al*., 2018). SUMOylation is involved in copper homeostasis and tolerance in *Arabidopsis* regulating metal transporters such as YSL1 and YSL3. SUMOylation-deficient *siz1-2* and *siz1-3* mutants had shown a copper excess hypersensitivity phenotype, being unable to regulate YSL1 and YSL3 expression under copper excess stress (Chen *et al.,* 2011). The same results were observed in OTS1, a protease that cleaves SUMO from its substrate proteins, where mutants of this protease also showed increase sensitivity to excess Cu (Zhan *et al.,* 2017). SUMO E3 ligase SIZ1 also positively regulates plant Cd tolerance modulating ROS homeostasis by regulating the activities of antioxidant enzymes. In Zheng *et* al., 2022, the lost-of-function mutant *siz1-2* showed reduced resistance to Cd exposure and accumulated more ROS.

Although not significant, SUMOylable variants of ERF2 and ERF6 show a greater metal resistance than the SUMO-deficient variants. The non-significance of these results may be due to the fact that the toxicity of heavy metals is regulated by the ethylene pathway, there are other factors that influence tolerance.

ERF-transcription factors regulate various stress responses, in which they also respond to hormones with improved plant survival during stress conditions. Plant hormones affect abiotic stress by activating a wide range of physiological processes (Xie *et al*., 2019) where post-translational modifications (PTM) are a key player to modulate them. Cross-alk between PTM and different hormone pathways is crucial to integrate exogenous environmental signals into plant development. Transcription factors form an important component of the hormone signaling pathway as they regulate downstream genes in response to activation of hormone signaling pathways (Srivastava *et al.,* 2021). A microarray analysis of the double mutant SUMO protease *spf1-1/spf2-1* showed upregulation of genes associated with the hormones auxin, brassinosteroids, cytokinin, gibberellin, jasmonate and salicylic acid. Another double mutant of the SUMO *ots1-1/ots2-1* protease resulted in decreased sensitivity to jasmonic treatment (Morrell and Sadanandom, 2019).

In our results, the effect of the treatment with different hormones and inhibitors seems to depend on the expression levels of the gene in question, so doses of hormones can't be compared. The ethylene inhibitor appears to have an effect independent of the gene expression.

Among the biological processes in which the genes that showed differences in expression levels are involved, cold stress stands out. To analyze whether the SUMOylation of ERF2, ERF6 or ERF104 can regulate resistance to cold stress, two assays were performed, one with a 7-day acclimatization period at 4 ºC and the other without acclimation. In the experiment with acclimatization, anthocyanins levels were analyzed, observing less accumulation in SUMOylation-deficient variants. SUMOylation has been shown to play an important role in the regulation of anthocyanins against cold stress and light stress (Zhou *et al.,* 2017; Zheng *et al.,* 2020; Jiang *et al.*, 2022), which is consistent with the lower levels of anthocyanins observed in SUMO-deficient variants. In the experiment without acclimation, a greater resistance to these conditions was observed by the lines that overexpress ERF6. ERF6 upregulation is markedly induced by cold treatments and its combined activity with ERF5 is also required for a full cold acclimation response (Sewelam *et* al., 2013; Wang *et al*., 2013; Illgen *et al.,* 2020). The ERF2 lines were affected at these temperatures but to a lesser degree, while none of the ERF104 lines survived.

In previous studies (Park *et al.,* 2015; Bolt *et al.,* 2017; Illgen *et al.,* 2020) it has been observed that several ERF genes are involved in response to low temperatures. Exposure of *Arabidopsis* plants to low temperatures, not to freezing, causes an increase in resistance to freezing in which the CBF (C-repeat binding factor) regulatory pathway is involved, as well as the genes ERF5, ERF6, ERF104 and ERF105 (Park *et al.*, 2015). It has been seen that the mutation of these genes doesn't cause any alteration in cold tolerance, except in the case of ERF105, where its mutation and overexpression result in a lower and higher tolerance, respectively (Park *et al.,* 2015; Bolt *et al.,* 2017; Illgen *et al.,* 2020). In our results, we have observed that both ERF2 and ERF6 SUMOylation positively regulate this gene. The gene STZ is also upregulated by SUMOylation of ERF2 and ERF6 and downregulated by ERF104. STZ is a transcriptional repressor that increases under stress conditions such as cold (Sakamoto *et al.*, 2004). Expression of C-repeat-binding factor (CBF) transcription factors is induced by cold stress. These transcription factors activate downstream cold response (COR) genes which are required for freezing tolerance. ICE1 is an important regulator of CBF and its stability is crucial for its function. MPK3, downregulated by SUMOylation of ERF6, and MPK6 phosphorylate ICE1, reducing its stability by attenuating freezing tolerance (Li *et al.,* 2017). ICE1 is downregulated by ubquination-mediated proteolysis and upregulated by SUMOylation (Chinnusamy *et al.,* 2007). WRKY33, upregulated by SUMOylation of ERF6, is also related to cold resistance. In tomato (Guo *et al*., 2022) the silenced mutants *wrky33* reduce cold tolerance. All these results reveal that ERF2 and ERF6

regulate genes related to cold response that provide greater resistance to these conditions, although SUMOylation doesn't seem to have a direct influence.

In recent years, post-translational modification mechanisms have been described as the main regulatory elements of defense responses to pathogens in plants. In fact, phosphorylation, ubiquitination, SUMOylation, nitrosylation and glycosylation play an important role in plant immunity (Lee *et al*., 2007; Stulemeijer and Joosten, 2008). The *siz1* mutant plants are characterized by accumulating high levels of salicylic, which increases the expression of PR genes, that constitutively induce systemic acquired resistance (SAR) conferring greater resistance to bacterial pathogens such as *Pseudomonas syringae* pv. Tomato (Pst) (Lee *et al*., 2007; van den Burg *et al*., 2010). Lines with altered levels of SUMOylation have shown increased susceptibility to *Botrytis* infection*,* confirming the hypothesis that SUMOylation is required for fungal resistance (Castaño-Miquel et al., 2017).

Constitutive overexpression of ERF1, ERF2, or ORA59 activates the expression of several defense-related genes, including PDF1.2 and ChiB (PR-3) (McGrath *et al.,* 2005, Moffat *et al.,* 2012, Meng *et al.,* 2013), and was shown to confer resistance to a variety of pathogens (Berrocal-Lobo *et al*., 2002; Brown *et al*., 2003; McGrath *et al.,* 2005, Xu *et al.,* 2008). In Moffat *et al.,* 2012, the *erf5* and *erf6* single mutants didn't show susceptibility to *B. cinerea* infection, while the *erf5/erf6* double mutant shows a significant increase in susceptibility to infection. It was complemented by increased resistance to *Botrytis* and susceptibility to *P. syringae* of the ERF5 and ERF6 overexpressed plants. In Bethke *et al*., 2009, overexpression of ERF104 didn't reduce susceptibility to *Botrytis cinerea* infection. Both *erf104* mutant and ERF104^{OE} plants result in increased susceptibility to the infection, meaning that any variation could lead to disturbances in signaling balance.

These results don't correspond to those observed in this project where the non-SUMOylable variants of ERF2 and ERF6 are more resistant to *Botrytis* infection. The observed susceptibility of altered levels of SUMOylation plants (Castaño-Miquel et al., 2017) could be related with the total reduction of SUMOylation in general and not in a specific defense-related protein. On the other hand, the differences with the susceptibility-resistance phenotype observed in the gain-of-function plants in Moffat *et al.,* 2012, compared to our results could be due to differences in the growth conditions of the plants and the post-infection. Gain-of-function phenotypes are not indicative of a requirement for an ERF gene in defense. According to Moffat *et al.,* 2012, constitutive expression of an ERF can lead to inappropriate binding to promoters that are not

normally regulated by the transcription factor. It would be necessary to study this phenotype related to *Botrytis* infection in different stages and conditions of the plants.

It would be necessary to analyze if the susceptibility-resistance phenotype varies according to different conditions, comparing the results in medium growth conditions with soil conditions and with different plant stages. Furthermore, this phenotype could change depending on the type of pathogen used. It would be interesting to also analyze infections with both biotrophic and hemibiotrophic pathogens.

Overall, this thesis had led to us to unravel the effect of SUMOylation of ERF2, ERF6 and ERF104 on development and defense and to better understand how this modification may alter the regulation of related genes. The generated results identify SUMOylation as an important regulatory mechanism of ethylene signaling, with implications in plant development and defense responses against fungal pathogens.

Conclusions

Conclusions

1. Under our experimental conditions, alterations of SUMOylation does not affect the capacity of the plant to inhibit root growth in response to auxin.
2. Based on literature review, 644 potential SUMOylation candidates were selected. Of those, only 25 presented a role in development and in defense.
3. From the selected candidates, ERF2, ERF6, ERF104 and ACO4 were confirmed to be SUMOylated. Those SUMO targets, including the previously validated as SUMO substrate EIN3, are involved in the ethylene signaling pathway, which is of special interest since ethylene is a hormone known to play a role in both development and in defense.
4. SUMO acceptor lysine residues were in identified in ERF2, ERF6 and ERF104, but not in ACO4, highlighting the challenges associated with the study of protein SUMOylation.
5. Since the proteins under study are transcriptional regulators of genes related to the response to ethylene, we analyzed the effect of the SUMOylation on the regulation of genes belonging to this pathway, resulting in the identification of 17 genes with different expression levels. ERF2 presents mainly an activating role, ERF6 has an activating and a repressor role depending on the target gene, whereas ERF104 is characterized by showing a repressor role of the genes analyzed. The SUMOylation deficient variants have compromised the activating or repressing activity of the transcription factor analyzed.
6. To study the effect of SUMOylation in plant phenotype, different parts of the plant were analyzed. It was observed that SUMOylation of ERF2 and ERF6 seems to affect the onset of flowering, advancing and delaying it, respectively, but the effect of the SUMOylation status was only significant in ERF6. Significant differences were also found in the number of lateral roots. SUMOylation of ERF2 affects positively to lateral roots development, as opposed to ERF6 in which has a negative effect. In the case of ERF104, it seems that non-SUMOylable variants have a greater number of lateral roots, although it is not statistically significant.
7. Since exposure to heavy metals affects ethylene biosynthesis in plants, we wanted to study whether SUMOylation had a role in regulation of heavy metal toxicity, screening with various heavy metals available in the laboratory, but no significant differences were found.
8. Since our genes of interest belong to the ethylene signaling pathway and this hormone participates in the cross-talk with other hormones, the analysis of the addition of different hormones to the medium was performed. We found that, in some cases, the effect of expression levels may mask the effect of the regulation by SUMO. Nonetheless, it is well shown that SUMOylation of ERF2 and ERF6 has an important role in the developmental processes analyzed, while SUMOylation of ERF104 seems not to be relevant for the parameters analyzed.
9. Under optimal growth conditions, SUMO deficient variants of ERF2, ERF6 and in ERF104 possess significant more photosynthetic capacity compared to the SUMOylable variants.

10. To analyze whether the SUMOylation of ERF2, ERF6 and ERF104 can regulate resistance to cold stress, two assays were carried out, one with a period of acclimation and the other one without acclimation.

   a. With the experiment of cold stress with acclimation, the effect of this stress through the accumulation of anthocyanins was studied directly, where the SUMOylation seems to induce accumulation.

   b. With the experiment of cold stress without acclimation, greater resistance to these conditions was observed by the ERF6 overexpressing lines, obtaining a survival rate of 100%. On the contrary, the ERF2 lines were affected at these temperatures, but the recovery of some plants was observed, while in the ERF104 lines, showed a mortality of 100%. Nevertheless, we couldn't find significant differences between native or SUMO deficient variants.

11. To study the effect of SUMOylation in defense, an infection with *Botrytis cinerea* was performed. Non-SUMOylable variants of ERF2 and ERF6 were significantly more resistant to infection with *B. cinerea*, showing larger lesion area and amount of fungal biomass in infected leaves. On the contrary, SUMOylation of ERF104 doesn't seem to affect the susceptibility-resistance phenotype.

12. Overall, the generated results identify SUMOylation as an important regulatory mechanism of ethylene signaling, with implications in plant development and defense responses against fungal pathogens.

Materials and Methods

1. Bacterial strains and growth conditions

1.1. Microbiology material

1.1.1. Bacterial strains

The following bacterial strains have been used in this study:

- TOP10: *Escherichia coli* strain used for vector cloning.
- BL21 (DE3): A strain of *Escherichia coli* used for protein expression.
- GV3101: *Agrobacterium tumefaciens* strain for cloning binary vectors used for agroinfiltration in *Nicotiana benthamiana* and for plant transformation.

1.1.2. Bacterial culture media

Bacterial culture media is prepared with distilled water. Supplements or antibiotics required on each occasion were added after tempering the medium a 50 ºC. The same media were used for solid petri dish culture, adding 1.5% agar.

- Medium LB pH 7.5 (autoclave): Bactotriptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L. Adjust the pH with NaOH. For the solid medium, 15 g/L is added.
- Medium YEB pH 7.2 (autoclave): Beef extract 5 g/L, yeast extract 1 g/L sucrose 5 g/L and $mgSO_4$ 0.48 g/L. Adjust the pH with NaOH. For the solid medium, 15 g/L is added.
- Medium 2xTY pH 7.5 (autoclave): Bactotriptone 16 g/L, yeast extract 10 g/L and NaCl 5 g/L. Adjust the pH with NaOH.

1.1.3. Bacterial culture media supplements

The **Table M1** show the different supplements used, the concentrations of the stock solutions and de solvent in which they are prepared with the working solution.

Table M.1 – Table of supplements used with bacteria and plants.

			Working solution		
Antibiotic	**Stock**	**Solvent**	***E. coli***	***A. tumefaciens***	***A. thaliana***
Kanamycin (Km)	25 mg/ml	H_2O	25 µg/ml	25 µg/ml	25 µg/ml
IPTG	1 M	H_2O	0.2 mM	-	-
Chloramphenicol (Cl)	25 mg/ml	EtOH	10 µg/ml	-	-
Rifampicin (Rf)	50 mg/ml	DMSO	-	50 µg/ml	-
Hygromycin (Hyg)	50 mg/ml	H_2O	-	-	30 µg/ml
Basta/ppt	16 mg/ml	H_2O	-	-	16 µg/ml
Spectinomycin (Sp)	50 mg/ml	H_2O	100 µg/ml	100 µg/ml	-

1.1.4. Bacterial culture conditions

Liquid culture of *E. coli* cells is performed at 37 ºC with stirring at 220 rpm. The solid culture is carried out in plate to 37 ºC. Liquid culture of *A. tumefaciens* cells is performed at 28 ºC with stirring at 200 rpm. The solid culture is carried out in plate to 28 ºC. Bacterial cultures or strains are permanently stored in 15% glycerol at -80 ºC.

1.2. Plant material, transformation and growth conditions

1.2.1. Wild-type ecotype

Arabidopsis thaliana plants of Columbia-0 (Col0) ecotype and *Nicotiana benthamiana* plants have been used in this project.

1.2.2. Transgenic lines

1.2.2.1. Overexpressing lines

- SUMO1^{ox}: Overexpressing plants if SUMO1 under the constitutive 35S promoter in Col0 background (Lois *et al*., 2003).
- SAE2^{UFDCt}: Lines that overexpress the UFD-Ct domain of the large subunit of the activating enzyme (AtSAE2) under the control of the 35S constitutive promoter in Col0 background, giving rise to different levels of SUMO conjugate accumulation, inversely related to the expression levels of the SAE2^{UFDCt} domain (Castaño-Miquel *et al.,* 2017).
- ERF2^{ox}: Overexpressing plants of ERF2 native protein under the constitutive 35S promoter in Col0 background.
- ERF2^{K87,228R_ox}: SUMOylation deficient overexpressing plants of ERF2 K87,228R mutated protein under the constitutive 35S promoter in Col0 background.
- ERF6^{ox}: Overexpressing plants of ERF6 native protein under the constitutive 35S promoter in Col0 background.
- ERF6^{K53,63,72,121,231R_ox}: SUMOylation deficient overexpressing plants of ERF6 K53,63,72,121,231R mutated protein under the constitutive 35S promoter in Col0 background.
- ERF104^{ox}: Overexpressing plants of ERF104 native protein under the constitutive 35S promoter in Col0 background.
- ERF104^{K183R_ox}: SUMOylation deficient overexpressing plants of ERF104 K183R mutated protein under the constitutive 35S promoter in Col0 background.

1.2.2.2. Complemented lines

- SUMO RV: 6His-SUMO1(H89R) *sumo1-1 sumo2-1.* Double mutant *sum1-1 sum2-1* complemented with the variant His:SUMO1 H89R under expression of the endogenous SUMO1 promoter (Miller *et al*., 2010).

- ERF6: Complemented lines under 35S constitutive promoter of ERF6 native protein in *erf5/erf6* background.
- ERF6^{K53,63,72,121,231R}: SUMOylation deficient complemented lines under 35S constitutive promoter of ERF6 K53,63,72,121,231R mutated protein in *erf5/erf6* background.
- ERF104: Complemented lines under 35S constitutive promoter of ERF104 native protein in *erf104* background.
- ERF104^{K183R}: SUMOylation deficient complemented lines under 35S constitutive promoter of ERF104 K183R mutated protein in *erf104* background.
- EIN3/EIL1: Complemented lines under 35S constitutive promoter of EIN3 native protein in *ein3/eil1* background.
- EIN3/EIL1^{K334,336,446R}: SUMOylation deficient complemented lines under 35S constitutive promoter of EIN3 K334,336,446R mutated protein in *ein3/eil1* background.

1.2.3. Mutant lines

- *erf6:* T-DNA insertion mutant in the sequence encoding for ERF6 gene (SALK_087356) (**Figure M.1**).
- *erf104:* T-DNA insertion mutant in the sequence encoding for ERF104 gene (SALK_057720) (**Figure M.1**).
- *aco4:* T-DNA insertion mutant in the sequence encoding for ACO4 gene (SALK_014965) (**Figure M.1**).
- *erf5/erf6:* Double T-DNA insertion mutant in the sequence encoding for ERF5 (GK-681E07) and to ERF6 (SALK_087356) genes (**Figure M.1**).
- *ein3/eil1:* Double mutant for EIN3 and EIL1 genes, for EIN3, mutant mutation induced by the mutagenic agent ethyl methanesulfonate, known as MS. It provokes the conversion of a nucleotide from G to A forming a STOP codon which gives to a truncated protein (from 1501 bp to 1477 bp). For EIN3 gene, En-I transposon insertion at position 697. Gen-Bank adhesion AF004214 (Alonso *et al.,* 2003).
- *siz1-3*: T-DNA insertion mutant in the sequence encoding for the A ligase AtSIZ1 (Catala *et al*., 2007).

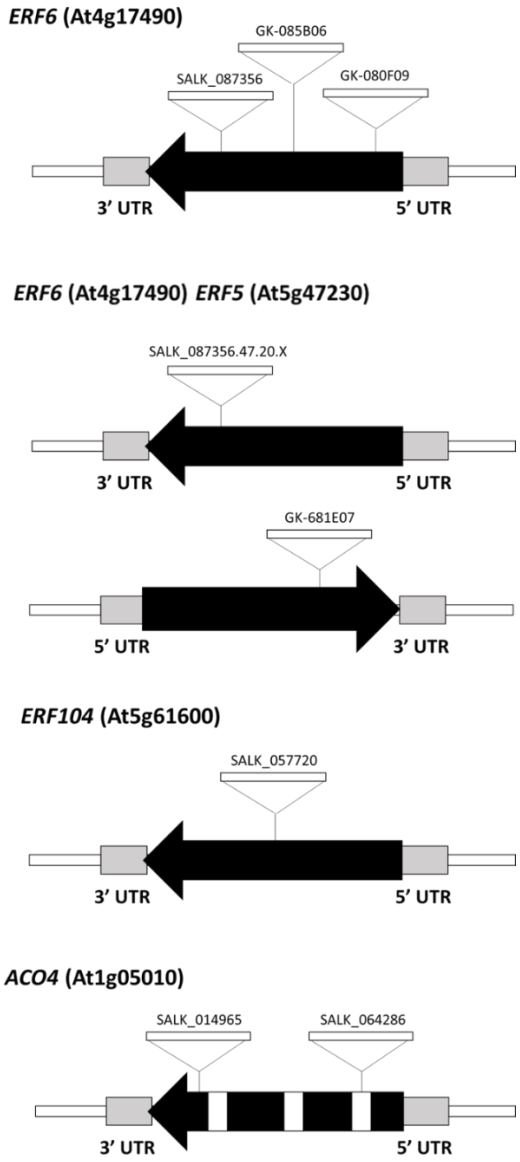


Figure M.1 – Graphic representation of the identified T-DNA insertion mutants from the public collections of the genes erf6, erf104 and the double mutant erf5/erf6.

1.2.4. Plant cultivation conditions

The plants have been grown in pots with a mixture of peat 3:5, perlite 1:5 and vermiculite 1:5. The growth period has been developed in greenhouses or in controlled growing rooms.

Greenhouses: the plants were grown in conditions of 14 hours of light and 10 hours of darkness. The conditions of crop have been 22 ± 2 ºC with a humidity of 40-60%. The plants have been watered by flooding the trays containing the pots, 3 times a week.

Controlled culture chambers: The culture condition in chambers have been in long day conditions (16/8), 22 ± 2 ºC with a humidity of 40-60%. The plants have been watered by flooding the trays containing the pots, 3 times a week.

1.2.5. Plant culture media

Murashige and Skoog media (MS) has been used to grow plants in petri dishes and in sterile conditions. Composition MS pH 5.7 (0.5x): Murashige & Skoog (Duchefa) medium including vitamins 2.2 g/L, MES 0.5 g/L. Adjust the pH with KOH. 8 g/L agar is added.

1.2.6. Supplements to plant culture media

Supplements added to the media have been antibiotics and herbicides. The **Table M.1.** shows the different supplements used, the concentrations of the stock solutions and the solvent in which they are prepared, along with the working concentrations.

1.2.7. In vitro culture conditions

Under *in vitro* conditions, the plants were grown in neutral conditions, 12 hours of light and 12 hours of darkness at 22 ± 2 ºC. In case of a dark treatment, previously and once the seed have been sterilized and stratified (48 hours in water at 4 ºC), a light treatment of 2 hours is carried out in order to activate germination.

1.3. Bacterial and plant cloning vectors

1.3.1. Expression vectors in E. coli

- pCR™-Blunt II- TOPO® (Invitrogen): Vector used for the expression of heterologous proteins in *E. coli* cells under the control of the phage promoter T7 and SP6. Kanamicin^R.
- pET28 (Novagen): Vector used for the expression of heterologous proteins in *E. coli* cells under the control of the phage promoter T7. Kanamicin^R.
- pACYCDuetTM-1 (Novagen): Vector used for the expression of heterologous proteins in *E. coli* cells under the control of the phage promoter His. Chloramphenicol^R.
- pCDFDuetTM-1 (Novagen): Vector used for the expression of heterologous proteins in *E. coli* cells under the control of the phage promoter His. Spectinomycin^R.
- pENTR™ Directional TOPO (Invitrogen): Vector used for the expression of heterologous proteins in *E. coli* cells under the control of the phage promoter T7. Kanamicin^R.

1.3.2. Expression vectors in plants

- pBA002 (Kost *et al.* 1998): Vector used for Agrobacterium transformation that allows cloning the gene of interest under the control of the 35S constitutive promoter. Spectinomycin^R in bacterium and BASTA^R in plants.
- pGWB512 (Gateway): Vector used for Agrobacterium transformation that allows cloning the gene of interest under the control of the 35S constitutive promoter and a Flag-tag. Spectinomycin^R in bacterium and BASTA^R in plants.

1.3.3. Constructions

Table M.2 – Constructions used in this work.

Type of vector	Cloning vector	Promoter	Fusion	Gene	Construction	Insert	Name
Expression vector in *E. coli*	pACYCDuetTM-1	T7	His/ S-tag	SAE2 (AT2G21470) SAE1a (AT4G24940)	pACYCDuetTM-1_AtSAE2_AtSAE1a	AtSAE2_AtSAE1a	SAE2
	pCDFDuetTM-1	T7	His/ S-tag	SUMO1 (AT4G26840) SCE1a (AT3G57870)	pCDFDuetTM-1_AtSUMO1GG_AtSCE1a	AtSUMO1GG_AtSCE1a	SUMO1_GG
					pCDFDuetTM-1_AtSUMO1AA_AtSCE1a	AtSUMO1AA_AtSCE1a	SUMO1_AA
	pET28a	T7	His, T7/ His	MYB30 (AT3G28910)	pET28a_MYB430	MYB30	MYB30
				MYC2 (AT1G32640)	pET28a_MYC2	MYC2	MYC2
				ERF6 (AT4G17490)	pET28a_ERF6	ERF6	ERF6
					pET28a_ERF6_K53R	ERF6_K53R	ERF6_K53R
					pET28a_ERF6_K63R	ERF6_K63R	ERF6_K63R
					pET28a_ERF6_K72R	ERF6_K72R	ERF6_K72R
					pET28a_ERF6_K121R	ERF6_K121R	ERF6_K121R
					pET28a_ERF6_K231R	ERF6_K231R	ERF6_K231R
					pET28a_ERF6_K63,72R	ERF6_K63,72R	ERF6_K63,72R
					pET28a_ERF6_K63,121R	ERF6_K63,121R	ERF6_K63,121R
					pET28a_ERF6_K63,231R	ERF6_K63,231R	ERF6_K63,231R
					pET28a_ERF6_K72,121R	ERF6_K72,121R	ERF6_K72,121R
					pET28a_ERF6_K72,231R	ERF6_K72,231R	ERF6_K72,231R
					pET28a_ERF6_K121,231R	ERF6_K121,231R	ERF6_K121,231R
					pET28a_ERF6_K63,72,121R	ERF6_K63,72,121R	ERF6_K63,72,121R
					pET28a_ERF6_K63,72,231R	ERF6_K63,72,231R	ERF6_K63,72,231R
					pET28a_ERF6_K63,121,231R	ERF6_K63,121,231R	ERF6_K63,121,231R
					pET28a_ERF6_K72,121,231R	ERF6_K72,121,231R	ERF6_K72,121,231R
					pET28a_ERF6_K63,72,121,231R	ERF6_K63,72,121,231R	ERF6_K63,72,121,231R
					pET28a_ERF6_K53,63,72,121,231R	ERF6_K53,63,72,121,231R	ERF6_K53,63,72,121,231R

				ERF104 (AT5G61600)	pET28a_ERF104	ERF104	ERF104
					pET28a_ERF104_K183R	ERF104_K183R	ERF104_K183R
					pET28a_ERF104_K81,183R	ERF104_K81,183R	ERF104_K81,183R
					pET28a_ERF104_K172,183R	ERF104_K172,183R	ERF104_K172,183R
				CA1 (AT3G01500)	pET28a_CA1	CA1	CA1
				ACO4 (AT1G05010)	pET28a_ACO4	ACO4	ACO4
					pET28a_ACO4_K191R	ACO4_K191R	ACO4_K191R
					pET28a_ACO4_K295R	ACO4_K295R	ACO4_K295R
					pET28a_ACO4_K191,295R	ACO4_K191,295R	ACO4_K191,295R
					pET28a_ACO4_K120R	ACO4_K120R	ACO4_K120R
					pET28a_ACO4_K11R	ACO4_K11R	ACO4_K11R
					pET28a_ACO4_K23R	ACO4_K23R	ACO4_K23R
					pET28a_ACO4_K151R	ACO4_K151R	ACO4_K151R
					pET28a_ACO4_K289R	ACO4_K289R	ACO4_K289R
	pET28b	T7	His, T7/ His	ERF1 (AT3G23240)	pET28b_ERF1	ERF1	ERF1
				ERF2 (AT5G47220)	pET28b_ERF2	ERF2	ERF2
					pET28b_ERF2_K87R	ERF2_K87R	ERF2_K87R
					pET28b_ERF2_K228R	ERF2_K228R	ERF2_K228R
					pET28b_ERF2_K87,228R	ERF2_K87,228R	ERF2_K87,228R
				ICE2 (AT1G12860)	pET28b_ICE2	ICE2	ICE2
				NHL10 (AT2G35980)	pET28b_NHL10	NHL10	NHL10
				ERF5 (AT5G47230)	pET28b_ERF5	ERF5	ERF5
Transient transformation vector in plant	pBA002	p35S	FLAG	SUMO1 (AT4G26840	pBA002_3xHis-HA-SUMO1GG	3xHis-HA-SUMO1GG	SUMO1_GG
					pBA002_3xHis-HA-SUMO1AA	3xHis-HA-SUMO1AA	SUMO1_AA
Transient transformation vector in plant /Stable plant transformation vector	pGWB512	p35S	FLAG	ERF2 (AT5G47220)	pGWB512_ERF2	ERF2	ERF2
					pGWB512_ERF2_K87,228R	ERF2_K87,228R	ERF2K
				ERF6 (AT4G17490)	pGWB512_ERF6	ERF6	ERF6
					pGWB512_ERF6_K53,63,72,121,231R	ERF6_K53,63,72,121,231R	ERF6K
				ERF104 (AT5G61600)	pGWB512_ERF104	ERF104	ERF104
					pGWB512_ERF104_K183R	ERF104_K183R	ERF104K
				ACO4 (AT1G05010)	pGWB512_ACO4	ACO4	ACO4
				MYC2 (AT1G32640)	pGWB512_MYC2	MYC2	MYC2
				NHL10 (AT2G35980)	pGWB512_NHL10	NHL10	NHL10

1.3.4. SUMO substrate cloning design

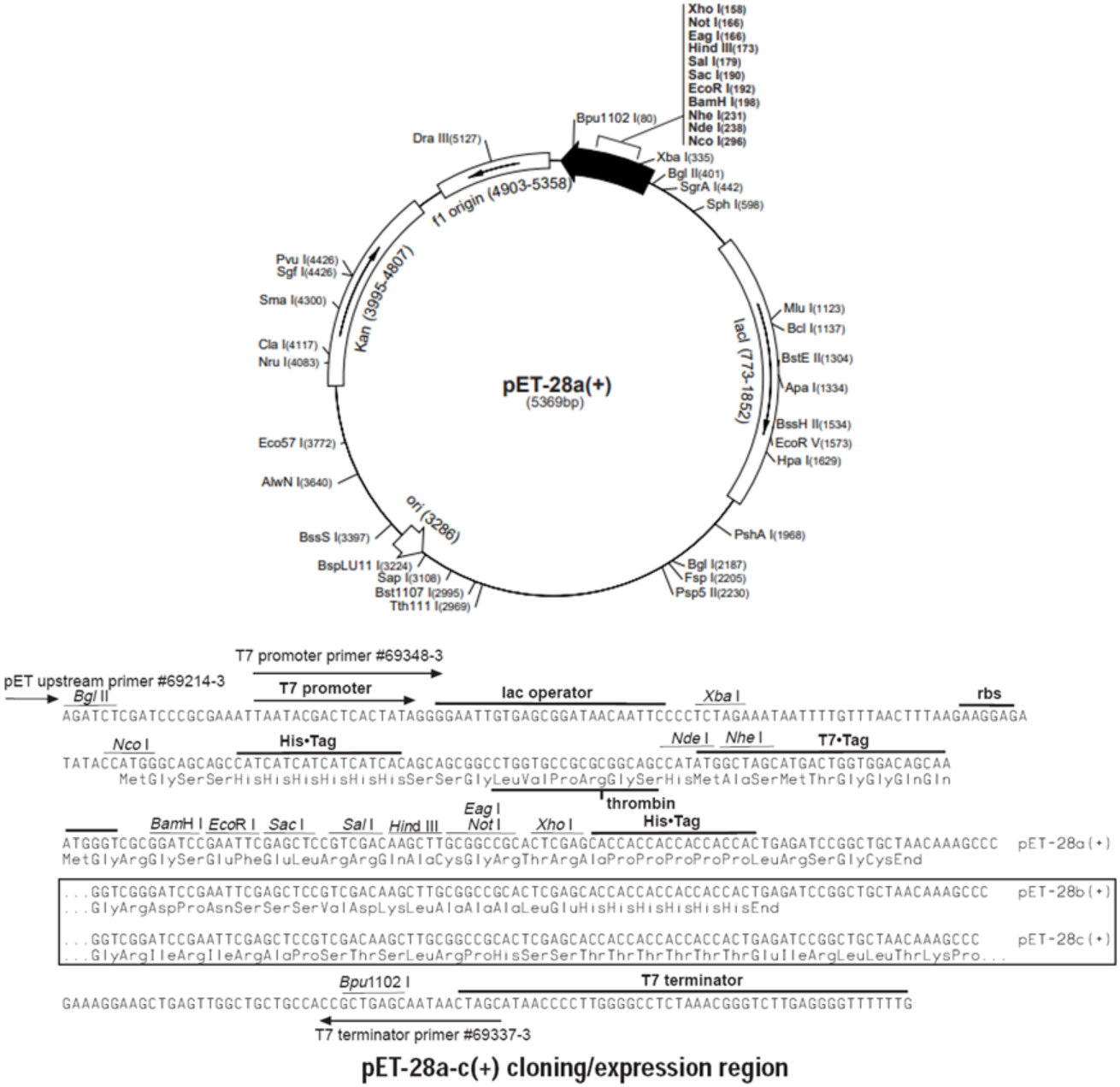


Figure M.2 – Description of the pET-28a-c expression plasmid with its corresponding cloning/expression region. *(Complete description available in https://www.helmholtz-muenchen.de/fileadmin/PEPF/pET_vectors/pET-28a-c_map.pdf).*

The SUMO substrate to validate were cloned in a pET28 vector as described in **Table M.2**. The **Figure M.2** shows the map of this vector with its corresponding cloning/expression region. The cloning was performed by restriction enzymes based on most genes with the same restriction enzyme, avoiding selecting too close restriction enzymes and avoid removing His-Tag or T7-Tag from the vector. Once two restriction enzymes were selected for each gene, the percentage of compatibility of each pair was compared with the Promega Buffers. For the MYC2

gene, the enzymes to be used are found just before the T7-Tag, so we would run out of that sequence. In this case, it would depend on the His-Tag located just after the cut (**Table M.3**).

Table M.3 – Summary of genes with possible restriction enzymes to use for the cloning in pET-28a-c expression plasmid. *Restriction enzymes available for each gene are marked with an X. Those selected for use are marked in bold and grey. The selection of the enzymes was made based on most genes with the same restriction enzyme, avoiding selecting too close restriction enzymes and avoiding removing His-Tag and T7-Tag from the vector. Once two restriction enzymes were selected for each gene, the percentage of compatibility of each pair was compared with the Promega Buffers.*

	XhoI	NotI	EagI	HindIII	SalI	SacI	Eco RI	BamHI	NheI	NdeI	NcoI
MYC2		**X**	X						X	**X**	X
ERF2	**X**	X	X	X	X	X	X	**X**	X	X	X
ERF6	**X**	X	X	X	X	X	X	**X**	X	X	X
ERF104		**X**	X		**X**	X			X	X	
NHL10	**X**	X	X		X	X	X	**X**	X	X	X
CA1	**X**	X	X				X	**X**	X	X	X
ACO4		**X**	X		X	X	X	**X**	X	X	
ERF1	**X**	X	X	X	X	X	X		X	X	
ICE2		**X**	X		X		X	**X**	X	X	

1.3.5. Primers

As 257 primers have been designed for this project, all of them are collected in the supplementary material section in **Table S.1**.

2. Molecular biology techniques of nucleic acids

2.1. Preparation of DNA

Plasmid DNA minipreparation. It is prepared with the Promega Miniprep kit, following the manufacturer's instructions.

2.2. DNA cloning

For DNA modification reactions performed, such as digestion of DNA fragments with restriction enzymes, ligations, etc. different restriction enzymes (Promega, Roche, NewEngland Biolabs, Gibson, Invitrogen) have been used, and the conditions recommended by the manufacturer in each case have been followed. That is why in the present work only those that have been of relevant importance are detailed or if the protocol followed involves some modifications over the original.

2.3. Transformation of competent *E. coli* cells by heat shock

E. coli cell transformation is used to amplify plasmid DNA or for heterologous strain-dependent protein expression. The competent cells were transformed by heat shock according to the following protocol:

1. Thaw in ice the tube containing an aliquot of competent cells.
2. Add 1-5 µL (10-20 ng) of DNA and mix gently.
3. Keep the mixture in ice for at least 15 minutes.
4. Perform a thermal shock for 1 minute at 42 ºC.
5. Transfer the tube to ice for 1 minute.
6. Perform again a thermal shock for 1 minute at 42 ºC.
7. Add 1 mL of fresh LB medium without antibiotics. Stir the tube at 220 rpm at 37 ºC for 1 hour.
8. Plate different volumes of the transformation in two solid LB plates with the appropriate selective antibiotic.
9. Incubate the bacteria at 37 ºC O/N.
10. Colony PCR to look for properly transformed colonies.

2.4. Transformation of *Agrobacterium* cells (for Floral Dip) by heat shock

The strain of *Agrobacterium* GV3101 were used for the obtention of transgenic plants of *Arabidopsis* by Floral Dip (section 4.3.). The *Agrobacterium* cells were transformed according to the following protocol:

1. Thaw the competent cells carefully on ice (5-15 minutes).
2. Add 500 ng – 2 µg of DNA to cells and mix gently.
3. Incubate on ice for 30 minutes.
4. Freeze the cells in liquid nitrogen for 5 minutes.
5. Thaw the cells at 37 ºC for 5 minutes.
6. Add 500 µL – 1 µL of YEB liquid culture media and incubate at 28 ºC for 2-4 hours while shaking (160 rpm).
7. Place the cells on YEB plates containing the proper antibiotic and incubate at 28 ºC for 2 days.
8. Colony PCR to look for properly transformed colonies.
9. Liquid culture of the positive ones (2 mL YEB + proper antibiotics). Incubate at 28 ºC for 1-2 days.
10. Glycerinate and storage at -80 ºC.

2.5. Polymerase chain reaction (PCR)

To amplify DNA fragments, the polymerase chain reaction (PCR) method has been used (Saiki *et al.*, 1988), using specific oligonucleotides and complementary plasmid DNA (cDNA) or genomic (depending on the case) as a substrate. Its theoretical basis is described in all molecular biology textbooks (Sambrook, 2001).

Commercial thermostable DNA polymerase enzymes were used to amplify the fragments by PCR. Pfu DNA Polymerase (Stratagene), Taq Polymerase (Promega) and Go Taq Polymerase (Promega) were used according to their availability. All of them are characterized by having a low error rate (HF, High Fidelity). These enzymes have their own buffers and were used following the manufacturer's instructions.

The primers used for the PCRs were produced by Sigma-Aldrich. Their sequences are specified in the supplementary material section in **Table S.1**.

2.5.1. Retrotranscription

This technique has been used to amplify the first strand of specific cDNAS from total RNA. In this work, primer homologous to the sequence of interest were used. For the synthesis of the first strand of cDNA, Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) were used according to the manufacturer's procedures.

2.5.2. PCR amplification

In each PCR cycle there are three stages that require different temperatures and are carried out in the PCR apparatus or thermal cycler, which allows a very fast transition between one temperature and another. The three stages of each cycle are:

1. Denaturation of DNA in presence of primer's oligonucleotides. It's carried out at a high temperature, usually 94 ºC, for 30 seconds.
2. Hybridization of the primers to the complementary sequences of DNA template to be amplified. It's carried out at the TM temperature of the oligonucleotide, which is given by its sequence and length. It tends to be between 45 and 65 ºC. The hybridization time is about 45 seconds.
3. Once the primers have joined the DNA, the thermostable DNA polymerase acts, synthesizing the complementary DNA strands in the 5' to 3' direction, with exonuclease activity in the 3' to 5' proofreading direction to correct possible errors in the previous activity. This stage is carried out at 72 ºC for a time that depends on the size of the fragment to be amplified (approximately 1 minute per 1 kb of DNA).

In the case of colony PCR, the PCR template DNA is added by pricking the colony with a tip and dipping the tip into the PCR mix. The verification of the amplified fragments is carried out by loading a part of the volume obtained on an agarose gel (section 2.1.5.3.). When necessary, the fragments obtained were cloned a subsequently sequenced (section 2.1.6.).

2.5.3. DNA separation in agarose gels

PCR products or DNA fragments from digestions have been separated in agarose/TAE gels with 1% (w/v) agarose and 0.05% (w/v) ethidium bromide, in 1X TAE, at side of an appropriate molecular weight marker for the expected band size.

Solutions:

- Loading buffer 6X: Glycerol 30% (v/v), xylene FF 0.25% (w/v), bromophenol blue 0.25% (w/v), EDTA 0.5 µM pH 8.
- TAE buffer 10X: Tris acetate 0.4 M, EDTA 20 mM pH 8.

2.6. DNA sequencing

The determination of the prepared DNA sequences has been carried out by the Sequencing Service of the Centre for Research in Agrigenomics (CRAG) through automatic sequencing. This system is based on the dye terminator method. The average read with this method is 600-700 bp.

The sequences obtained have been computer processed using DNAstar bioinformatic programs and free programs on the Internet such as NCBI's BLAST and Genedoc.

2.7. Purification of DNA fragments

DNA fragments resolved on agarose/TAE gels and PCR products were purified using the kit Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions.

2.8. PCR-directed mutagenesis

By means of a PCR reaction with the appropriate oligonucleotides and the *DpnI* enzyme (Promega), directed mutagenesis was carried out, following the manufacturer's specifications. The restriction enzyme *DnpI* has the particularity of cutting only methylated sites, exclusively attacking the parental chains.

2.9. Preparation of total RNA

Total RNA from plant tissues was extracted using the Maxwell® RSC Plant RNA Kit AS1500 (Promega) according to the manufacturer's instructions. Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) were used to generate cDNA according to the manufacturer's procedures.

2.10. Quantification of nucleic acids

The amount of DNA or RNA present in a sample was estimated form the measurement of absorbance at 260 nm. The purity of the nucleic acid samples was determined by calculating the ratios between the absorbances at 260 nm and 280 nm. The ratio between these two absorbances indicates the presence of proteins outside the range of 1.7-2.

2.11. Quantitative PCR

The relative abundance of mRNA was assessed by quantitative PCR from reverse transcription (RT-qPCR) in a total reaction volume of 10 µL using LightCycler 480 SYBR® Green I Master (Roche) in a LightCycler 480 Real-Time PCR System (Roche) with 0.3 µM of each of the corresponding primers. The primer sequences are specified in the supplementary material section in Table S.1.

3. Molecular biology of proteins

3.1. Protein extraction from plant tissues

The following method was used for protein extraction:

1. Triturate the sample in liquid nitrogen using a mortar.
2. Weigh the sample (about 100 mg).
3. Add 2 µL of PE extraction buffer per mg of sample.
4. Vortex until completely homogenized.
5. Place on the wheel for 30 minutes at 4 ºC.
6. Centrifuge for 20 minutes at maximum speed at 4 ºC and recover the supernatant.
7. Measure the amount of protein present in the sample using the Bradford method.
8. Freeze with liquid nitrogen and store at -80 ºC.

Solutions:

- PE extraction buffer: Tris-HCl 100 mM pH 7.7, Urea 8 M, Triton X-100 0.2%, Sarkosyl 0.2 % (w/v) and protease inhibitors (PMSF (phenylmethanesulphonylfluoride) 1 mM, Pepstatin 1 µg/mL, Leupeptin 1 µg/mL, NEM (N-Ethylmaleimida) 2 mM, Iodoacetamida 10 mM).

3.2. Quantification of proteins

The samples extracted according to the previous protocol were quantified according to the Bradford spectrophotometric method with the Biorad Protein Assay reagent. In each case, replicas of the samples to be measured have been made. The measures were carried out in a spectrophotometer at a wavelength of 595 nm.

3.3. Electrophoretic separation of proteins

Proteins were separated by protein denaturing electrophoresis (SDS-PAGE) using the Biorad vertical apparatus, following the manufacturer's instructions.

Procedure:

1- Assemble the gel preparation system.

2- Add the separating gel up to a height of approximately 7 cm. Add a few drops of isopropanol to cover the surface exposed to atmospheric oxygen, which would inhibit polymerization. Leave to polymerize for 1 hour.
3- Remove the isopropanol. Add the stacking gel to the upper end of the glasses and immediately fit the corresponding comb with the appropriate number of wells. Let the gel polymerize for 1 hour. 0.75-1.5 mm thick glass has been used.
4- Place the gel in the corresponding support in the electrophoresis cuvette. Add electrophoresis buffer 1X until the wells overflow.
5- Prepare the samples as described before and add the appropriate volume of 6X loading buffer. Boil the samples for 5 min at 95 ºC.
6- Load the gel with the samples. Apply the appropriate voltage for the correct separation of the proteins. Amperages of 20-40 mA were set, depending on the number of gels that were migrating in the system (1 or 2). Once the migration front reaches the lower end of the gel, stop the electrophoresis.

Solutions:

- 10X electrophoresis buffer: Tris base 0.25 M, Glycine 1.92 M, SDS 1% (w/v).
- Loading buffer 6X: Tris-HCl 0.35 M pH 6.8, 30 % glycerol (v/v), SDS 10% (w/v), DTT 0.6 M, bromophenol blue 0.012% (w/v).

3.4. Protein analysis by transfer and immunodetection (Western-blot)

From an SDS-PAGE gel, the proteins were transferred to PVDF membranes following the semi-dry method. The Trans-Blot Semi-Dry Electrophoretic Cell apparatus from BioRad was used following the manufacturer's instructions.

Protein detection was by chemiluminescence using a specific primary antibody against the protein to be detected and a secondary anti-primary antibody conjugated with a horseradish peroxidase (GE Healthcare), which hydrolyses the ECL PLUS chemiluminescent reagent (ECL PLUS Western Blotting detection reagents, Ge Healthcare).

Procedure:

1. Recover the SDS-PAGE gel and discard the stacking gel.
2. Wash the PVDF membrane in methanol absolute for 30 seconds. Wash the excess methanol from the membrane for 30 seconds with H_2O milliQ and finally keep in transfer buffer.

3. Attach the membrane to the gel between pieces of thick absorbent paper of the same size and previously equilibrated with the transfer buffer.
4. Place the assembly in the transfer device and set a voltage of 25 V for 30 min.
5. Recover the membrane.
6. Block with TBST 1X + 3% (w/v) of skimmed milk powder for 1 hour at RT with agitation.
7. Remove the block and add the primary antibody to the corresponding dilution in TBST buffer 1X + 3% (w/v) of skimmed milk powder O/N at 4 ºC and according to the manufacturer's recommendations.
8. Wash the membrane with TBST with three washes of 10 minutes at RT and stirring.
9. Incubate the membrane for 45 minutes at RT with the corresponding secondary antibody in TBST buffer 1X + 3% (w/v) of skimmed milk powder.
10. Wash the membrane with TBST with three washes of 10 minutes at RT and stirring.
11. Wipe the membrane to remove excess TBST and add the ECL Plus reagent. Reveal the membrane with the help of the LAS4000 equipment (Fujifilm Life Science Products) varying the exposure times to achieve optimal image resolution and contrast.

Solutions:

- Transfer buffer: Tris 48 mM, glycine 39 mM, methanol 10% (v/v).
- TBST buffer: Tris-HCL 20 mM pH 7.6, NaCl 20 mM, Tween-20 0.1% (v/v).

3.5. Antibodies used

3.5.1. Primary antibodies

- Anti-SUMO (Ref. A S08308): Polyclonal antibody. Work dilution 1:2000.
- Anti-T7 (Ref. AB3790): Polyclonal antibody. Work dilution 1:2000.
- Anti-His (Ref. H1029): Polyclonal antibody. Work dilution 1:6000.
- Anti-FLAG (Ref. F1804): Monoclonal antibody. Work dilution 1:2000.
- Anti-ACO4 (Ref. AS18 4240): Polyclonal antibody. Work dilution 1:1000.

3.5.2. Secondary antibodies

- Anti-Rabbit (Ref. NA934): Anti-rabbit serum combined with horseradish peroxidase. Work dilution 1:10000 or 1:25000 for transient expression in *N. benthamiana* experiment.
- Anti-Mouse (Ref. NA931): Anti-mouse serum combined with horseradish peroxidase. Work dilution 1:10000 or 1:25000 for transient expression in *N. benthamiana* experiment.

3.6. Protein staining

Staining of gels and membranes was performed with Coomassie staining solution.

Procedure:

1. Place the membrane in staining solution for 10 minutes at RT with agitation.
2. Remove the staining solution and add the destaining solution. Keep stirring and replace the solution as often as necessary until the ratio between the intensity of the bands and the background noise is optimal.

Solutions:

- Coomassie: Methanol 40% (v/v), glacial acetic acid 10% (v/v), Coomassie blue R-250 0.1% (w/v).
- Coomassie destaining solution: Ethanol 40% (v/v), glacial acetic acid 3% (v/v).

3.7. Reconstitution of SUMOylation machinery in *E. coli*

For the *SUMOylation* assay in *E. coli,* the protocol is showed in **Figure M.3**.

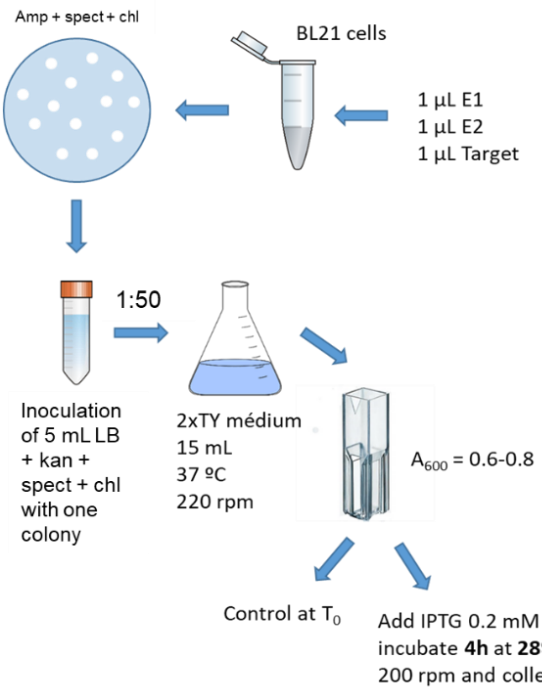


Figure M.3 – Protocol of the bacterial culture conditions for de reconstitution of SUMOylation machinery in E. coli.

Procedure:

1. Transform the BL21 with the E1, E2 and the target substrate to validate.
2. Transformed BL21 cells are growth in a LB medium plate with kanamycin, spectinomycin and chloramphenicol O/N at 37 ºC.

3. The liquid culture is carried out in 5 mL LB with kanamycin, spectinomycin and chloramphenicol O/N at 37 ºC with stirring at 220.
4. Culture is transferred to a 2xTY medium in a 1:50 relationship at 37 ºC with stirring at 220 rpm until an OD of 0.6-0.8.
5. IPG 0.2 mM is added and incubated for 4 hours at 28 ºC with stirring at 200 rpm.
6. Centrifuge at 2000 rpm for 5 minutes.
7. Remove the supernatant and add Loading Buffer SDS 4X and resuspend with 100 µL in T_o and 200 µL in T_4 samples.

4. Plant Methods

4.1. Seeds sterilization

Arabidopsis seeds were sterilized for those experiments that involved sowing them for plate cultivation or infection assay.

Procedure 1:

1. Carry out 3 washes with sterilization solution. Between one wash and the next, shake vigorously with vortex.
2. Leave the seeds in the sterilization solution for 10 minutes in agitation.
3. Perform 3 washes with sterile milliQ water.
4. Remove the water.

Solutions:

- Sterilization solution: Bleach 20% (v/v), Triton X-100 0.01% (v/v).

Procedure 2:

Put the seeds in an opened Eppendorf tube with bleach vapours for 4 hours in a vacuum chamber.

Solutions:

- 100 mL of bleach with 3 mL of HCl.

Procedure 3:

1. Add 500 µL of Et-OH 70% with Triton X-10 0.05% (v/v).
2. Shake for 5 minutes and remove the supernatant.
3. Add 500 µL of Et-OH absolute.
4. Shake for 5 minutes and remove the supernatant.

5. Leave drying in a laminar flow hood.

Regardless of the sterilization method used, we can store the seeds with water at dark at 4 ºC for between 2 and 5 days. This process, known as stratification, allows the dormancy of the seeds to be broken, ensuring synchronized germination.

4.2. Seed sowing

Depending on the type of experiment that was carried out, the seeds were sown as follows.

4.2.1. Sow seeds in groups on the growing medium

About 1000-2000 seeds are sown in a group directly on the culture medium when it comes to seeds of the T0 generation, from plants transformed with agrobacterium, from which we want to select those that contain the transgene.

4.2.2. Sow seeds one by one on the growing medium

The seeds are sown one at a time directly on the culture medium when it comes to facilitating their counting. The seeds density depends on the experiment.

Procedure:

1. Resuspend the seeds in sterile water.
2. Pipette the seeds with a P1000 pipette, collecting around 200 µL of the mixture seeds and water and mixing to avoid the compaction of the seeds.
3. Drop them one by one onto the corresponding solid medium by contacting the tip of the pipette with the medium.

Regardless of the sowing system used, once the seeds have been sown and if we haven't stratified them before, the plates are sealed with porous micropore tape and stored in the dark at 4 ºC for between 2 and 5 days for the stratification.

4.3. Generation of transgenic plants

Arabidopsis plants of the wild Col0 Ecotype, and *erf5/erf6, erf104* and *aco4* mutants described in section 4.3.1., were transformed by immersion according to the method described by Clough and Bent in 1998 and Zhang *et al.* in 2006, with a culture of *A. tumefaciens* carrying the construct that in each case wanted to be introduced into the plant (section 1.3.2.).

Procedure:

1. Grow the *Arabidopsis* plants to be transformed in pots in the greenhouse. In 11.5 cm diameter pots with the substrate mixture covered by a grid. About 8-10 seeds are sown, a density that will allow optimal growth of the plant.

2. When the plants have developed inflorescences of 5-7 cm (approximately 6 weeks after planting), cut the inflorescences close to the rosette. Cutting the main inflorescence of the plant causes new lateral flower stems to form, thus increasing the number of flower stalks susceptible to being transformed. After 9-10 days these flower stalks are at an optimal stage for transformation.
3. Liquid culture of the transformed *Agrobacterium* (5 mL YEB + appropriate antibiotics). If the culture origin is a colony, incubate at 28 ºC for 2 days. If the culture origin is a glycerinate, incubate only O/N.
4. Inoculate 2 mL in 500 mL of YEB + antibiotics. Incubate 16-24 hours at 28 ºC to obtain cells grown until the stationary state (OD_{600} ~1.5-2.0).
5. Centrifuge at 4000 x g for 10 min at RT.
6. Discard SN and resuspend gently the cells in 1 volume (~200 mL) of freshly made 5% (w/v) sucrose (20 g of sucrose for 400 mL) by adding a part of the volume to the bottle and vortex.
7. Pour the mix into the container where the Floral Dip is going to be performed.
8. Add Silwet L-77 to a concentration of 0.02% (v/v) (80 µL per 400 mL of solution). 400-500 mL of solution should be enough to transform at least 6 pots of plants.
9. Invert plants and dip aerial parts of plants in the *Agrobacterium* cell suspension for 10 seconds with gentle agitation. Dip the rosette as well to soak axillary inflorescences. Remove dipped plants form the solution and rain the treated plants for 3-5 seconds. Flower buds will be damaged if they are soaked in the solution for too long.
10. Cover dipped plants with a plastic cover or wrap them with plastic film and lay down the treated plants on their sides for 16-24 hours in darkness to maintain high humidity.
11. Remover the cover and allow the plants to develop normally.
12. Collect the seeds of the transformed plants (T0) and sow them in MS medium with the appropriate selection antibiotic (**Figure M.4**).
13. Repeat the previous step with the next generation of seeds (T1) selecting individuals with 75% of resistance, individuals who have a single insertion and are therefore heterozygous for the transgene (**Figure M.4**).
14. Repeat the previous step once more to analyze the T2 generation and select individuals homozygous for the transgene, those with 100% or resistance (**Figure M.4**).

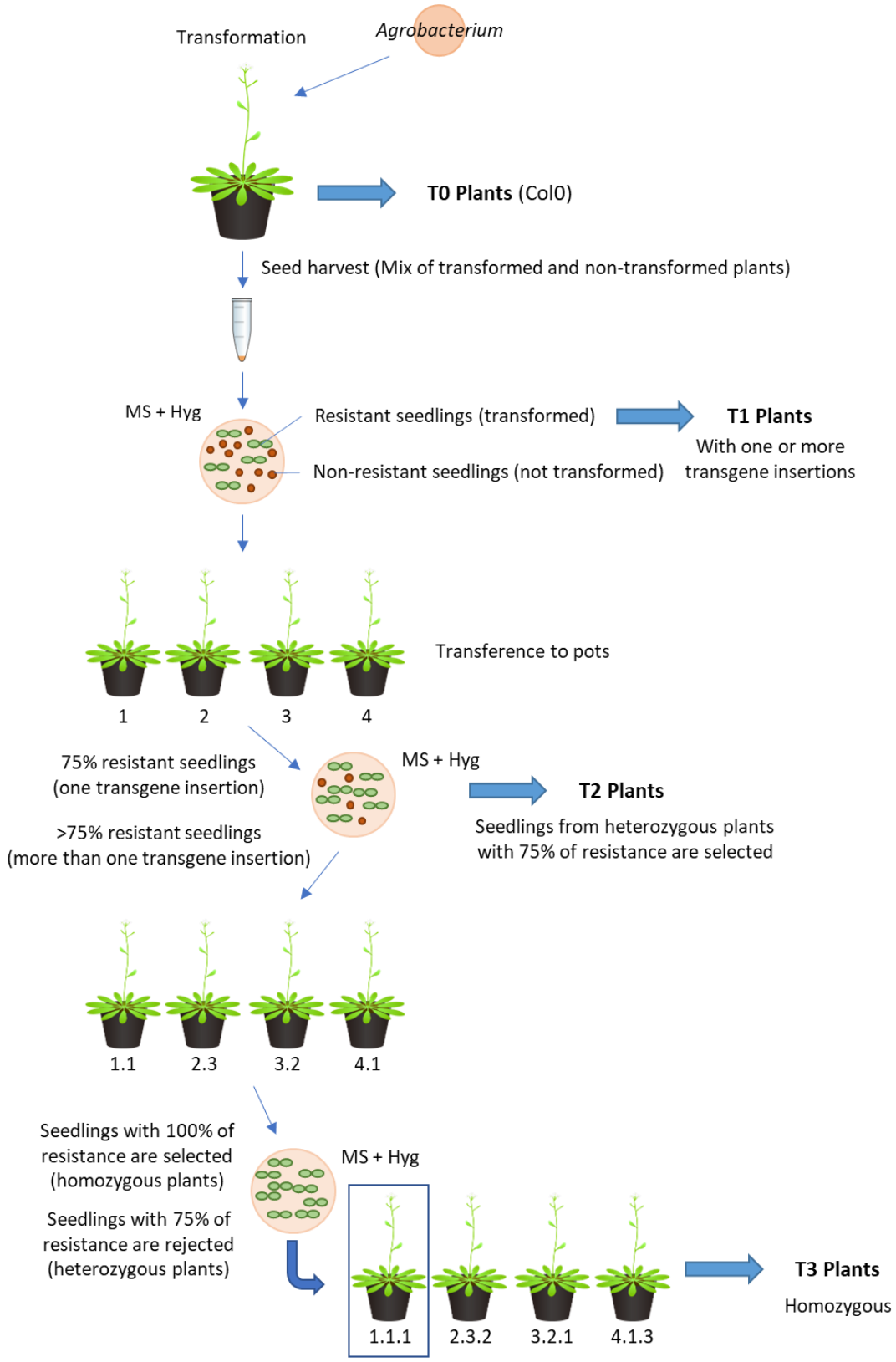


Figure M.4 – Generation of homozygous transgenic lines. *Wild ecotype plants (Col0) and erf5/erf6, erf104 and aco4 mutants represented in section 4.3.1 were transformed with Agrobacterium using constructs detailed in section 1.3.2. and according to the method described by Clough and Bent in 1998 and Zhang et al. in 2006. The different generations and selections carried out to obtain homozygous plants with a single insertion of the transgene of interest are represented.*

4.4. Transient expression in *Nicotiana benthamiana*

4.4.1. Agroinfiltration

N. benthamiana plants were agroinfiltrated with a culture of *A. tumefaciens* carrying the construct and the conjugable or the non-conjugable SUMO variant and the target protein to analyze (section 1.3.2.) according to the method described by Valli *et al.* in 2006. P1B, an ipomovirus from the Cucumber Vein Yellowing Virus (CVYV), is used as silencing inhibitor.

Procedure:

1. Transformed *Agrobacterium* preculture (5 mL YEB + appropriate antibiotics). Incubate O/N at 28 ºC.
2. Final *Agrobacterium* culture (100 µL of the preculture in 5 mL). Incubate O/N at 28 ºC.
3. Measurement of the OD_{600} of each culture as reference using 1 mL of the culture.
4. Centrifuge at 4000 rpm for 15 min at RT and remove supernatant.
5. Resuspend in 4 mL of induction buffer to maintain the original OD.
6. Lead to a final OD of 0.2 and wait for 3 hours.
7. For the agroinfiltration, hold the leaf, turn over and inoculate on the underside. Put the fingers behind the point where the syringe is placed to prevent it from breaking. Avoid infiltrating venous points.

Solutions:

- Induction buffer: $MgSO_4$ 10 mM, MES 10 mM, Acetosyringone 150 µM.

4.4.2. ACC and MG132 treatment

3 days after the agroinfiltration of the plants described in 4.4.1 section, the same plants were infiltrated with the proteasome inhibitor MG132 50 µM or with the ethylene precursor ACC 10 µM following the step 7 of the same procedure. The samples were collected 6 hours after the infiltration.

4.5. Molecular analysis of mutant plants

4.5.1. Obtaining plant genomic DNA

For the extraction of genomic DNA from the plants the following protocol has been used:

Procedure:

1. Cut 1-2 young leaves, not too big, and put them into an Eppendorf tube with two lyser balls per tube.
2. Grind the leaves with the Tissue-lyser 2 x (1 minute – 1 minute 30 seconds, F = 30.0).
3. Add 400 µL of the extraction buffer. Vortex intensively and maintain at RT,

4. Centrifuge at 14000 rpm for 10 minutes.
5. Transfer 300-350 µL of supernatant to a new tube and add the same amount of isopropanol. Invert twice and keep them 2 min at RT.
6. Centrifuge 10 min at 14000 rpm. Remove the SN by inverting the tube.
7. Wash the pellet in 500 µL Et-OH 70%.
8. Centrifuge 5 min at 14000 rpm to dry the pellet.
9. Resuspend pellet in 30-50µL of MQ water.
10. Keep at -20 ºC.

Solutions:

- DNA extraction buffer: Tris-HCl 200 mM pH 7.5, NaCl 250 mM, EDTA 25 mM and SDS 0.5%.

4.5.2. Genotype verification by PCR

The PCR technique is used to rapidly detect the presence of a transgene introduced into the plant and for the genotyping of mutant plants from T-DNA insertion collections, using oligonucleotides from the insertion element of each line and from the genomic region flanking its insertion site.

The PCR reaction uses as a template 0.5 – 1 µL of DNA from the extraction of genomic DNA (section 4.5.1.) for PCR final volume of 10 µL and a suitable pair of primers.

4.6. Measurement of phenotypes

4.6.1. Analysis of rosette area

Plants were grown under long day conditions at 22 ºC for 21 days. Rosette area analysis of plants was performed by Image J free software v1.53k drawing the outline of the rosette using the oval selection option and adjusting three points (**Figure M.5 A**). To avoid the effect of the variability due the growth cabin, the relative measure per tray was calculated.

4.6.2. Analysis of plant length

Plants were grown under long day conditions at 22 ºC for 21 days. The measure length of the main stem was performed by Image J free software v1.53k following the stem using the segmented line option (**Figure M.5 B**). To avoid the effect of the variability due the growth cabin, the relative measure per tray was calculated.

4.6.3. Flowering measurement

Plants were grown under long day conditions at 22 ºC for 21 days. The flowering of the different lines was analyzed by counting the number of plants with floral stem per line when it

is 1 cm high. To avoid the effect of the variability due the growth cabin, the relative measure per tray was calculated.

4.6.4. Analyzing of root length

Seedlings were grown vertically in plates under neutral conditions (12-12) at 22 ºC for 8 days. The measure length of the main root was performed by Image J free software v1.53k following the root using the freehand line option (**Figure M.5 C**). To avoid the effect of the variability due the plate position, the relative measure per plate was calculated.

4.6.5. Number of lateral roots measurement

Seedlings were grown vertically in plates under neutral conditions (12-12) at 22 ºC for 8 days. The number of lateral roots was analyzed by counting and it's graphically represented as number of lateral roots per line or calculating the relative measure per plate to avoid the effect of the variability due the plate position.

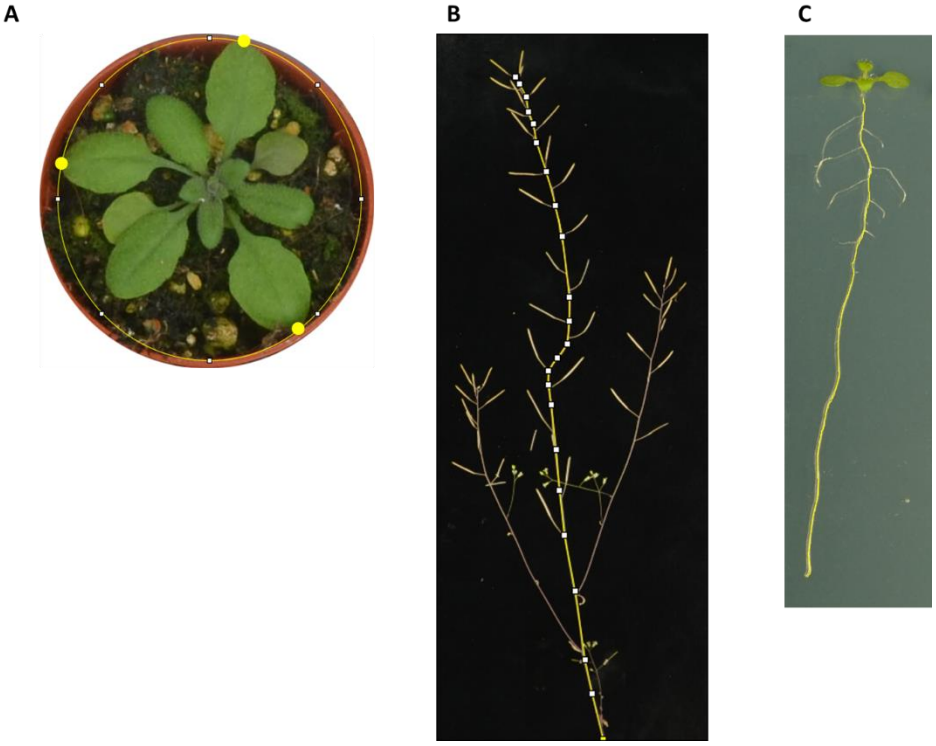


Figure M.5 – Use of the Image J free software v1.53k for the phenotype measures. *(**A**) The rosette area was measure drawing the outline of the rosette using the oval selection option and adjusting three points. (**B**) The length of the main stem was measured following the stem using the segmented line option. (**C**) The length of the main root was measured following the root using the freehand line option.*

4.7. Senescence analysis

To perform the senescence analysis, 7 days old seedlings growth under neutral conditions (12-12) at 22 ºC were transferred to darkness 22 ºC conditions for 3, 4 or 5 days with their corresponding light controls. The photosynthetic capacity was measured using Imaging PAM-

MAXI equipment available in images room and performing a period of adaptation of 15 minutes before starting with the measurement. All the measurements were taken at the same time, 16:00 with Spanish time.

4.8. Heavy metals treatment

Seedlings were grown vertically in plates with 0.5 MS with different concentrations of heavy metals or without as a control for 8 days. The main root length for each condition was measured and graphically represented along as the control condition. The percent of inhibition rate is also represented taking the control condition as reference.

A screening with different heavy metals available in the laboratory with different concentrations were performed selecting those in which a resistance/sensibility phenotype tendency was observed. The **Table M.4** represents the final selection of metals, concentrations, and lines for the treatment.

Table M.4– Table with the different heavy metals used with the selected concentrations and the analyzed lines in each case.

Treatment	*Final concentration (µM)*	*Analyzed lines*
Control	0	ERF2, ERF6, ERF104
$CuSO_4$	30	ERF2, ERF6, ERF104
$CdCl_2$	15	ERF2, ERF104
H_3BO_4	750	ERF2, ERF104

4.9. Hormones treatment

4.9.1. Effect of auxins in different levels of SUMOylation variants

Seedlings were growth vertically in plates with MS for 3 days and transferred to new plates with different concentrations of auxins and the inhibitor of auxin transport (TIBA). The root length was measured the day 0 and the day 4 after the transference. The root growth in those 4 days is graphically represented. The **Table M.5** represents the concentration of auxins and inhibitor used for the treatment.

Table M.5 – Table with the different concentrations of auxins and the inhibitor of auxin transport (TIBA).

Hormone	*Concentrations*
*Indole-3-acetic acid (**IAA**)*	0.0075 µM
	0.01 µM
	0.05 µM
*2,3,5-Triiodobenzoic acid (**TIBA**)*	1 µM
	5 µM
	10 µM
	30 µM

4.9.2. Effect of different hormones in the SUMOylation of ERF2, ERF6 and ERF104

Seedlings were growth vertically in plates with 0.5 MS for 4 days and transferred to new plates with different hormones and inhibitors. The root length was measured the day 0 and the day 3 after the transference. The root growth in those 3 days is graphically represented. The **Table M.6** represents the concentration for the different hormones and inhibitors used for the treatment.

Table M.6 – Table with the different hormones and inhibitors used along as the correspondent concentrations.

Hormone	*Activator*		*Inhibitor*	
Auxins	1-naphthylacetic aid (**NAA**)	**0.1 µM**	2,3,5-Triiodobenzoic acid (**TIBA**)	**10 µM**
Ethylene	Aminocliclopropan-1-carboxilic acid (**ACC**)	**0.5 µM**	Silver nitrate (**$AgNO_3$**)	**10 µM**
Jasmonic	Methyl jasmonate (**MeJA**)	**20 M**		

4.10. Cold treatment

Plants were subjected to two different cold conditions, one with acclimation of 7 days at 4ºC (**Figure M.6 A**), in which the amount of accumulated anthocyanins was measured after a recovering time of 7 days at normal conditions, and other without acclimation (**Figure M.6 B**), in which the recovered rate was measured.

The protocol for the acclimated treatment was adapted from Catalá *et al.* 2003, Miura *et al.,* 2007 and Song *et al.,* 2016 and the protocol without acclimation was adapted from Bolt *et al.* in 2017.

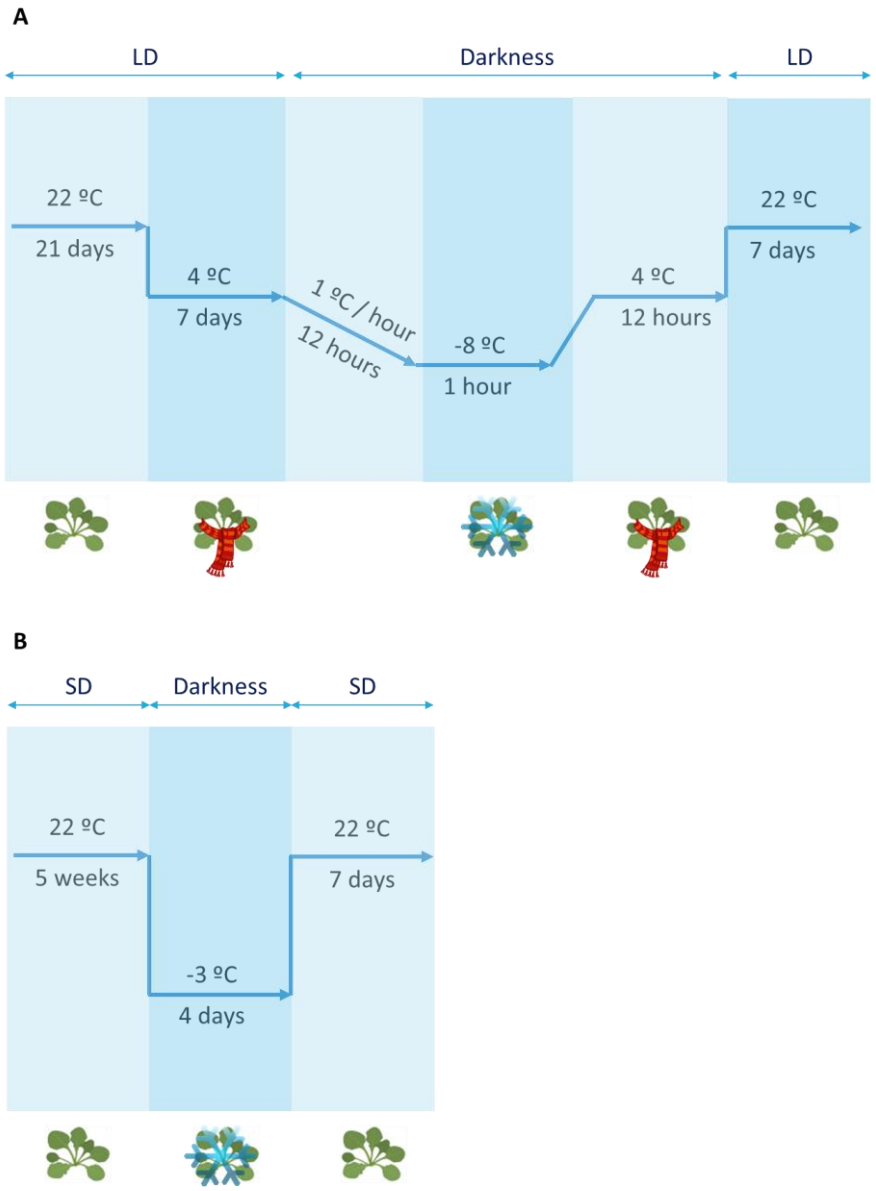


Figure M.6 – Protocols of the cold treatments performed in ERF2, ERF6 and ERF104 SUMOylable and non-SUMOylable variants. *(**A**) The plants were growth in long day conditions for 21 days followed of a temperature acclimatization of 7 days at 4 ºC, after that, the temperature was reduced until -8 ºC keeping it for 1 hour. A new acclimatization of 12 hours at 4 ºC (from -8 ºC to 4 ºC in one hour) was performed before return the plants to normal Long Day conditions for their recovering for 7 days. (**B**) The plants were growth in Short Day conditions for 5 weeks followed of a sudden drop in temperature until -3 ºC for 4 days. After that, the plants return to normal short day conditions for its recovering for 7 days.*

4.11. *Botrytis cinerea* infection

4.11.1. Preparation of inoculum for fungal infection

Botrytis cinerea were grown on potato dextrose agar (PDA) plates for 15 days at room temperature. Spores were harvested in a 50 mL centrifuge tube with 40 mL of sterile water and filtering through Miracloth (Calbiochem). After filtering through the cotton 2-3 times and centrifuging the mixture at 4000 rpm for 10 minutes at room temperature, the supernatant was discarded. The spores were resuspended in B5 + 2% sucrose medium. The collected spores were

quantified using a counting chamber and a working solution containing the appropriate spore concentration was inoculated in dark for 2 hours before the infection.

4.11.2. Fungal infection procedure

Plants were growth on soil in short day conditions at 22 ºC for 4 weeks. Infection was performed by drop-inoculation on top of 3 leaves of each plant placing a 10 µL spore suspension ($3.95x10^6$ spores/mL) using a pipette. The inoculated plants were kept in a plastic box under high humidity for 3 days.

4.11.3. Fungal infection analysis

Area of infection was measured by Image J free software v1.53k drawing the outline of the affected area of the leaf using the oval selection option.

The relative abundance of fungal biomass was assessed by quantitative PCR in a total reaction volume of 10 µL using LightCycler 480 SYBR® Green I Master (Roche) in a LightCycler 480 Real-Time PCR System (Roche) with 0.3 µM of each of the corresponding primers. The primer sequences are specified in the supplementary material section in **Table M.7**.

Table M.7 – Primers used with the analysis of fungal biomass.

Gene	Gene code	Sequence
Actin2	AT3G18780	GATTCAGATGCCCAGAAGTCTTGT
		TGGATTCCAGCAGCTTCCAT
Inf. β-tubulin	B. cinerea β-tubulin DNA (accession no. KC620303)	GTTACTTGACATGCTCTGCCATT
		CACGGCTACAGAAAGTTAGTTTCTACAA

5. SUMO targets identification and analysis

5.1. Creation of a database to store the identified SUMO target

For the store of all the possible SUMO targets identified by bibliography, a database was created using FileMaker Pro Advanced v17 with the data fields: ID, Name, Annotation, Biological function, References, Type of analysis by which the substrate was identified, Validation references, SUMO acceptor Lysine with its Mutation Lysine Reference and the SUMO isoform, lending us an easy way to have together all the information and the possibility to update it whenever we needed it (**Figure M.7**).

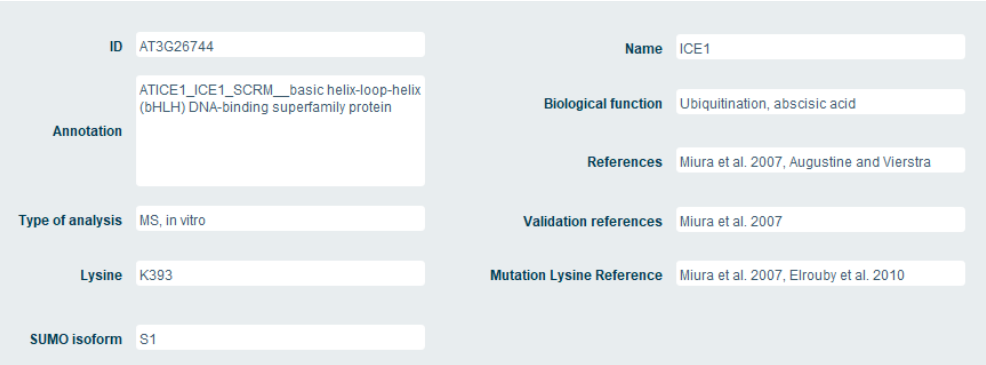


Figure M.7 – Representation of an example database entry. *The database was created using FileMaker Pro Advanced v17 with the data fields: ID, Name, Annotation, Biological function, References, Type of analysis by which the substrate was identified, Validation references, SUMO acceptor Lysine with its Mutation Lysine Reference and the SUMO isoform.*

5.2. Identification of the SUMO targets with a role in development and in defense

The possible SUMO candidates collected in 5.1. section were introduced into the GO Term Enrichment from the TAIR website:

https://www.*arabidopsis*.org/tools/go_term_enrichment.jsp

By using the R programming language and starting from the list obtained by GO, a new list of unique proteins related to development and another with those related to defense were created. Both lists were unified to form a new one that contained all proteins with developing and / or defense function. Finally, the proteins that appeared more than once were analyzed, understanding that these proteins were those that appeared in the list because they have a role in development and a role in defense.

5.3. Selection of the SUMO targets to validate

The genes identified in 5.2. section were introduced into Genevestigator v.8.0.1. to analyze the effect of the perturbations related with biotic stresses and fungal elicitors. STRING v11 (Szklarcyk *et al.*, 2019) and Cytoscape v3.7.1. (Shannon *et al.,* 2003) network analysis tools were used to identify the possible connections of those genes.

5.4. Selection of the lysine residues to validate

The implemented strategy to identify the possible lysines was:

1. Identify potential lysine candidates *in-silico*
2. Infer number of modified lysines from SUMOylation assay results
3. Conservation analysis in homologs
4. Search for structural information and how they are exposed.
5. Validation of lysine acceptor candidates by mutagenesis

Using GPS-SUMO v1.0 and JASSA (available in http://jassa.fr/), sensitive lysines to be SUMOylated were introduced with the aim of analyze the possible SUMOylation motifs, identify the SUMOylable lysine or lysines and generate non-SUMOylable variants by directed mutagenesis. The SUMOylation bands obtained by Western-blot were compared against candidate to SUMOylation lysines identified in each protein. Using the protein sequence as a query and land plants (taxid: 3193) as organism to compare with, a BLASTp alignment was performed to see if the SUMOylation motifs are conserved through the plant families. At last, the protein structure was analyzed with PyMOL v2.3.4. in case it was available at SWISS-MODEL webpage (https://swissmodel.expasy.org/).

6. Graphical representation and statistical analysis

For the realization of final assemblies, Adobe® Photoshop® CS3 v10.0, CorelDRAW 2018 and Microsoft 365 PowerPoint were used.

For the creation of the graphs and the statistical analysis, GraphPad Prism 9.4.1 and RStudio (from v1.1.463) along as R (from R-3.5.3 version) were used. The visualization of the Biological Process of the genes from the transcriptomic analysis was carried out with GOplot package (Walter *et al.*, 2015), with the GOChord function used for the graphic visualisation of the dataset. The representation of the KEEG pathway and the Heatmaps were performed using enrichplot (Yu, 2022) and gplots (Warnes, 2016) package respectively.

The Wilcoxon no-parametric test (p.value ≤ 0.05) was performed to determine if there're significant differences between the SUMOylable and the non-SUMOylable variants.

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Supplementary Materials

Table S.1 – Primers used in this work.

Code	Gene	Gene code	Sequence	Observations
DF01	MYC2	AT1G32640	agcc cat ATGACTGATTACCGGCTACAAC	fwd; NdeI addtion before ATG to clone into pET28a
DF02			agtc gcggccgc TTAACCGATTTTTGAAATCAAACTTG	rev; NotI addition after STOP codon to clone into pET28a
DF03	ERF6	AT4G17490	TGGACAGCAAATGGGTCGCGATGGCTACACCAAACGAAG	fwd; BamHI addtion before ATG to clone into pET28a using NEBuilder system; it doesn't work properly
DF04			AGTGGTGGTGGTGGTGGTGCTCAAACAACGGTCAATTG	rev; XhoI addition after STOP codon to clone into pET28a using NEBuilder system; it doesn't work properly
DF05	ERF104	AT5G61600	GGATCCGAATTCGAGCTCCGATGGCAACTAAACAAGAAG	fwd; SalI addtion before ATG to clone into pET28a using NEBuilder system; it doesn't work properly
DF06			TGGTGGTGGTGCTCGAGTGCTTAAGTGACGGAGATAACG	rev; NotI addition after STOP codon to clone into pET28a using NEBuilder system; it doesn't work properly
DF07	CA1	AT3G01500	TGGACAGCAAATGGGTCGCGATGTCGACCGCTCCTCTC	fwd; BamHI addtion before ATG to clone into pET28a using NEBuilder system; it doesn't work properly
DF08			AGTGGTGGTGGTGGTGGTGCCTACAGCTTCCAATGTAGTATGG	rev; XhoI addition after STOP codon to clone into pET28a using NEBuilder system; it doesn't work properly
DF09	ACO4	AT1G05010	TGGACAGCAAATGGGTCGCGATGGAGAGTTTCCCGATCATCAATC	fwd; BamHI addtion before ATG to clone into pET28a using NEBuilder system; it doesn't work properly
DF10			TGGTGGTGGTGCTCGAGTGCTCACGCAGTGGCCAATGG	rev; NotI addition after STOP codon to clone into pET28a using NEBuilder system; it doesn't work properly
DF11	ERF6	AT4G17490	CACCGGATCCATGGCTACACCAAACGAAGTATC	fwd; BamHI addtion before ATG to clone into pET28a
DF12			AGTCCTCGAGTCAAACAACGGTCAATTGTGGATAACC	rev; XhoI addition after STOP codon to clone into pET28a
DF13	ERF104	AT5G61600	CACCGTCGACATGGCAACTAAACAAGAAGCTTTAG	fwd; SalI addtion before ATG to clone into pET28a
DF14			AGTCGCGGCCGCTTAAGTGACGGAGATAACGGAAAAG	rev; NotI addition after STOP codon to clone into pET28a
DF15	CA1	AT3G01500	CACCGGATCCATGTCGACCGCTCCTCTCTC	fwd; BamHI addtion before ATG to clone into pET28a
DF16			AGTCCTCGAGCTACAGCTTCCAATGTAGTATGG	rev; XhoI addition after STOP codon to clone into pET28a
DF17	ACO4	AT1G05010	CACCGGATCCATGGAGAGTTTCCCGATCATCAATC	fwd; BamHI addtion before ATG to clone into pET28a
DF18			AGTCGCGGCCGCTCACGCAGTGGCCAATGGTCC	rev; NotI addition after STOP codon to clone into pET28a
DF19	ERF1	AT3G23240	AGCAAATGGGTCGGGATCCGATGGATCCATTTTTAATTCAGTC	fwd; EcoRI addtion before ATG to clone into pET28b using NEBuilder system
DF20			AGTGGTGGTGGTGGTGGTGCTCACCAAGTCCCACTATTTTC	rev; XhoI addition after STOP codon to clone into pET28b using NEBuilder system

DF21	ERF2	AT5G47220	GTGGACAGCAAATGGGTCGGATGTACGGACAGTGCAATATAG	fwd; BamHI addtion before ATG to clone into pET28b using NEBuilder system
DF22			AGTGGTGGTGGTGGTGGTGCTTATGAAACCAATAACTCATCAAC	rev; XhoI addition after STOP codon to clone into pET28b using NEBuilder system
DF23	ICE2	AT1G12860	GTGGACAGCAAATGGGTCGGATGAACAGCGACGGTGTTTG	fwd; BamHI addtion before ATG to clone into pET28b using NEBuilder system
DF24			TGGTGGTGGTGCTCGAGTGCTCAAACCAAACCAGCGTAAC	rev; NotI addition after STOP codon to clone into pET28b using NEBuilder system
DF25	NHL10	AT2G35980	GTGGACAGCAAATGGGTCGGATGGCTGCTGAACAACCTC	fwd; BamHI addtion before ATG to clone into pET28b using NEBuilder system
DF26			AGTGGTGGTGGTGGTGGTGCTCAGAAGTCGAAGTCGCAC	rev; XhoI addition after STOP codon to clone into pET28b using NEBuilder system
DF27	ERF104_K183R	AT5G61600	GAAGGCGGCTAGGGTTAGAGTGGAGGAAGAAGAG	fwd; ERF104 K183R
DF28			CTCTTCTTCCTCCACTCTAACCCTAGCCGCCTTC	rev; ERF104 K183R
DF29	ACO4_K295R	AT1G05010	CTCTACTCTGCTGTCAGGTTTCAGGCCAAGGAAC	fwd; ACO4 K295R
DF30			GTTCCTTGGCCTGAAACCTGACAGCAGAGTAGAG	rev; ACO4 K295R
DF31	NHL10_K222R	AT2G35980	CTCCACTGTTTTCCCGATCAGGTGCGACTTCGAC	fwd; NHL10 K222R
DF32			GTCGAAGTCGCACCTGATCGGGAAAACAGTGGAG	rev; NHL10 K222R
DF33	ERF2_K87R	AT5G47220	GATTTTCCGGCGGTTAGAGTCGAGCCAACTGAG	fwd; ERF2 K87R
DF34			CTCAGTTGGCTCGACTCTAACCGCCGGAAAATC	rev; ERF2 K87R
DF35	ERF2_K228R		GAGGTGGTGCAGGTGAGGTGTGAGGTTGGTGATG	fwd; ERF2 K228R
DF36			CATCACCAACCTCACACCTCACCTGCACCACCTC	rev; ERF2 K228R
DF37	ERF6_K63R	AT4G17490	CGATCTCGTCACTCCCAGACCGGAGATTTTTG	fwd; ERF6 K63R
DF38			CAAAAATCTCCGGTCTGGGAGTGACGAGATCG	rev; ERF6 K63R
DF39	ERF6_K72R		GATTTCGATGTGAGATCTGAAATTCCATCTG	fwd; ERF6 K72R
DF40			CAGATGGAATTTCAGATCTCACATCGAAATC	rev; ERF6 K72R
DF41	ERF6_K121R		CGCAACCGGAAATCCTAGACCGGAACTTCCCG	fwd; ERF6 K121R
DF42			CGGGAAGTTCCGGTCTAGGATTTCCGGTTGCG	rev; ERF6 K121R
DF43	ERF6_K231R		GAGAAAGTGCTAAGGACGGAGGAGAGTTACGACG	fwd; ERF6 K231R
DF44			CGTCGTAACTCTCCTCCGTCCTTAGCACTTTCTC	rev; ERF6 K231R

DF45	NHL10	AT2G35980	GTGGACAGCAAATGGGTCGGGTCCGACCTCGTGCCATC	fwd; removing of the membrane domain and cloning into pET28b using NEBuilder system
DF46	ERF2	AT5G47220	CACCATGTACGGACAGTGCAATATAGAATCCG	fwd; Gateway
DF47			TGAAACCAATAACTCATCAACACGTGTCTCATC	rev; Gateway without codon STOP
DF48	ERF6	AT4G17490	CACCATGGCTACACCAAACGAAGTATCAGCTC	fwd; Gateway
DF49			AACAACGGTCAATTGTGGATAACCAAACGG	rev; Gateway without codon STOP
DF50	ERF104	AT5G61600	CACCATGGCAACTAAACAAGAAGCTTTAGCC	fwd; Gateway
DF51			AGTGACGGAGATAACGGAAAAGTTGGGAGACG	rev; Gateway without codon STOP
DF52	ACO4	AT1G05010	CACCATGGAGAGTTTCCCGATCATCAATCTCG	fwd; Gateway
DF53			CGCAGTGGCCAATGGTCCAACATTG	rev; Gateway without codon STOP
DF54	ACO4_K191R	AT1G05010	CCTCTTCCAAGACGACAGAGTCAGTGGACTTCAG	fwd; ACO4 K191R
DF55			CTGAAGTCCACTGACTCTGTCGTCTTGGAAGAGG	rev; ACO4 K191R
DF56	SALK	LBb1.3	ATTTTGCCGATTTCGGAAC	SALK mutants
DF57	SALK	LBa1	TGGTTCACGTAGTGGGCCATCG	SALK mutants
DF58	SALK	LB_6313R	TCAAACAGGATTTTCGCCTGCT	SALK mutants
DF59	GK	o8474	ATAATAACGCTGCGGACATCTACATTTT	GK mutants
DF60	ACO4.1	SALK_005704	CTTTTGTTGATTATTAGTCCAAAAC	fw; ACO4.1 SALK mutant
DF61			CTTTGTCCAAAAGCTCGAGTG	rev; ACO4.1 SALK mutant
DF62	ACO4.2	SALK_014965	CCTTATTTGTCTGCAGTTTATTCGTATTG	fw; ACO4.2 SALK mutant
DF63			GGAGCTACTGGATCTGCTGTG	rev; ACO4.2 SALK mutant
DF64	ACO4.3	SALK_064286	GGTTCAGTAGTTACCTCAAGTTGATCGCCG	fw; ACO4.3 SALK mutant
DF65			CTCGAGCTTTTGGACAAAGTG	rev; ACO4.3 SALK mutant
DF66	ERF104	SALK_024275, SALK_152806, SALK_057720	GAAGTGTTTAGATATGGACTTAACGAAAC	fw; ERF104 SALK mutants
DF67	ERF104.1	SALK_024275	AAGAAAGTCCGATTCCTCGTC	rev; ERF104.1 SALK mutant
DF68	ERF104.2,3	SALK_152806, SALK_057720	GAAACAGGCCCTAGAACCATC	rev; ERF104.2,3 SALK mutants

DF69	ERF6.1,4	GK-080F09.01, GK-085B06.01	CTAATCATCTGCCAAAGAAACCC	fw; ERF6.1,4 GK mutants
DF70			ATTGGATTGAACAGTAACGCGAGG	rev; ERF6.1,4 GK mutants
DF71	ERF6.2,3	SALK_087357, SALK_087356	GACGTTTGAGACGGCGATCG	fw; ERF6.2,3 SALK mutants
DF72			CGAGCATATTACATGCCATTG	rev; ERF6.2,3 SALK mutants
DF73	ERF6.4	GK-085B06.01	CCTCCTCCTCGCCGTCTCTC	rev; ERF6.4 GK mutant
DF74	eil1-1_FW	AT2G27050	GGGAATGGTGGAAAGATAAG	fw; eil1-1 mutant
DF75	eil1-1_RV	AT2G27050	CTTTCGCCGTCATCTTATCC	rev; eil1-1 mutant
DF76	SALK_LB	SALK_LB	GGCAATCAGCTGTTGCCCGTCTCACTGGTG	SALK mutants
DF77'	ein3-1_FW	AT3G20770	GTGGAAAGATAAGGTTAGGTTTG	fw; ein3-1 mutant
DF78'	ein3-1_RV	AT3G20770	AAGATGAAATCTACTACCAAGAC	rev; ein3-1 mutant
DF77	ERF104_K81R_FW	AT5G61600	CAACAAAGACAGAGAGAGAGGAAGAAGAGAGGCAC	fwd; ERF104 K81R
DF78	ERF104_K81R_RV		GTGCCTCTCTTCTTCCTCTCTCTCTGTCTTTGTTG	rev; ERF104 K81R
DF79	ERF104_K178R_FW		CTCCGCCGGAAGAGGAGAGGGCGGCTAGGG	fwd; ERF104 K178R
DF80	ERF104_K178R_RV		CCCTAGCCGCCCTCTCCTCTTCCGGCGGAG	rev; ERF104 K178R
DF81	ACO4_K120R_FW	AT1G05010	GAAAGACTTCGCCGGAAGGATAGAGAAGTTG	fwd; ACO4 K120R
DF82	ACO4_K120R_RV		CAACTTCTCTATCCTTCCGGCGAAGTCTTTC	rev; ACO4 K120R
DF83	ERF5_ERF6_FW	SALK_087356 GK-681E07	ATTTTATCAATGGCGACTCCTAACG	fw; ERF5 ERF6 mutants
DF84	ERF5_ERF6_RV		GTTCAGACAGAATCACGGTTATGC	rev; ERF5 ERF6 mutants
DF85	ACO4_K11R_FW	AT1G05010	GATCATCAATCTCGAGAGGCTTAATGGAGAAGAG	fwd; ACO4 K11R
DF86	ACO4_K11R_RV		CTCTTCTCCATTAAGCCTCTCGAGATTGATGATC	rev; ACO4 K11R
DF87	ACO4_K23R_FW		GAGCAATCACTATGGAGAGGATCAAAGACGCTTGTG	fwd; ACO4 K23R
DF88	ACO4_K23R_RV		CACAAGCGTCTTTGATCCTCTCCATAGTGATTGCTC	rev; ACO4 K23R
DF89	ACO4_K151R_FW		GGTGTTTTACGGGTCGAGAAGACCGACTTTTGG	fwd; ACO4 K151R
DF90	ACO4_K151R_RV		CCAAAAGTCGGTCTTCTCGACCCGTAAAACACC	rev; ACO4 K151R
DF91	ACO4_K289R_FW		GTTTGAAGATTACATGAGACTCTACTCTGCTGTC	fwd; ACO4 K289R
DF92	ACO4_K289R_RV		GACAGCAGAGTAGAGTCTCATGTAATCTTCAAAC	rev; ACO4 K289R
DF93	ERF6_K53R_FW	AT4G17490	CTGAATTTGAAACCAGACCGGAAATAATCGATC	fwd; ERF6 K53R

DF94	ERF6_K53R_RV		GATCGATTATTTCCGGTCTGGTTTCAAATTCAG	rev; ERF6 K53R
DF95	ERF5	AT5G47230	GTGGACAGCAAATGGGTCGGATGGCGACTCCTAACGAAG	fwd; BamHI addtion before ATG to clone into pET28b using NEBuilder system
DF96			AGTGGTGGTGGTGGTGGTGCTCAAACAACGGTCAACTG	rev; XhoI addition after STOP codon to clone into pET28b using NEBuilder system
DF97	6xHis-HA-SUMO1_fw	AT4G26840	acgggggactctagaggatcAGATGGGCCATCATCATC	fwd; XhoI addition before 6xHis to clone into pBA002 using NEBuilder system
DF98	6xHis-HA-SUMO1GG_rev		atcggggaaattcgagctcaTTAGCCACCAGTCTGATG	rev; SpeI addition after STOP codon to clone into pBA002 using NEBuilder system
DF99	6xHis-HA-SUMO1AA_rev		atcggggaaattcgagctcaTTAGGCAGCAGTCTGATG	rev; SpeI addition after STOP codon to clone into pBA002 using NEBuilder system
DF100	MYC2	AT1G32640	CACCATGACTGATTACCGGCTACAACCAACG	fwd; Gateway
DF101			ACCGATTTTTGAAATCAAACTTGCTCTGAGCTG	rev; Gateway without codon STOP
DF102	NHL10	AT2G35980	CACCATGGCTGCTGAACAACCTCTCAATGGC	fwd; Gateway
DF103			GAAGTCGAAGTCGCACTTGATCGGGAAAACAG	rev; Gateway without codon STOP
DF104	ERF2	AT5G47220	TTATGAAACCAATAACTCATCAACACGTGTCTCATC	rev; Gateway with codon STOP
DF105	ERF6	AT4G17490	TCAAACAACGGTCAATTGTGGATAACCAAACGG	rev; Gateway with codon STOP
DF106	ERF104	AT5G61600	TTAAGTGACGGAGATAACGGAAAAGTTGGGAGACG	rev; Gateway with codon STOP
DF107	ACO4	AT1G05010	TCACGCAGTGGCCAATGGTCCAACATTG	rev; Gateway with codon STOP
DF108	MYC2	AT1G32640	TTAACCGATTTTTGAAATCAAACTTGCTCTGAGCTG	rev; Gateway with codon STOP
DF109	NHL10	AT2G35980	TCAGAAGTCGAAGTCGCACTTGATCGGGAAAACAG	rev; Gateway with codon STOP
DF110	GST11_qFW	At1g02920	GTTTACGAACACAGGCTTGG	qPCR Fw
DF111	GST11_qRV		GGTGTACCAAGCAAGTACTGA	qPCR Rv
DF112	GST1_qFW	At1g02930	AGAGGCTAAGCTAGCCAAAG	qPCR Fw
DF113	GST1_qRV		ATCGACCAAAGTGAAGTGGT	qPCR Rv
DF114	ABCI19_qFW	At1g03905	CAATGGATCTGGCAAGACAAC	qPCR Fw
DF115	ABCI19_qRV		ACCACTGCAAACAAGTTGAG	qPCR Rv
DF116	MYB51_qFW	At1g18570	CGTTCTCTGATTCCTCCGTT	qPCR Fw
DF117	MYB51_qRV		CAGAAATGTGGAGAACCCGA	qPCR Rv
DF118	JAZ1_qFW	At1g19180	GCAAACCAAGTTCCTCATCC	qPCR Fw

DF119	JAZ1_qRV		TGCCTTTGACGTAACTCTGT	qPCR Rv
DF120	CLH1_qFW	At1g19670	AAGAGTGTAAGGCGACGAAA	qPCR Fw
DF121	CLH1_qRV		CATACAACCGGCCATAAACC	qPCR Rv
DF122	TGA3_qFW	At1g22070	ACTTGTAAGAGCTAGGCAGC	qPCR Fw
DF123	TGA3_qRV		AGCCAGTGTGTGTATTCCAT	qPCR Rv
DF124	STZ_qFW	At1G27730	TCAACACTAGTAGCGTGTCC	qPCR Fw
DF125	STZ_qRV		TGACCATCGAGAATTCAGGG	qPCR Rv
DF126	MYC2_qFW	At1g32640	GGCGATGAAGGTAAACGAAG	qPCR Fw
DF127	MYC2_qRV		GGCGGTTTTATCTCCGAATG	qPCR Rv
DF128	MKK4_qFW	At1g51660	CAAGGTGATTGGGCTAGTCT	qPCR Fw
DF129	MKK4_qRV		GCAATAGCTGCATAGCACTC	qPCR Rv
DF130	GLIP2_qFW	At1g53940	CTAGAGCGTGAACTATCGGG	qPCR Fw
DF131	GLIP2_qRV		ATTGCAAAAGGTTTTGTTACCG	qPCR Rv
DF132	PDF1.5_qFW	At1g55010	CTGATTTTGAAGCACCGACAA	qPCR Fw
DF133	PDF1.5_qRV		CCAGCGCAATATCCATCATTT	qPCR Rv
DF134	JAZ9_qFW	At1g70700	ATTCAATGCAGCTCCTCGTA	qPCR Fw
DF135	JAZ9_qRV		CTCATAAGCCTCTCTTTGCG	qPCR Rv
DF136	THI2.1_qFW	At1g72260	ACTGCAAGTTAGGGTGTGAA	qPCR Fw
DF137	THI2.1_qRV		GAACATCCCTTGGCACATTG	qPCR Rv
DF138	TN9_qFW	At1g72920	TTGGTTGAAGGTTGGTTGGT	qPCR Fw
DF139	TN9_qRV		AGAGTGATGTGATCTATGAAGCA	qPCR Rv
DF140	MKK9_qFW	At1g73500	TGAGCTTCTCGTTGGTCATT	qPCR Fw
DF141	MKK9_qRV		CTCTTCAGAACATCCCTCCG	qPCR Rv
DF142	JAZ2_qFW	At1g74950	AGAGCCAATTCAGCCTAACC	qPCR Fw
DF143	JAZ2_qRV		GCCTTTGATGTGATCCTATCCT	qPCR Rv
DF144	PR5_qFW	At1g75040	AGCTAACGATAAGCCGGAAA	qPCR Fw
DF145	PR5_qFW		AGTTAGCTCCGGTACAAGTG	qPCR Rv

DF146	PDF1.1_qFW	At1g75830	TGGTGGAAGCACAGAAGTTG	qPCR Fw
DF147	PDF1.1_qRV		GTTGCAAGATCCATGTCGTG	qPCR Rv
DF148	GAL1_qFW	At1g78850	AAAGAGTTCGAGGTGTTGGT	qPCR Fw
DF149	GAL1_qRV		TGTTTGCATTAGGAGCTTTGA	qPCR Rv
DF150	GAL2_qFW	At1g78860	GAAGAGTTCGAGGTGTTGGT	qPCR Fw
DF151	GAL2_qRV		CTAGCATTCGGAGCCTTGAC	qPCR Rv
DF152	WRKY40_qFW	At1g80840	AGAAGTAGCTTGACTGTGCC	qPCR Fw
DF153	WRKY40_qRV		GGAAGAAGCCATTTGCTCCA	qPCR Rv
DF154	PRX16_qFW	At2g18980	TATAACGAAGTTGGGTCGGG	qPCR Fw
DF155	PRX16_qRV		ACGTGAACAATCCCGTCTTA	qPCR Rv
DF156	PDF1.3_qFW	At2g26010	TGCTGCTTTTGAAGCACCGATA	qPCR Fw
DF157	PDF1.3_qRV		AGATCCATGTTTTGCCCCCTC	qPCR Rv
DF158	PDF1.2b_qFW	At2g26020	ATCACCTTTATCTACGCTGC	qPCR Fw
DF159	PDF1.2b_qRV		GCAAACTCCTGACCAAGTA	qPCR Rv
DF160	PLP2_qFW	At2g26560	CGGGTTGTAACGAAAATGCT	qPCR Fw
DF161	PLP2_qRV		AGCTTTTGCATGAGGTGAAC	qPCR Rv
DF162	EIL1_qFW	At2g27050	GGACAGATCAGCAGGTTACA	qPCR Fw
DF163	EIL1_qRV		TGCTCCATACGCTAAACGAT	qPCR Rv
DF164	ERF15_qFW	At2g31230	TTTGCAGCGGAGATAAGAGA	qPCR Fw
DF165	ERF15_qRV		CAAGAGATCCTTTTGTGGCG	qPCR Rv
DF166	WRKY33_qFW	At2g38470	ACAGGATTCGTCTTCAGTCC	qPCR Fw
DF167	WRKY33_qRV		AACCAAAAGGCCCGGTATTA	qPCR Rv
DF168	SZF2_qFW	At2g40140	CTTGAGTGGGGAATGCAAAG	qPCR Fw
DF169	SZF2_qRV		GTTGTTTCCATGAATGCCGA	qPCR Rv
DF170	MPK6_qFW	At2g43790	CCTTATCCTCGCCAATCCAT	qPCR Fw
DF171	MPK6_qRV		GTGCAACGAGTTCAGGTATG	qPCR Rv
DF172	PEN2_qFW	At2g44490	AGGAGGATCAGAGTGGAGTT	qPCR Fw

DF173	PEN2_qRV		GACTCTGTTCCGCTACTTCA	qPCR Rv
DF174	WRKY46_qFW	At2g46400	CAGCGAGGTTTTATCTGCAC	qPCR Fw
DF175	WRKY46_qRV		TACGACCACAACCAATCCTG	qPCR Rv
DF176	TN13_qFW	At3g04210	CGGAAAATGTCAAAACCGGA	qPCR Fw
DF177	TN13_qRV		TATGAGCTTTCTGTGGGTCC	qPCR Rv
DF178	PR4_qFW	At3g04720	ATTTGTCGACTGTGGCAATG	qPCR Fw
DF179	PR4_qRV		CGATCAATGGCCGAAACAAG	qPCR Rv
DF180	PR3_qFW	At3g12500	TTTGGTTCTGGATGACTGCT	qPCR Fw
DF181	PR3_qRV		CCATAACCCGGTAATCTCCC	qPCR Rv
DF182	ERF4_qFW	At3g15210	GATGGGGATCGGTAACGTAG	qPCR Fw
DF183	ERF4_qRV		CACCTTCGAAATCAACGACC	qPCR Rv
DF184	JAZ3_qFW	At3g17860	TCTAGAGATTCAGCTCCCAA	qPCR Fw
DF185	JAZ3_qRV		GGCCTGAAGGATAGAAACTG	qPCR Rv
DF186	CAF1a_qFW	At3g44260	GTCGGGGCTTGTTTGTAATG	qPCR Fw
DF187	CAF1a_qRV		TCCCCAAACAACACTCTCAT	qPCR Rv
DF188	MPK3_qFW	At3g45640	GCCAATCTGTCAAAAGCCAT	qPCR Fw
DF189	MPK3_qRV		CCGTATGTTGGATTGAGTGC	qPCR Rv
DF190	PRX33_qFW	At3g49110	GTCCTCGCAATGGTAATCAA	qPCR Fw
DF191	PRX33_qRV		TCTTGGTCGCTCTGGATAAG	qPCR Rv
DF192	SZF1_qFW	At3g55980	GATGAAAGCGAGATCAACCG	qPCR Fw
DF193	SZF1_qRV		TGCTTGAGTAGCTGGTTTGA	qPCR Rv
DF194	RAP2.9_qFW	At4g06746	CTTTAAGGATGGAAACGGCG	qPCR Fw
DF195	RAP2.9_qRV		TTCTCTAACCGCAACTCTGC	qPCR Rv
DF196	ACS6_qFW	At4g11280	TGAACCGGGATGGTTTAGAG	qPCR Fw
DF197	ACS6_qRV		CTAGAGCTGTCTCCATCGTC	qPCR Rv
DF198	OSM34_qFW	At4g11650	CGAACTGCCATCGGATACTA	qPCR Fw
DF199	OSM34_qRV		ATCGCTACATGATCCCTGAC	qPCR Rv

DF200	CBL1_qFW	At4g17615	GCAGACGTGAATCAAGATGG	qPCR Fw
DF201	CBL1_qRV		GACCTCCGAATGGAAGACAA	qPCR Rv
DF202	DIC2_qFW	At4g24570	ATCAGGAGCATGGTTAAGGG	qPCR Fw
DF203	DIC2_qRV		GATCGTAAGACGCTAGCTGA	qPCR Rv
DF204	CMI1_qFW	At4g27280	GACTGATGAAGATGTCCGGT	qPCR Fw
DF205	CMI1_qRV		CTCCATCTGATTCAACGCAC	qPCR Rv
DF206	CAPE3_qFW	At4g33720	CCAATACATGTGCATGGGAC	qPCR Fw
DF207	CAPE3_qRV		TCACTTTTGCACATCCCAAC	qPCR Rv
DF208	AP2_qFW	At4g36920	AAGTCAAGATATGCGGCTCA	qPCR Fw
DF209	AP2_qRV		AAGCATTCCGGTTTGACCTA	qPCR Rv
DF210	FLA17_qFW	At5g06390	TGACGGTGTTTTGTTTCCAC	qPCR Fw
DF211	FLA17_qRV		TAAGTGTCCTTGCCAAAAGC	qPCR Rv
DF212	JAZ10_qFW	At5g13220	TTTCGGTAATTCTTCCGACC	qPCR Fw
DF213	JAZ10_qRV		GAGATGTTGATACTAATCTCTCCT	qPCR Rv
DF214	THI2.2_qFW	At5g36910	ACGATATGTCCTCCGGGAT	qPCR Fw
DF215	THI2.2_qRV		TTTTGGAGAGTGGTCAAGGC	qPCR Rv
DF216	PDF1.2_qFW	At5g44420	GACGCACCGGCAATGG	qPCR Fw
DF217	PDF1.2_qRV		GCTGGGAAGACATAGTTGCAT	qPCR Rv
DF218	PDF1.2c_qFW	At5g44430	TGCTACCATCATCACCTTCC	qPCR Fw
DF219	PDF1.2c_qRV		TGCATGCATTACTGTTTCCG	qPCR Rv
DF220	ERF5_qFW	At5g47230	AGTAAGACAAAGACCGTGGG	qPCR Fw
DF221	ERF5_qRV		TCGTAGTCTAAACGCTGCTT	qPCR Rv
DF222	RBOHD_qFW	At5g47910	TCTTGCTGTACCCATCCTTC	qPCR Fw
DF223	RBOHD_qRV		GGCTTCGTCATGTGTAGAGA	qPCR Rv
DF224	ERF105_qFW	At5g51190	AGGACTTGGGAGACAACAAG	qPCR Fw
DF225	ERF105_qRV		TAATGGACAAGCATCAGCCT	qPCR Rv
DF226	SCL8_qFW	At5g52510	CAACGTGGTGACGAGTTTAC	qPCR Fw

DF227	SCL8_qRV		TTCGTCTCTTGGATTCTCCG	qPCR Rv
DF228	RLK_qFW	At5g67280	ACAGGTTAATGGGCTTGTGA	qPCR Fw
DF229	RLK_qRV		CACAAGCTAGGCCCATTTTC	qPCR Rv
DF230	UBC_qFW	At5g25760	TCAAATGGACCGCTCTTATC	qPCR Fw
DF231	UBC_qRV		CACAGACTGAAGCGTCCAAG	qPCR Rv
DF232	EIN3_qFW	At3g20770	CCGAGTCATGTCCACCTCTT	qPCR Fw
DF233	EIN3_qRV		TGGCTTGAGCTCTTCCACTT	qPCR Rv
DF234	ERF1_qFW	At3g23240	ACCGCTCCGTGAAGTTAGATAATG	qPCR Fw
DF235	ERF1_qRV		ATCCTAATCTTTCACCAAGTCCCAC	qPCR Rv
DF236	ERF2_qFW	At5g47220	CAAGGATGACGATGACAAGG	qPCR Fw flag
DF237	ERF2_qRV		ACGTGTTATCGACTCCAACA	qPCR Rv flag
DF238	ERF6_qFW	At4g17490	ACTACAAGGATGACGATGACA	qPCR Fw flag
DF239	ERF6_qRV		GAGCTGATACTTCGTTTGGTG	qPCR Rv flag
DF240	ERF104_qFW	At5g61600	GGATGACGATGACAAGGCT	qPCR Fw flag
DF241	ERF104_qRV		TGAGAAGGTGTTGGCTTATGA	qPCR Rv flag
DF242	ERF2_qFW	At5g47220	ATGCCTTCCATTTTGACACG	qPCR Fw
DF243	ERF2_qRV		ATCGCCGTAAAGTTCTCAGT	qPCR Rv
DF244	ERF6_qFW	At4g17490	GCTTACGACAAAGAAGCGTT	qPCR Fw
DF245	ERF6_qRV		CGTCTCTCTTCCGTTTGTTG	qPCR Rv
DF246	ERF104_qFW	At5g61600	GCTGGAAGAGCTTATGACCA	qPCR Fw
DF247	ERF104_qRV		CGCTTTCGTTTCCCTAATCC	qPCR Rv
DF248	THI2.1_qFW	At1g72260	GTATGCAAGTGAGTGGATGC	qPCR Fw
DF249	THI2.1_qRV		CACACAGAAGTTTCACACCC	qPCR Rv
DF250	TN9_qFW	At1g72920	TTTGAGAGTGGTTGGGATTTG	qPCR Fw
DF251	TN9_qRV		AACCTTCAACCAATGACAGAT	qPCR Rv
DF252	PRX16_qFW	At2g18980	TATCCGGTAGAGCTAGGGAG	qPCR Fw
DF253	PRX16_qRV		AAACTAGGCTGGGGTAGACT	qPCR Rv

DF254	pdf1.2b_qFW	At2g26020	GTTGTGCGAGAAGCCAAGTG	qPCR Fw
DF255	pdf1.2b_qRV		TGCATGCATTGCTGTTTCCG	qPCR Rv
DF256	ERF1_qFW	At3g23240	TCGATGAGACGGAGAATGAC	qPCR Fw
DF257	ERF1_qRV		CCCAAAAGCTCCTCAAGGTA	qPCR Rv