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In vitro assays on the enzymatic activity of the three tomato XERICO proteins and the Arabidopsis SUMO protease OTS1

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In vitro μελέτη της ενζυματικής ενεργότητας των τριών πρωτεϊνών XERICO από την τομάτα και της SUMO πρωτεάσης OTS1 από Arabidopsis

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ABSTRACT

Tomato crop production has been always faced with the challenge to overcome abiotic stresses such as salt stress. Two major posttranslational modifications of proteins in eukaryotes, ubiquitination and SUMOylation, have emerged as significant regulatory mechanisms in salt stress responses. They both include the attachment of small molecules, ubiquitin and SUMO (small ubiquitin-like modifier), on protein substrates and involve a highly similar 3-step mechanism that recruits a set of analogous E1, E2 and E3 enzymes.

The generation of mature SUMO molecules is a work of SUMO proteases which cleave SUMO precursors. Out of the three SUMO proteases tested, results confirm AtSUMO1 cleavage by protease AtOTS1 but not by AtESD4 or SlOTS2. In tomato there are four SUMO homologs (SlSUMO1-4) which were expressed and purified.

E3 enzymes function as ubiquitin ligases, transferring ubiquitin to the substrate. The most studied class of these enzymes are the RING finger E3 ligases, with important roles in a variety of biological processes. Here, it is tested whether three RING-H2 proteins identified in tomato (SlXERICO1, SlXERICO2, SlXERICO3) can act as ubiquitin ligases in vitro. Most E3 enzymes are known to autoubiquitinate. Taking advantage of that, an in vitro ubiquitination assay was performed using SlUBA1 (E1), SlUBC8 (E2) and SlXERICO1-3, which showed all three of SlXERICOs exhibiting E3 ubiquitin ligase activity in vitro.

1. INTRODUCTION

Tomato (*Solanum lycopersicum)* is one of the most consumed and economically important crop plants around the world. It contains a valuable compound, lycopene, which possesses anti-oxidative and anticancer properties. Therefore, tomato production and consumption are permanently increasing (Raiola, Rigano et al. 2014). However, cultivation and crop productivity are always challenged by a variety of factors. One of the most severe of them is high salinity in soil, leading to salt stress for tomato plants (Apse, Aharon et al. 1999, Tester and Davenport 2003). Harmful effects on plants because of salt stress include osmotic stress, ionic toxicity and associated oxidative stress, resulting in the malfunction of the photosynthetic apparatus and the slowing down of the developmental processes (Zhu 2002). That is why researchers are becoming more and more interested in elucidating the mechanisms underlying salt stress responses in plants.

1.1. The novel role of DELLA proteins and their downstream target XERICO in plant abiotic stress signaling

Plants, as sessile organisms, must be able to adapt to the various environmental conditions. The endogenous and exogenous signals perceived by plant cells during environmental adversity are integrated by the function of hormonal pathways. Particularly, the phytohormone abscisic acid (ABA) has a major role in the activation of plant cellular adaptation to abiotic stresses (Raghavendra, Gonugunta et al. 2010).

Despite the distinct function of the ABA pathway in abiotic stress responses, the focus has recently shifted on emerging evidence of the crosstalk between the ABA pathway and other hormonal pathways, specifically the gibberellic acid (GA) pathway. This evidence emphasizes a primary role of GA in modulating ABA responses (Golldack, Li et al. 2013). In *Arabidopsis*, GA signalling is blocked by a family of DELLA repressor proteins. The binding of GA to the GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptor on DELLA leads to a conformational change, which allows the DELLA-GA-GID1 complex to be recruited by the SCF E3 ligase for ubiquitination, after which this complex is sent for degradation through the 26S proteasome (Willige, Ghosh et al. 2007, Wang and Deng 2011). A linking function of DELLA proteins at the interface of ABA-mediated abiotic stress responses and GA-controlled developmental signaling has been supported by

modified salt tolerance of the quadruple DELLA mutant with functional losses of *rga, gai, rgl1, and rgl2* (Achard, Cheng et al. 2006). Interestingly, a transcriptional downstream target of DELLAs, XERICO, is a RING-H2 zinc finger protein that confers drought tolerance in *Arabidopsis* (Ko, Yang et al. 2006), rice (Zeng, Hou et al. 2015) and maize (Brugiere, Zhang et al. 2017) through subsequent ABA accumulation, revealing its role in abiotic stress responses as a link between ABA and GA pathways (Zentella, Zhang et al. 2007).

Considering ABA pathway is involved in salt stress responses (Raghavendra, Gonugunta et al. 2010), investigating the role of XERICO under salt stress conditions is becoming rather intriguing.

1.2. Ubiquitination can regulate abiotic stress responses with a class of E3 ubiquitin ligases containing a RING domain

Ubiquitin (Ub), a small regulatory protein consisting of 76 amino-acids and a molecular weight of about 8.6 kDa, found in most tissues of eukaryotic organisms, is a key player in a complex system of regulatory mechanisms involving posttranslational protein modifications. It acts as a tag, getting attached to proteins in a process called ubiquitination, promoting protein-protein interactions that determine the cellular fate of these peptides. In plants, ubiquitination has been shown to regulate the activity of many proteins in response to salt stress (Vierstra 2009).

The first step of the process involves an enzymatic cascade of sequential activation of three enzyme families, E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (ubiquitin ligase), which leads to the ligation of ubiquitin to the specific targets (Smalle and Vierstra 2004). E1 enzymes of the cascade catalyze an ATP-dependent reaction which results in a thioester bond between an E1 Cys residue and the C-terminal Gly of ubiquitin. This activated ubiquitin is conjugated to a cysteine in an E2 enzyme by *trans*-esterification. This way, E2 serves an intermediate donor using a specific E3 enzyme which eventually transfers the ubiquitin to the substrate (Fig. 1). The final product is a ubiquitin conjugate which is produced by forming an isopeptide bond between the C-terminal Gly carboxyl group of ubiquitin and one or more amino groups of the target, typically a Lys ε-amino group (Vierstra 2009).

By attaching ubiquitin differentially on the target protein, distinct target fates can be achieved. In some cases, one or more single ubiquitin molecules can get attached (monoubiquitination); more often it is a polymeric ubiquitin chain that gets attached to the substrate (polyubiquitination) through reiterative ubiquitination rounds, where ubiquitin molecules are linked to each other through one of their seven Lys residues (K6, K11, K27, K29, K33, K48, K63) (Fig. 1). The type of chain that is created determines the fate of the ubiquitin conjugate. The most abundant ones in plants are K48-linked polymers, which lead to the 26S proteasome degradation of the target (Smalle and Vierstra 2004, Vierstra 2009).

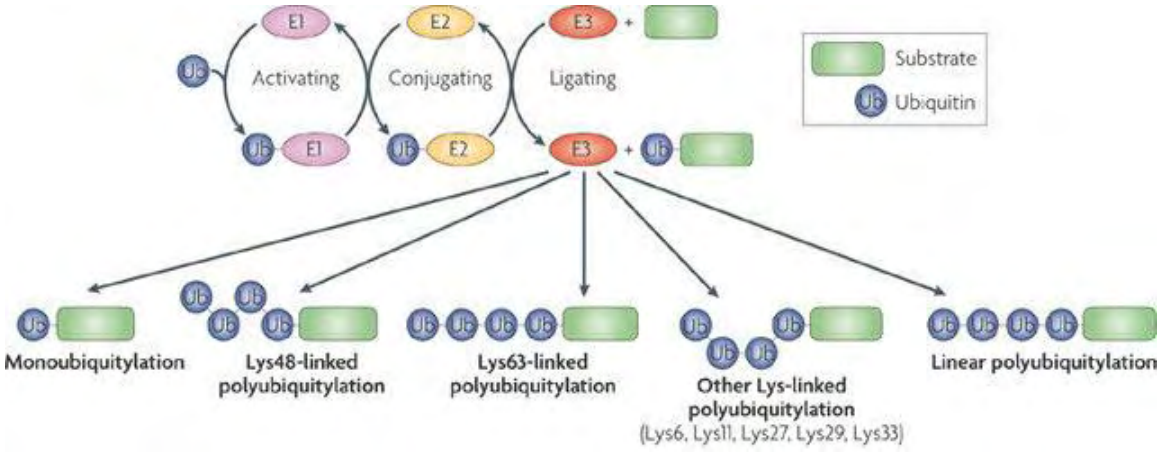


Figure 1: Enzymatic reactions that lead to ubiquitination. Substrates are ubiquitinated as a result of the activity of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme , and an E3 ubiquitin ligase. Different types of ubiquitin chains can be produced which depends on the Lys residue (K6, K11, K27, K29, K33, K48, K63) that is used to form a bond between the ubiquitin molecules. The type of ubiquitin change determines the fate of the ubiquitinated substrate, e.g. K48-linked polyubiquitination leads to proteasomal degradation (Dikic, Wakatsuki et al. 2009)

Arabidopsis encodes for only two E1 isoforms (Hatfield, Gosink et al. 1997), at least 37 E2 enzymes (Bachmair, Novatchkova et al. 2001), while the number of genes encoding for E3 ligases has significantly expanded and includes more than 1300 genes (Vierstra 2003). With such abundancy in the genome it is no surprise that E3 ligases are the ones that confer substrate specificity. Based on their type of interaction domain they use to bind to E2 enzymes and whether they act as single subunits or complexes, E3 ligases are subdivided into 4 families: HECT, RING, U-box and cullin-RING ligases (CRLs) (Moon, Parry et al. 2004). The RING E3 ligases are the most studied ones and have been reported to possess

important roles in abiotic stress tolerance in plants (Zhang, Yang et al. 2007, Bu, Li et al. 2009, Li, Jiang et al. 2011).

Tomato (*Solanum lycopersicum*) is a popular model plant, especially for fruit development studies, but relatively unexplored regarding ubiquitination. There is one gene encoding for the Ub-activating enzyme (E1) and at least 40 genes encoding for Ub-conjugating enzymes (E2) (Zhou, Mural et al. 2017).

In tomato, three *Xerico* genes homologous to *AtXerico* were identified, *SlXerico1* (Solyc05g018760), *SlXerico2* (Solyc07g045190) & *SlXerico3* (Solyc12g006230) with all three encoding for a RING-H2 finger protein. RING finger protein ZmXERICO1 functions as an E3 ligase in vitro controlling ABA homeostasis in drought stress (Brugiere, Zhang et al. 2017) making SlXERICOs putative candidates to also function as E3. Also, given its interaction with the ABA pathway, this may provide with an insight in their potential role in salt stress response.

1.3. SUMO proteases in salt stress responses

After ubiquitination, another type of posttranslational modification of proteins includes SUMO molecules (small ubiquitin-like modifiers) which have emerged as significant regulators in plants. SUMO's structure is similar to ubiquitin but differs in primary sequence (about 20% similarities) and contains ~15 additional N-terminal amino-acid residues; SUMO and ubiquitin are ~92–103 and 76 aa, respectively (Bayer, Arndt et al. 1998).

SUMOylation has also been found to play a key role in a variety of plant processes and responses like abiotic stresses, hormone signalling, flowering time (Miura, Jin et al. 2007). The attachment of SUMOs exhibits high similarity to ubiquitination in the sense that it requires a set of analogous E1, E2 & E3 enzymes. DeSUMOylation, deconjugation of SUMO molecules, is catalyzed by enzymes known as ubiquitin-like SUMO-specific proteases (ULPs) which are also responsible for generation of mature SUMO (Johnson 2004, Mukhopadhyay and Dasso 2007).

Three SUMO proteases that have been identified in *Arabidopsis,* EARLY IN SORT DAYS4 or ESD4, (Murtas, Reeves et al. 2003), and OVERLY TOLERANT IN SALT1 (OTS1) & OTS2 (Conti, Price et al. 2008) regulate SUMO conjugation in developmental

processes and abiotic stresses. Specifically, both *esd4* mutants and *ots1 ots2* double mutants show early flowering, while *ots1 ots2* double mutants exhibit also increased salt sensitivity inferring the role of OTS1 & OTS2 in salts stress responses.

A lot of major components in SUMOylation have also been identified in a variety of plant species, including tomato (Novatchkova, Tomanov et al. 2012). Specifically, two OTS type SUMO proteases have been described in tomato, SlOTS1 and SlOTS2, as well as 4 SUMO homologs (SlSUMO1-4) compared to Arabidopsis' two.

1.4. Aim of the project

In this project, the enzymatic activity of the SlXERICOs and the SUMO proteases, AtESD4, AtOTS1, and SlOTS2 is studied in vitro. By performing an ubiquitination assay, it is demonstrated that all three SlXERICOs function as E3 ubiquitin ligases in vitro. For this purpose, this assay takes advantage of the self-catalyzed ubiquitination (autoubiquitination) feature displayed by most of the E3 ligases (de Bie and Ciechanover 2011). In addition, after making use of a SUMO protease assay to test the protease activities of AtESD4, AtOTS1 and SlOTS2, only AtOTS1 is shown to cleave AtSUMO1. To extend the study of SUMOylation in tomato, all four SlSUMO homologs are expressed and purified.

2. MATERIALS & METHODS

2.1 In vitro ubiquitination assay

Plasmid construction

The open reading frames (ORFs) of the tomato E1 gene *SlUBA1* (Solyc06g007320) and E2 gene *SlUBC8* (Solyc12g056100) were amplified from tomato cDNA using the Phusion DNA polymerase (New England Biolabs). In tomato, 3 *XERICO* genes were found to be homologous to Arabidopsis *XERICO*: *SlXERICO1* (Solyc05g018760), *SlXERICO2* (Solyc07g045190), *SlXERICO3* (Solyc12g006230). Their ORFs were also amplified the same way from tomato cDNA. The primer sequences that were designed for this purpose are shown in Table 1. After successful amplification of the fragments, they were inserted using restriction cloning techniques to expression vector pGEX-6P-1. This vector carries a GST-tag (glutathione S-transferase) which is fused to the N-terminus of the expressed peptide of interest.

Table 1 : Forward and reverse primer sequences used to amplify SlUBA1, SlUBC8, SlXERICO1, SlXERICO2 & SlXERICO3

	Forward primer	Reverse primer
SlUBA1	5'ATGTCGACATATGGCTTTTGATGATGGAAATCC	5'ATGCGGCCGCTCAACGGAAGTATACAGACACCAGAG
SlUBC8	5'ATGCGGCCGCTCATCCCATGGCAAATTTTTGAGTC	5'ATGTCGACATATGGCATCCAAGCGGATTCTC
SlXER1	5'GGATCCATGGGACTCTCACCATATACGACT	5'GCGGCCGCTCACATTGGACAAGTATCTTCCTCA
SlXER2	5'GCGGCCGCATGGGACTCTCACAGTATCCAACT	5'CTCGAGTCACATAGGACAAGTATCTTCTTCGC
SlXER3	5'GCGGCCGCATGGGCCTCTCACAATATC	5'CTCGAGTCACATTGGACATGTATCGTC

Expression and purification of recombinant proteins

Once the sequence of the constructs was confirmed, they were transformed to *E. coli* strain BL21 (DE3). Liquid cultures (500ml) were grown at 37°C to an OD_{600} of 0.4-0.5 before

adding isopropylthio-β-galactoside (IPTG) to a final concentration of 0.5 mM to induce expression. Protein expression was performed at 28°C for 2h for UBA1, at 22°C for 4h for UBC8 and at 18°C overnight for XERICO1-3.

Cells were pelleted and resuspended in 25ml of extraction buffer (50 mM Tris-HCl, pH 7.5; 200mM NaCl; 10% glycerol; 2mM PMSF; 2mM DTT; 1% Triton X-100; 100μg/ml lysozyme). The soluble fractions were recovered through centrifugation and the GST-tagged proteins were purified with Protino® Glutathione Agarose 4B beads. Beads were washed twice using 5ml of washing buffer (50 mM Tris-HCl, pH 7.5; 200mM NaCl; 5% glycerol; 2mM PMSF; 2mM DTT; 0.1% Triton X-100). Proteins were eluted in 75μl of elution buffer (50mM Tris-HCl, pH 7.5; 5% glycerol; 2mM DTT; 20mM reduced GSH; 200mM NaCl). The purified proteins were stored at -80°C after adding glycerol to a final concentration of 10% and their quality was analyzed by 10% SDS-PAGE gel. Different concentrations of BSA were also analyzed on the same gel as a standard to measure the concentration of the eluted proteins and the results were quantified using the ImageJ software.

In Vitro Ubiquitination assay

The ubiquitination assay was performed for all three SlXERICOs as previously described with modifications (Furlan and Trujillo 2017). The reaction mixture included 2μg of ubiquitin, 0.2μg of UBA1, 0.6μg of UBC8 and 2μg of each of XERICO1-3. The samples were incubated in reaction buffer (25mM Tris-HCl, pH 7.5; 5mM $MgCl_2$; 50mM KCl; 0.6mM DTT; 2mM ATP) to a total volume of 20μl for 90min at 30°C. Reactions were stopped by adding 10μl of 5X Laemmli buffer and proteins were resolved on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose or PVDF membranes for Western blot analysis using the PIERCE G2 Fast Blotter. Membranes were eventually incubated with anti-XERICO3 and anti-XERICO1-3 primary antibodies (1:1000 dilution for both, Eurogentec) for 90min and with an HRP-conjugate goat anti-rabbit IgG secondary antibody (1:5000 dilution, ThermoFisher) for 60min to analyze ubiquitination.

2.2 In vitro SUMO protease assay

Plasmid construction

The plasmid pET-19b was used to express AtSUMO1 which was fused with FLC protein in the C terminus (Murtas, Reeves et al. 2003). The result was adding a 10×His tail to the N-terminus of AtSUMO1:FLC to create HIS:AtSUMO1:FLC. The ORFs of SUMO proteases AtESD4, AtOTS1 & SlOTS2 were cloned to pGEX-6P-1 which fuses them N-terminally with a GST tag.

Expression and purification of recombinant proteins

All constructs were transformed to BL21(DE3) *E. coli* competent cells for expression. Expression of the recombinant proteins was induced by 0.5mM IPTG for 4-5h at 37°C and 30°C and overnight at 18°C in 500ml cultures, after reaching an OD_{600} of 0.4-0.5.

Cells were then harvested and resuspended in 25ml of extraction buffer. For HIS:AtSUMO1:FLC the extraction buffer consisted of 50mM Tris-HCl (pH 7.9), 200mM NaCl, 10mM imidazole, 10% glycerol, 2mM PMSF, 2mM β-mercaptoethanol, 100μg/ml lysozyme; for AtESD4, AtOTS1 the extraction buffer used consisted of 50 mM Tris-HCl, pH 7.5; 200mM NaCl; 10% glycerol; 2mM PMSF; 2mM DTT; 1% Triton X-100; 100μg/ml lysozyme. For SlOTS2, Tris-HCl pH 8.5 was used instead of Tris-HCl pH 7.9 in the above extraction buffer. Samples were centrifuged and the supernatant (soluble fractions) was incubated with the appropriate beads for protein purification; HIS:AtSUMO1:FLC using TALON® Metal Affinity Resin beads and Protino® Glutathione Agarose 4B beads for AtESD4, AtOTS1 & SlOTS2.

Beads for HIS:AtSUMO1:FLC were washed twice with 50mM Tris-HCl pH 7.9, 200mM NaCl, 5% glycerol, 2mM β-mercaptoethanol, 10mM imidazole, 0.1% Triton X-100 and beads for GST-tag purification were washed with Tris-HCl pH 7.9 (pH 8.5 for SlOTS2), 200mM NaCl, 5% glycerol, 2mM DTT, 2mM PMSF, 0.1% Triton X-100.

HIS:AtSUMO1:FLC was eluted in 75μl of elution buffer (50mM Tris-HCl pH 7.9, 200mM NaCl, 5% glycerol, 2mM β-mercaptoethanol, 500mM imidazole). The elution buffer for AtESD4, AtOTS1 & SlOTS2 consisted of 50mM Tris-HCl pH 7.9 (pH 8.5 for SlOTS2), 5% glycerol, 2mM DTT, 20mM reduced GSH, 200mM NaCl. Proteins were stored at -

80°C after adding glycerol to a final concentration of 10% and their quality was analyzed by 10% SDS-PAGE gel. Their concentration was determined with the Bradford reagent using BSA as a standard.

In Vitro SUMO protease assay

HIS:AtSUMO1:FLC substrates (500ng) were incubated with 2μg of AtESD4, AtOTS1 & SlOTS2 separately for 0, 60 and 90min at 37°C in reaction buffer (50mM Tris-HCl, pH 8; 150mM NaCl; 0.1% Triton X-100; 2mM DTT). Reactions were stopped by adding 5X Laemmli buffer and proteins were resolved on a 15% SDS-PAGE gel.

2.3 SlSUMO expression and purification

The ORFs of the four tomato *SUMO* orthologs, *SlSUMO1* (Solyc07g049360), *SlSUMO2* (Solyc07g064880), *SlSUMO3* (Solyc09g059970), *SlSUMO4* (Solyc12g006010), were amplified by tomato cDNA using the Phusion DNA polymerase (New England Biolabs). The set of primers for each *SlSUMO* ORF is shown in Table 1. Amplicons were cloned to pET-28a(+) vector so that the peptides carry a 6×His tail tag both in the N- and C-terminus. The constructs were cloned to *E. coli* BL21(DE3) cells for expression.

Table 2: Forward and reverse primer sequences used to amplify SlSUMO1-4 ORFs from cDNA

	Forward primer	Reverse primer
SlSUMO1	5'AGCATATGATGTCTCAAGCAGCGG	5'ATCTCGAGAATAGTTGAGCCTCCAGTTTG
SlSUMO2	5'ATGCGGCCGCAGACAAAGATCCACCAGTCTGATG	5'AGCATATGATGTCAGGCGTCACTCAACAGG
SlSUMO3	5'AGCATATGATGTCTGCTAGCGGCG	5'ATCTCGAGAAAATTAGAGAAACAAGTGCAGCAAC
SlSUMO4	5'AGCATATGATGTCGGGAGTAGCAGG	5'ATCTCGAGAATAGTTGTGCCTCCGGTT

SlSUMO1-4 cultures (100ml) were grown to an OD_{600} of 0.4-0.5 and were expressed for 4h at 28°C after induction with 0.5mM IPTG. Cells were harvested and resuspended in 2ml

of extraction buffer (50mM Tris-HCl pH 8, 200mM NaCl, 10mM imidazole, 10% glycerol, 2mM PMSF, 2mM β-mercaptoethanol, 100μg/ml lysozyme). After brief sonication, cell debris was removed by centrifugation at max rpm/rcf for 30min. The supernatant was incubated with TALON® Metal Affinity Resin beads for purification of the SlSUMOs.

Beads were washed twice with wash buffer (50mM Tris-HCl pH 8, 200mM NaCl, 5% glycerol, 2mM β-mercaptoethanol, 10mM imidazole, 0.1% Triton X-100) and the SlSUMOs were eluted in 75μl of elution buffer (50mM Tris-HCl pH 8, 200mM NaCl, 5% glycerol, 2mM β-mercaptoethanol, 500mM imidazole). Proteins were stored at -80°C after adding glycerol to a final concentration of 10% and their quality was analyzed by 12% SDS-PAGE gel.

3. RESULTS

3.1 AtOTS1 functions as AtSUMO1 protease in vitro

All recombinant proteins were successfully expressed, as shown by the SDS-PAGE gel analysis (Fig. 2). A set of different temperatures was used to assess optimal expression conditions. All proteins were successfully expressed at a temperature as high as 37°C, despite it not being the usually preferable one for recombinant proteins, since higher temperatures might not allow them to fold properly.

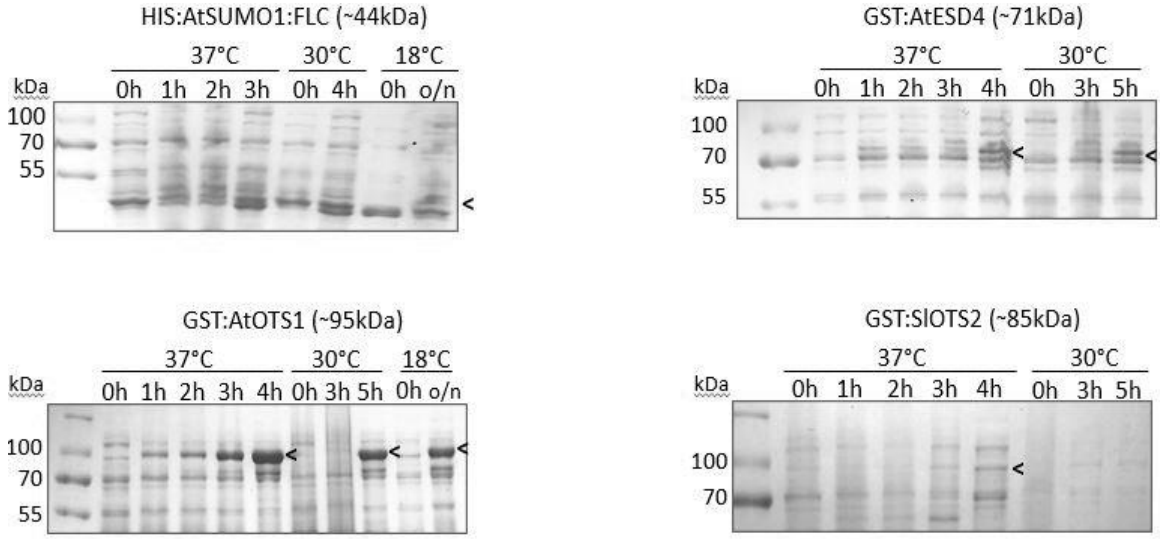


Figure 2: Expression of AtSUMO1:FLC, AtESD4, AtOTS1 & SlOTS2. Expression of the recombinant proteins was tested at different temperatures (18°C, 30°C & 37°C) to assess optimal conditions. 18°C-30°C are the most common temperatures used for expression because they allow proper folding of recombinant proteins, but proteins were also able to be expressed successfully at 37°C. Proteins were expressed for 1h to overnight (o/n) after induction.

The purification of these proteins displayed some degradation, but the size check suggests that some amount of the enzymes was still purified after two elution steps (Fig. 3). After quantification with the Bradford assay using BSA as standard, HIS:AtSUMO1:FLC substrates were incubated with equal amounts of soluble fractions of AtESD4, AtOTS1 & SlOTS2 for 60min and 90min at 37°C.

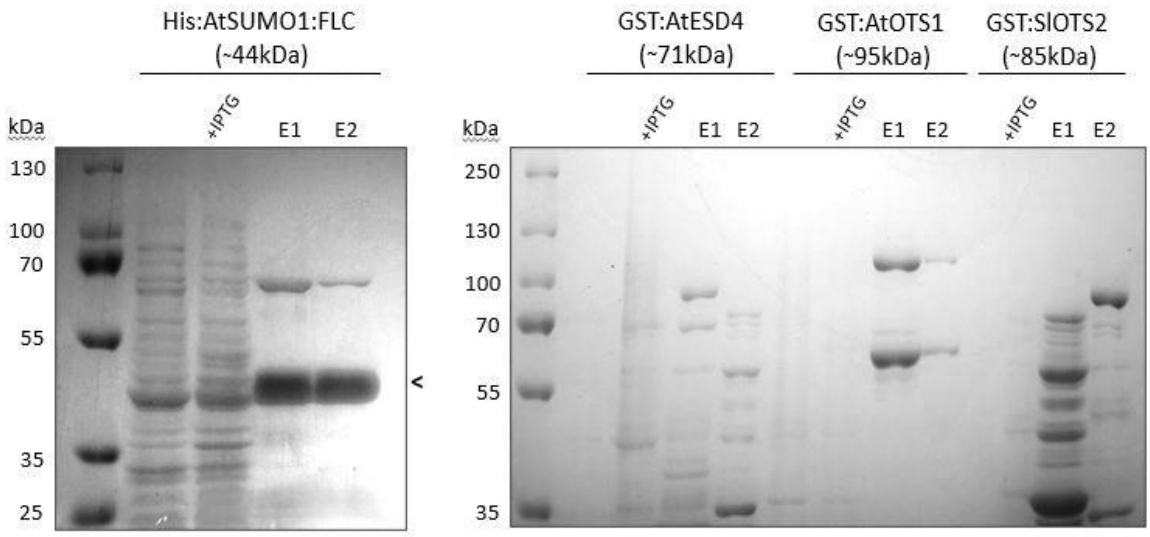


Figure 3: Purification of HIS:AtSUMO1:FLC, GST:AtESD4, GST:AtOTS1 & GST:SlOTS2. For the purification, two elution steps (E1 & E2) were performed, E1 for 90min elution and E2 for an overnight elution. Despite degradation of GST-purified proteins some amounts of the enzymes were still able to be recovered.

SUMO proteases cleave SUMO molecules in a conserved di-Glycine motif to generate mature SUMO. C-terminus fusion of transcription factor FLC to AtSUMO1 helps detection of SUMO cleavage by the proteases by increasing the size of the peptide (Murtas, Reeves et al. 2003).

After incubation, the products of the reaction were resolved in a 15% SDS-PAGE gel. Incubation of HIS:AtSUMO1:FLC with GST:AtESD4 or GST:SlOTS2 showed no cleavage activity since there was no difference at the band pattern. This may be due to the bad quality purification observed in the case of GST:AtESD4 and GST:SlOTS2. Incubation of HIS:AtSUMO1:FLC with GST:AtOTS1 for 90min resulted in a novel band at ~20kDa, which was not present after incubation with the other two proteases (Fig. 4). That could represent the C-terminus portion of HIS:AtSUMO1:FLC after cleavage by GST:AtOTS1, consisting of the cleaved part of HIS:AtSUMO1 which is now fused with FLC. The intensity of the band is also stronger as incubation time increases, while the band representing HIS:AtSUMO1:FLC is almost undetected. The results also show that SlOTS2 was not able to cleave AtSUMO1, but the quality of the SlOTS2 purification must be considered.

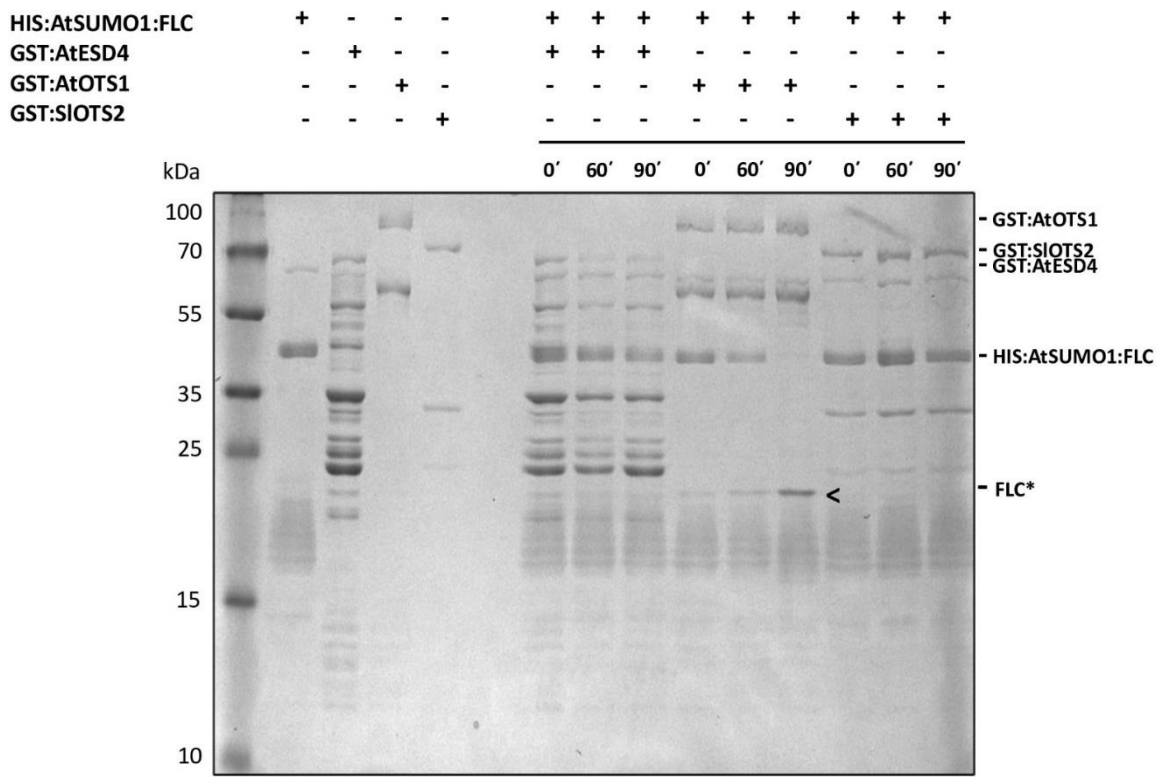


Figure 4: In vitro SUMO protease assay. Equal amounts (2μg) of GST:AtESD4, GST:AtOTS1 & GST:SlOTS2 were mixed with 500ng of purified HIS:AtSUMO1:FLC as SUMO substrate in the indicated combinations and the reaction products were resolved in by 15% SDS-PAGE gel. The asterisk on FLC refers to the band that could represent the cleaved C-terminal section of AtSUMO1 fused with the FLC.

3.2 SlSUMO1-4 cloning, expression & purification

In tomato, four SUMO homologs have been identified: *SlSUMO1, SlSUMO2, SlSUMO3* & *SlSUMO4* (Novatchkova, Tomanov et al. 2012) . The ORFs of all of them were amplified from tomato cDNA and were cloned to expression vector pET-28a(+). The cloning success was confirmed by sequencing (Fig. 5) using the T7 term sequencing primer (5'GCTAGTTATTGCTCAGCGG) and the final constructs were then used to transform *E.coli* BL21(DE3) competent cells. Despite some probable contamination in the purification process for SlSUMO3, the other 3 SlSUMOs were able to be expressed and purified successfully, as shown after analysis on a 15% SDS-PAGE gel (Fig. 6).

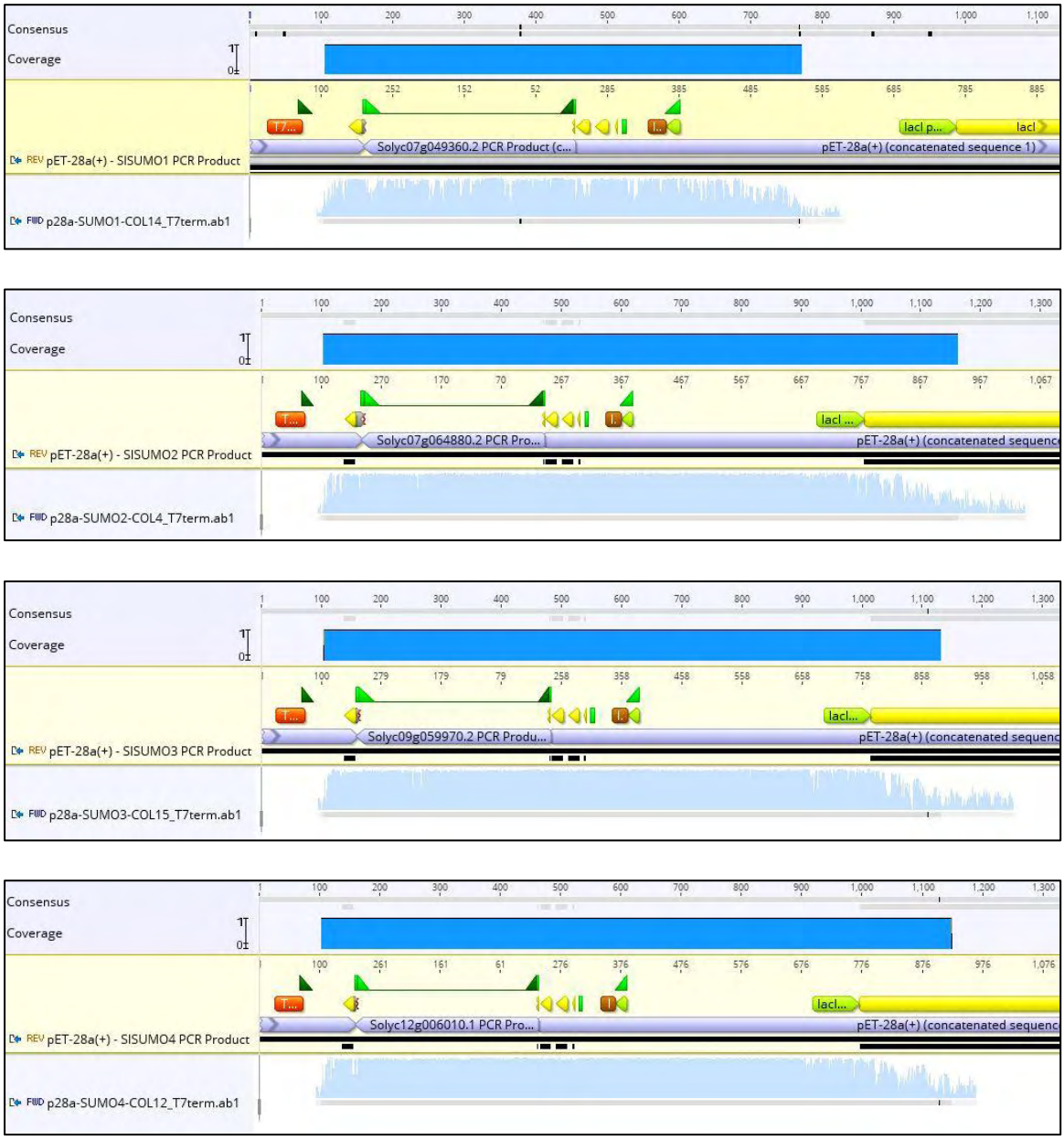


Figure 5: Sequencing results of SlSUMO1-4 cloning to pET-28a(+) expression vector. Constructs were sequenced using the T7 term primer to confirm the cloning of SlSUMOs to pET-28a(+) vector, which was successful for all four of them.

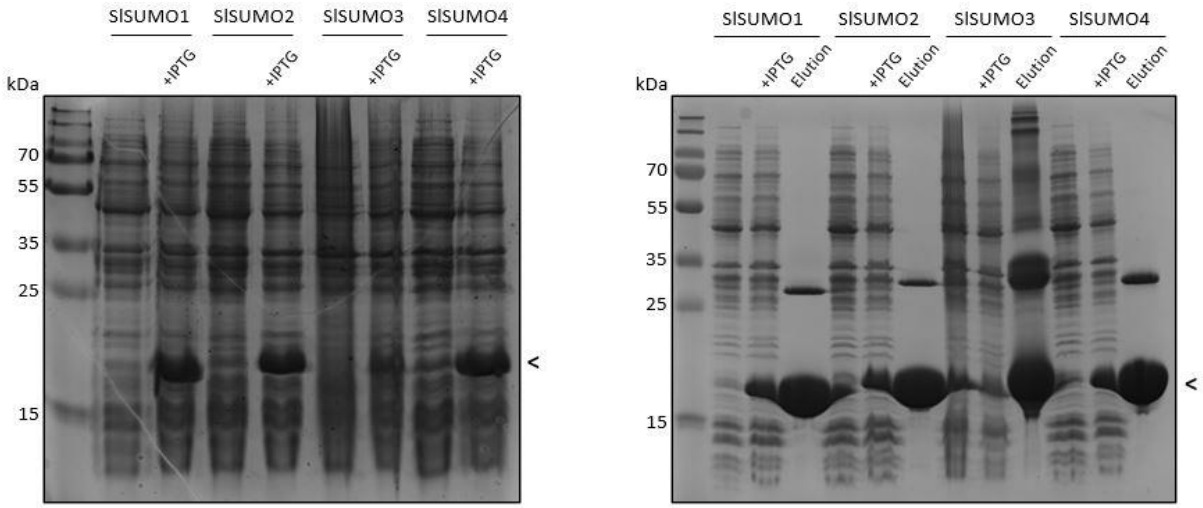


Figure 6: Expression and purification of SlSUMO1-4.
Left: Expression of SlSUMO1-4 after induction with IPTG. Arrowhead indicates the expected size of the SlSUMO proteins at ~18kDa.
Right: Purification of SlSUMO1-4. Purification of SlSUMO3 shows some contamination after elution while the quality of SlSUMO1, SlSUMO2 and SlSUMO4 purification was better.

3.3 Cloning of SlUBA1, SlUBC8 & SlXERICO1-3 to pGEX-6P-1 expression vector

SlUBA1 was amplified from tomato cDNA and was cloned to pGEX-6P-1 expression vector. After ligation, positive clones were sent for sequencing to confirm successful cloning. Because of *SlUBA1*'s big length coding sequence (CDS) a set of three sequencing reads were produced using three different primers, so that the biggest part of the CDS region is covered. The three reads were then assembled to one contig which confirmed the presence of the insert. The first read was performed using the pGEX forward sequencing primer (5'GGGCTGGCAAGCCACGTTTGGTG) and the second and third one using two primers (5'GGGTGCTTAACCCAATCAGG, 5'CTCAATGACATCATCATCAGTCAC) that target internal regions of the CDS in a way that all three reads overlap (Fig. 7). Similarly, after ligation of *SlUBC8* to the same vector the successful cloning was confirmed via sequencing using only the pGEX forward sequencing primer (Fig. 8). Finally, *SlXERICOs* were also successfully cloned to pGEX-6P-1 vector as confirmed by the sequencing results using the pGEX forward sequencing primer (Fig. 9).

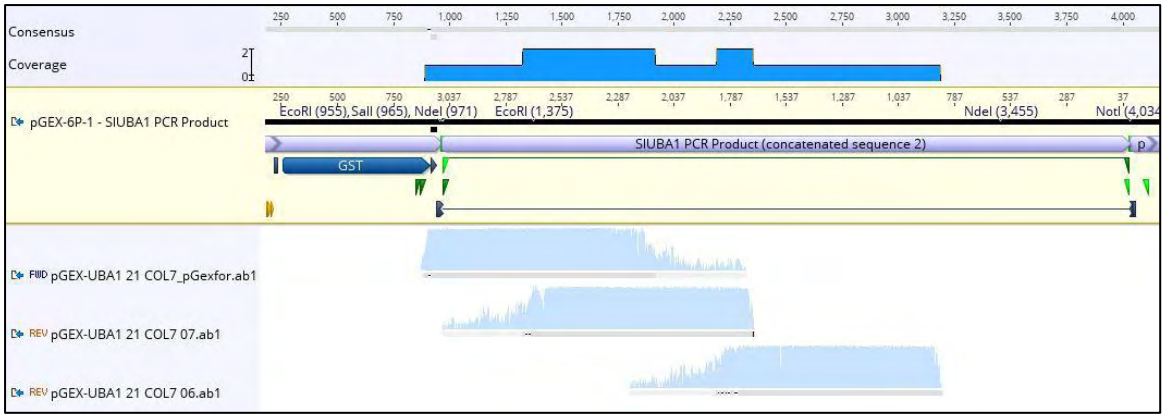


Figure 7: Creation of a contig from the sequencing results of the cloning of *SlUBA1* to pGEX-6P-1 expression vector. The contig which confirms the successful cloning of *SlUBA1* was created by assembling three overlapping reads from three different primers.

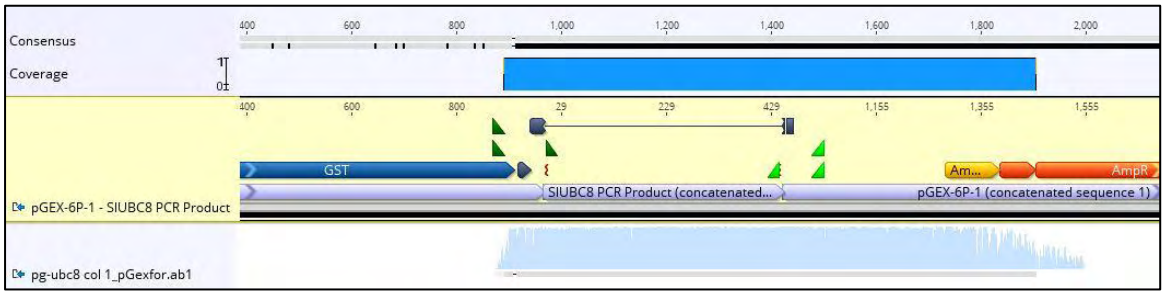
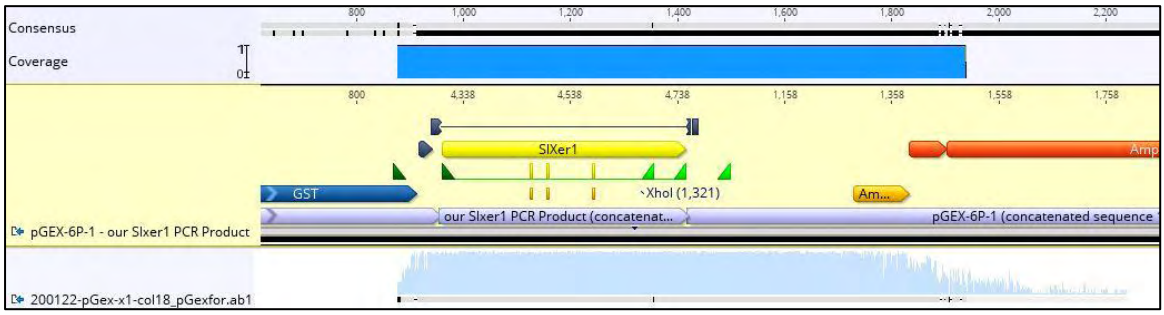
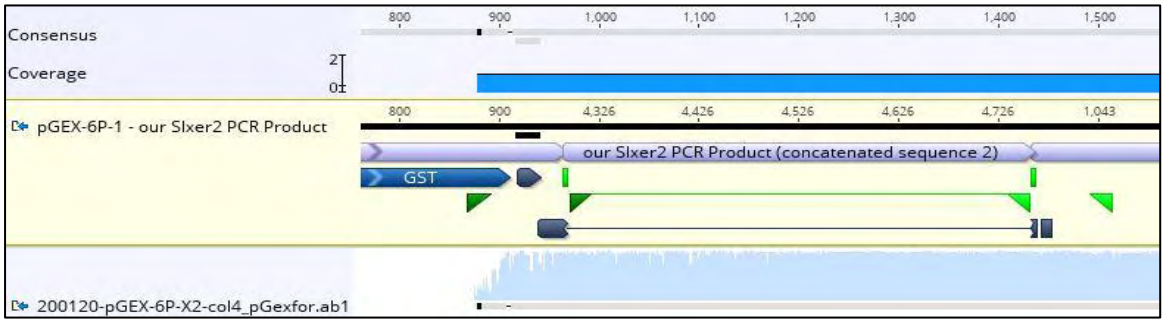


Figure 8: Sequencing results of the cloning of *SlUBC8* to pGEX-6P-1 expression vector. The successful cloning of *SlUBC8* was confirmed after sequencing using the pGEX forward sequencing primer.





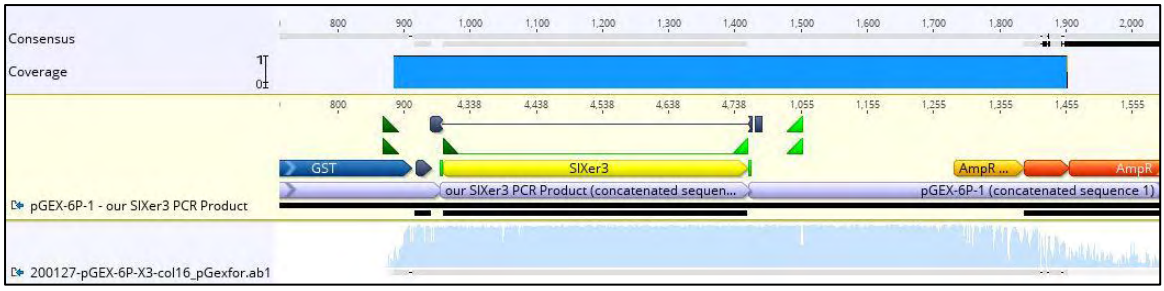


Figure 9: Sequencing results of the cloning of *SlXERICO1-3* to pGEX-6P-1 expression vector. Successful cloning of the three *SlXERICOs* was confirmed after sequencing using the pGEX forward sequencing primer.

3.4 SlXERICO1, SlXERICO2, SlXERICO3 exhibit E3 ubiquitin ligase activity in vitro

To test whether the tomato XERICO homologs exhibit E3 ubiquitin ligase activity, an in vitro ubiquitination assay was set up. Most E3 ligases, in the presence of all necessary components, can perform autoubiquitination, attaching ubiquitin molecules onto themselves (de Bie and Ciechanover 2011). This way, no further substrates are required to assess whether a protein of interest is a putative E3 ligase.

To reconstitute the ubiquitination cascade in vitro, all necessary enzymes had to be expressed and purified: SlUBA1 (E1 ubiquitin-activating enzyme), SlUBC8 (E2 ubiquitin-conjugating enzyme) and SlXERICO1-3. For the purification, an N-terminal fusion of glutathione S-transferase (GST) with all the above enzymes was produced and, as a result, all of them carry a GST-tag.

The attachment of the GST-tag increases the peptide sizes. The approximate size of GST:SlUBA1 is ~140kDa and ~44kDa for GST:SlUBC8. To determine the concentration of the enzymes a BSA curve was produced from different concentrations of BSA (from 0.1mg/ml to 1mg/ml) (Fig. 6). Despite some degradation in the case of GST:SlUBA1, the necessary amount of the enzyme for the assay was able to be recovered. The size of GST:SlXERICO1-3 is calculated at ~44kDa and their concentration was also determined with a BSA curve (Fig. 7).

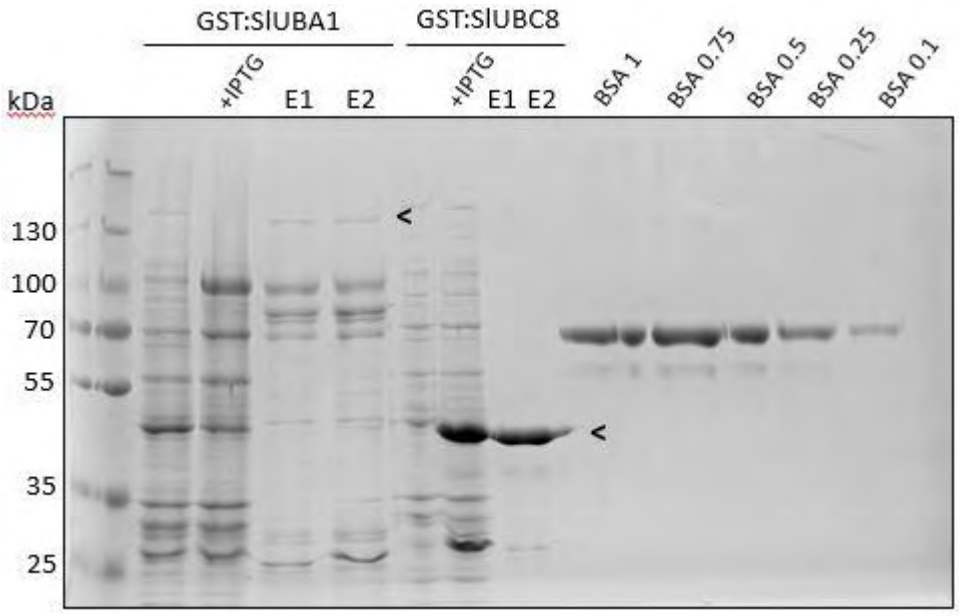


Figure 6: Expression and purification of GST:SlUBA1 and GST:SlUBC8. Two elution steps were performed, one after 90min (E1) and one overnight (E2). Arrowheads indicate the expected sizes of the proteins (~140kDa for GST:SlUBA1 and ~44kDa for GST:SlUBC8). Different concentrations of BSA as a standard (from 0.1mg/ml to 1mg/ml) were used to quantify the enzymes' concentration.

An immunoblot where the three GST:SlXERICOs (1μg) and GST:SlOTS2 (1μg) were incubated with anti-XERICO1-3 and anti-XERICO3 antibodies showed no detection of GST:SlOTS2 suggesting that these antibodies do not bind to either GST or SlOTS2 and that they are specific to SlXERICO1-3 as substrates (Fig.8).

Equal amounts of each of GST:SlXERICO1-3 (2μg) were mixed separately with SlUBA1 and SlUBC8 and the other required components, ATP and ubiquitin. After incubation, products were resolved on 10% SDS-PAGE gel and transferred on nitrocellulose membrane. To detect autoubiquitination, membranes were probed with anti-XER3 and anti-XER1-3 specific antibodies.

In the absence of GST:SlUBA1 or GST:SlUBC8 only one major band is detected which corresponds to the sizes of the GST:SlXERICOs. However, in the presence of GST:SlUBA1 and GST:SlUBC8 several bands of higher molecular weight are detected that indicate the successive addition of ubiquitin molecules on the GST:SlXERICOs (Fig. 9).

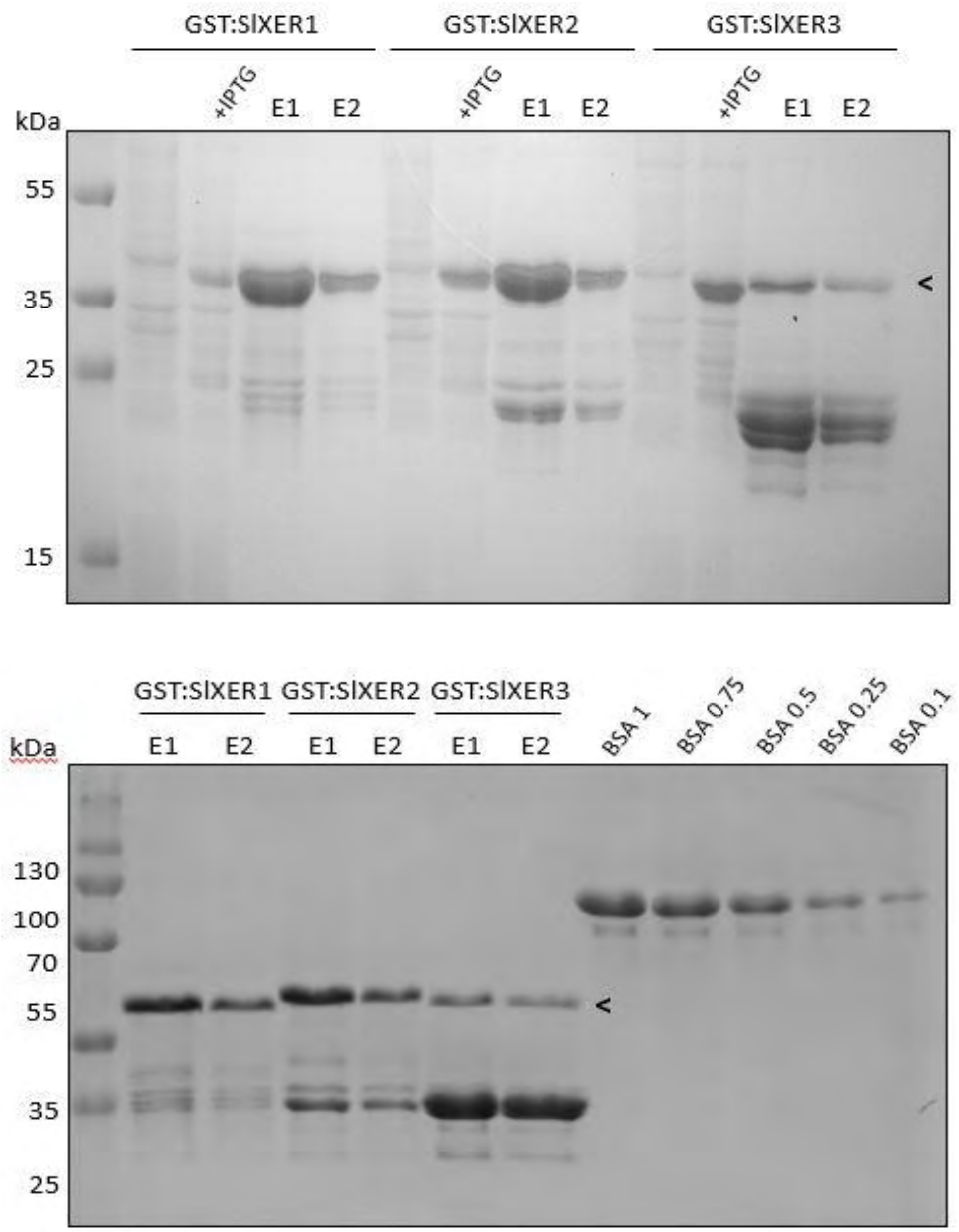


Figure 7: Expression and purification of GST:SlXERICO1-3. Arrowheads indicate the expected size of the SlXERICOs (~44kDa). Two elution steps were performed, one for 90min (E1) and one overnight (E2). The bottom image shows the two elutions of SlXERICOs placed next to a BSA curve for quantification.

After incubation with the anti-XERICO1-3 antibody ubiquitination could be detected for GST:SlXERICO1 and GST:SlXERICO2, while no signal could be detected with this antibody for GST:SlXERICO3. However, when the part of the membrane containing GST:SlXERICO3 was cut and was mixed with the anti-XERICO3 antibody ubiquitination signal could also be detected for this protein, too (Fig. 9). In conclusion, our results suggest that all three GST:SlXERICOs display E3 ubiquitin ligase activity in vitro.

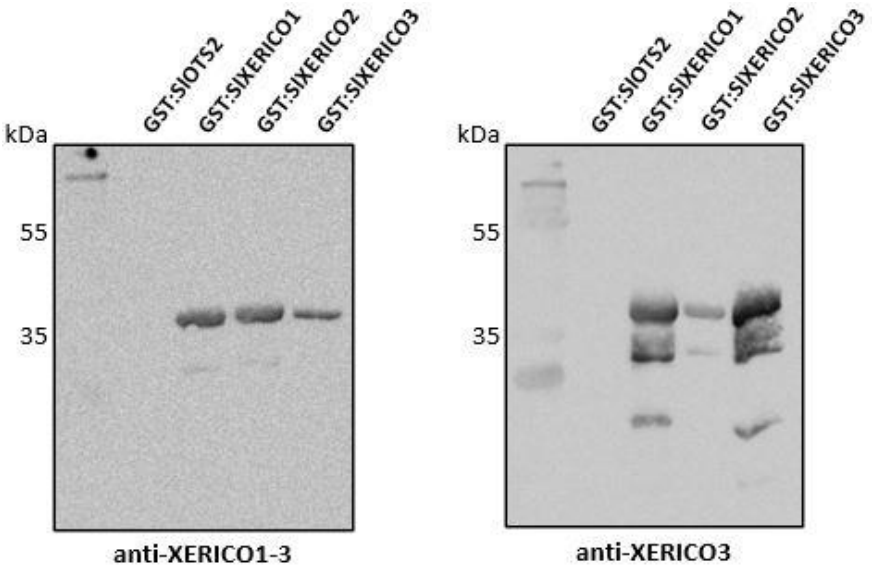


Figure 7: Incubation of GST:SlXERICO1-3 and GST:SlOTS2 with anti-XERICO1-3 and anti-XERICO3 specific antibodies. The two antibodies showed specificity to the SlXERICO1-3 as substrates since they did not bind to GST:SlOTS2.

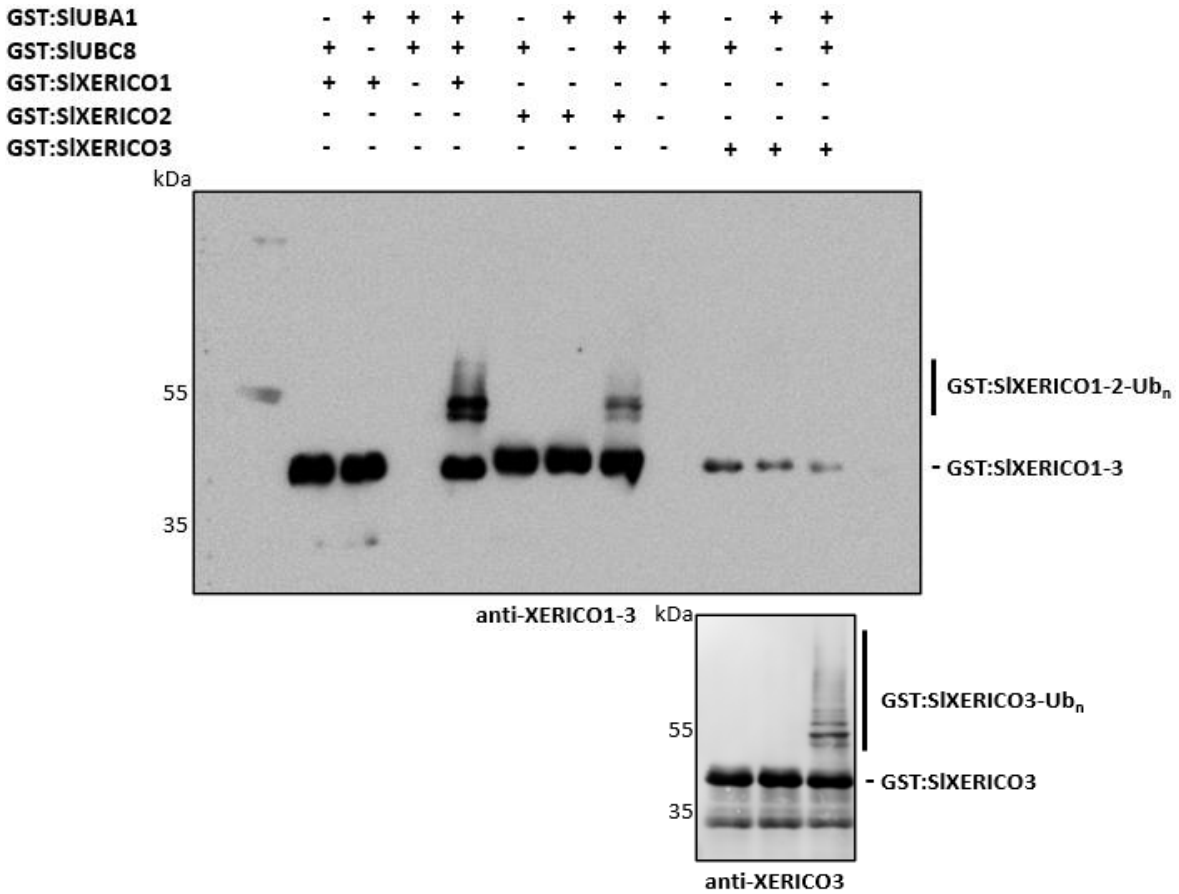


Figure 9: In vitro ubiquitination assay. Recombinant GST:SlXERICO1-3 proteins catalyze autoubiquitination in presence of GST:SlUBA1 and GST:SlUBC8, displaying this way a role as E3 ubiquitin ligases. Incubation of GST:SlXERICOs with anti-XERICO1-3 antibody detected autoubiquitination of GST:SlXERICO1 and GST:SlXERICO2. Subsequent incubation with anti-XERICO3 antibody showed autoubiquitination of GST:SlXERICO3.

4. DISCUSSION

4.1 AtOTS1 cleaves AtSUMO1 – Tomato OTS homologs can be tested for protease activity with the purified SlSUMO1-4

Our results from the in vitro SUMOylation assay indicate that AtOTS1 cleaves AtSUMO1 something that is in agreement to what has been shown by Conti et al. (2008). AtESD4 has also been shown to cleave AtSUMO1 (Murtas, Reeves et al. 2003) something that was not reproduced in our results since the quality of the purification was not high enough. SlOTS2 was also mixed with AtSUMO1 but no cleavage activity took place as a result of that, but again the low-quality purification must be considered. SlOTS2 is one of the two OTS homologs in tomato (Novatchkova, Tomanov et al. 2012) but SlOTS1 could not be amplified. Once obtained the same assay can be performed with the purified SlSUMOs to assess their SUMO protease activity and further investigate their function in salt stress responses.

4.2 All three SlXERICOs function as E3 ubiquitin ligases, exhibiting *bona fide* autoubiquitination in vitro

After testing the activity of the three SlXERICOs as E3 ubiquitin enzymes with the ubiquitination assay, it is shown that they are active E3 enzymes in vitro (Fig. 9). For detection of the ubiquitination signal, anti-XERICO1-3 and anti-XERICO3 antibodies had to be produced. To further enhance the validity of results, ubiquitination signal can also be detected using antibodies against ubiquitin. A potential disadvantage of the use of this antibody, however, is it can lead to the detection of unspecific ubiquitination of copurified impurities, or not fully translated E3 enzymes (Furlan and Trujillo 2017).

ZmXERICO1 does not contain a Lys residue, the usual site of ubiqitylation on the E3 substrates, inferring that ubiquitination was catalyzed on the MBP tag which was fused to the protein (Brugiere, Zhang et al. 2017). SlXERICOs contain Lys residues which could act as putative ubiquitination sites. Their *bona fide* autoubiquitination is confirmed after comparing the antibodies' specificity against SlXERICO1-3 with GST:SlOTS2 (Fig. 8). No binding to SlOTS2 or the GST moiety was observed suggesting that the signal detection in the ubiquitination assay is due to the autobiquitination of the SlXERICO domain.

4.3 RING domain mutations and ubiquitin mutants can provide information on the E3 ligase activity and the type of ubiquitin chain

The RING domain contains a highly conserved Cys-rich sequence motif (Freemont 1993). Since the E3 RING domain is necessary to catalyze ubiquitination, Brugière et al. (2017) also created a ZmXERICO1 mutant where cysteine sites on the zinc finger of the RING domain were mutated (C96G/C99G/C114G/H119F/C122G) and the result was the inactivation of E3 ligase activity. Similarly, mutations on the Cys-rich sites on the SlXERICOs' RING domain that could lead to the loss of their function to catalyze ubiquitination could further indicate their role as E3 ubiquitin ligases in vitro.

Proteasomal degradation is the most usual fate of ubiquitinated proteins, but the fate is primarily determined by the type of the ubiquitin chain attached on the target molecule (Vierstra 2009). Since these chains are produced by intermolecular linkages through seven Lys residues, ubiquitin mutants, such as ubiquitin lacking specific lysine residues (e.g. R48K), can be exploited as indicators of the type of synthesized ubiquitin chains.

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