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Characterization of the Interaction between RIN13 (RPM1-Interacting13) and Sumo Proteins in *Arabidopsis thaliana*

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Abstract

Small Ubiquitin-related MOdifier (SUMO) proteins can be found in many organisms, including *A. thaliana*, which possesses 9 SUMO genes. SUMO binds to various target proteins in a reversible reaction called SUMOylation. SUMOylation participates in transcription, chromosome organization, proteins localizations and stress responses. Our study showed that RIN13 (RPM1-Interacting13/At2g20310) is a target of SUMOylation, which was initially found by interaction between this protein and AtSCE1a (E2). Recent report showed that overexpression of RIN13 enhanced the resistance to pathogen without inducing hypersensitive response. However, the molecular interaction between RIN13 and SUMO proteins and its significance have not been studied yet. Thus, our study aimed to characterize the Interaction between RIN13 and SUMO proteins in *A. thaliana*. The result showed an isoform-specific SUMOylation between RIN13 and SUMO proteins. RIN13 is SUMOylated by SUMO1, 2, 3, and 5. Though expressed ubiquitously in *A.thaliana*, fluorescence microscopy showed that RIN13 localizes subcellularly in the nuclear body. Moreover, complete abolishment of SUMOylation with inactive E2 suggests the exclusion of RIN13 from nuclear body. These results showed that SUMOylation affected RIN13 localization, and indirectly influenced its interaction to other proteins and putative function. This paper presents evidence of RIN13 SUMOylation. Furthermore, RIN13 function in pathogenic resistance is shown to be supported by SUMOylation. Thus, this study enhanced the understanding of SUMO in plants and served as reference to molecular studies concerning post-translational modification of SUMO.

How to Cite

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INTRODUCTION

Posttranslational modifications are important integral mechanisms for eukaryote signaling cascades. Ubiquitin is the most familiar polypeptide modifier, and the enzymology of its activation and transfer has been extensively studied. Recently, a family of ubiquitin-like proteins (Ubls) has been identified to attach to target proteins through enzymatic processes similar to that of ubiquitination (Geiss-friedlander & Melchior, 2007; Meulmeester & Melchior, 2008). Small Ubiquitin-related MOdifier (SUMO) is one of the most intriguing Ubls and is the most widely studied to date. SUMO becomes attached to targets through a multistep process that requires an activating (E1-activating enzyme), a conjugating (E2-conjugating enzyme) and a ligating (E3-ligase) enzyme (Park et al., 2011). The E1 exists as one common enzyme for all SUMO substrates. This enzyme links to SUMO in ATP-dependent fashion and subsequently passes active SUMO to E2. SUMO covalently binds to E2 via thioester bond, similar to ubiquitination. The binding of SUMO to targets may be assisted directly by E2 or E3. Interestingly, E3 was proven to be an unessential requirement for SUMO conjugation to target proteins, displayed by the fact that SUMOylation can occur in reconstituted system (Okada et al., 2009).Although SUMOylation occurs only in a small portion of the total pool of the protein, SUMO has been shown to play important roles in diverse processes such as nucleo-cytoplasmic transporter, chromosome segregation, gene expression, chromatin structure, signal transduction, and genome maintenance (Geiss-Friedlander & Melchior, 2007). Unlike ubiquitination, SUMOylation is not known to target proteins for degradation, but rather is thought to regulate protein-protein interactions, alter the subcellular localization and/or activity of targets, and antagonize ubiquitin-dependent degradations (Martin, Wilkinson, Nishimune, & Henley, 2007; Miller & Vierstra, 2011; Xu & Yang, 2013).

Many of the core components of SUMOylation have been identified in *Arabidopsis thaliana* (Lois, Lima, & Chua, 2003; Novatchkova, Tomanov, Hofmann, Stuible, & Bachmair, 2012) such as ubiquitin, has emerged as a common and important mechanism for regulating protein function. Small ubiquitin-like modifier (SUMO. The *Arabidopsis* genome contains eight full-length SUMO genes (*AtSUMOs*), a single gene for the larger subunit of SUMO activating enzyme (SAE), SAE2 (*At2g21470*) and 2 genes for SAE smaller subunit, SAE1a (*At4g24940*) and SAE1b (*At5g50580*), one active gene SUMO-conjugating enzyme homolog to Ubc9 (AtSCE1a, for *A. thaliana* SUMO-conjugating enzyme, *At3g57870*), and 12 genes for putative SUMO protease Ulp-type (ubiquitin like protein) deconjugating enzymes. However, an understanding of SUMOylation in plants is still in rudimentary stage.

One of the found target protein for SUMO in *A. thaliana* was At2g20310. This protein is termed RIN13 (RPM1-Interacting Protein13) and was suggested to function as a positive regulator of RPM1 (Resistance to *P. syringae PV maculicola* 1). RPM1 belongs to the family of R (Resistance) protein, specialized in conferring immune response to avirulence (avr) protein of *Pseudomonas malicola* (Ashfield et al., 2014; Russell, Ashfield, & Innes, 2015)*the Pseudomonas syringae effector proteins AvrB and AvrRpm1 are both detected by the RESISTANCE TO PSEUDOMONAS MACULICOLA1 (RPM1*. The avr protein effectors (especially AvrRpm1) entered the host plants through type III secretion system (TTSS) and activated resistance pathways. RIN4, other cofactor of RPM1 became phosphorylated by AvrRpm1 and in turn activated RPM1 (Kim et al., 2005; Lee, Bourdais, Yu, Robatzek, & Coaker, 2015; Liu, Elmore, & Coaker, 2009; Selote & Kachroo, 2010; Takemoto & Jones, 2005)where they function to manipulate host defense and metabolism to benefit the extracellular bacterial colony. The activity of these virulence factors can be monitored by plant disease resistance proteins deployed to \"guard\" the targeted host proteins. The Arabidopsis RIN4 protein is targeted by three different type III effectors. Specific manipulation of RIN4 by each of them leads to activation of either the RPM1 or RPS2 disease resistance proteins. The type III effector AvrRpt2 is a cysteine protease that is autoprocessed inside the host cell where it activates RPS2 by causing RIN4 disappearance. RIN4 contains two sites related to the AvrRpt2 cleavage site (RCS1 and RCS2. RPM1 changes conformation, allowing RIN13 to bind to NB-ARC (nucleotide-binding-site-domain found in Apaf-1, R proteins, CED-4) and participate in race-specific normal defense signaling processes, resulted in hypersensitive response. Interestingly, ectopic expression of RIN13 conferred enhanced resistance to AvrRpm1 and AvrB in the absence of hypersensitive cell death, possibly by occupation of binding sites that activate bacterial restriction mechanisms (Al-Daoude, de Torres Zabala, Ko, & Grant, 2005).

Thus, this study aims to characterize the Interaction between RIN13 and SUMO proteins and its significance in *A. thaliana*, by assessing

the isoform-specificity of SUMOylation and RIN13 localization observation using fluorescence microscopy. As SUMO proteins are highly conserved in eukaryotes, the result signifies the importance of SUMO modulation in influencing the function of other proteins. This study presents the evidence of SUMO modulation of RIN13, a protein partially conserved in plants. Thus, this study enhanced the rudimentary understanding of plant SUMOs and may serve as a reference to molecular studies concerning post-translational modification by SUMO proteins in plants specifically and eukaryotes in general.

METHODS

Plant materials and growth conditions

The wild type used was of Columbia ecotype. Surface sterilized seeds were sown on germination medium containing 1% sucrose and were stratified for 2d at 4°C. Seeds were then incubated under 16 h light/8 h dark photoperiod at 22°C.

Yeast two-hybrid experiments

The basic procedure for the yeast two-hybrid system was based to the Clontech Yeast Protocols Handbook. The GAL4 DNA-binding domain (GBD) was fused to the *AtSUMO1*, *-2, -3, -5*, and *AtSCE1a* cDNAs as follows: the *AtSUMO1*, *AtSUMO2*, *AtSUMO3*, *AtSUMO5*, and *AtSCE1a* cDNAs were amplified by PCR from MATCHMAKER *A. thaliana* cDNA library. DNA extraction was done according to (Fibriana & Hadiyanti, 2016). *Nco*I-*Sal*I digested of each cDNA was inserted into pAS404 (Nakashima, Noguchi, & Nishimoto, 1999) to fuse in frame to the 3' end of the coding sequences of GBD. pAS404-AtSUMO1, AtSUMO2, AtSUMO3, AtSUMO5, and AtSCE1a were integrated into the *TRP* locus of yeast Y190 strain (Harper, Adami, Wei, Keyomarsi, & Elledge, 1993) respectively (Supplemental Table 1). Putative AtSCE1a-interacting clones were screened by the growth on the synthetic medium lacking His but containing 30 mM 3-amino-1,2,4-triazol (3-AT) and confirmed by blue color on medium containing the 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Transformation of *S. cerevisiae* was carried out by the lithium acetate method.

In vitro SUMO conjugation assay

The plasmid pET28a (Novagen, Madison, WI) was used for the expression of T7-His_6-recombinant proteins in *E. coli* BL21(DE3). The *At2g20310* cDNA was cloned into the *Nco*I/*Xho*I sites of pET28a vector and *Not*I/*Xho*I sites of pET28c vector to fuse in frame to the 3' end of the coding sequences of T7-His_6. This system contains E1 enzyme (50 ng of His_6-Fub2/Rad31), E2 enzyme (100ng of His_6-Hus5), E3 enzyme (200ng of His_6-Pli1), Pmt3-GG (1μg), 40 mM ATP and T7-His_6-At2g20310 protein substrate (100 ng) in SUMOylation buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP) (Okada et al., 2009). The products were separated and detected using anti-T7 monoclonal antibody (Novagen).

E.coli in vivo reconstituted SUMOylation assay

E.coli BL21(DE3) competent cells containing pACYCDuet-AtSAE1a/b-AtSAE2 was cotransformed with pET28a-RIN13 and pCDFDuet-AtSUMO1/2/3/5 (AA or GG)-AtSCE1a. Transformed cells were grown on Lactose selection plate containing appropriate antibiotics and then grown in Lactose Broth medium until OD_{600} was 1.0. Protein was induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) of final concentration of 0.1 mM. After 12-15 h incubation on 25°C, the protein was isolated and subjected to SDS-PAGE immunoblotting.

Western blotting analysis

The protein was extracted from *E. coli* using SDS buffer. Proteins were separated on 8-12.5 % SDS-PAGE gel and transferred onto PVDF membrane (Immobilon-P Millipore). Immunodetection was carried out using appropriate antibody, such as His polyclonal (Qiagen), T7 monoclonal (Novagen), or myc polyclonal antibody, followed by appropriate 2nd antibody (Promega). The proteins were then visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore) or Super Signal West Femto Maximum Sensitivity Substrate (Pierce).

Transient expression assay

Fluorescent proteins were expressed in *Allium* stem cells using particle-mediated DNA delivery. Coating of gold particle with desired plasmid was conducted as follows: gold particles were washed with 70% ethanol, followed by sterile water, and stored in 50% glycerol to the final concentration of 120 mg/ml. To 15 μl of well-mixed gold particle, 6 μg DNA was added, followed by 15 μl 2.5 M $CaCl_2$ and 7.5 μl 0.1 M spermidine. The supernatant was removed and washed by 70% ethanol and 100% ethanol subsequently. The particles were then stored in 20 μl 100% ethanol and spread equally onto the surface of 2 sterile microcarriers. Onion (*Allium cepa*) stem epidermal strips were placed on 2% agar plate and the vectors were introduced by gold particle

bombardment with a Biolistic Particle Delivery System-100/He (Bio-Rad). Bombardment was done twice for each sample under the following delivery conditions: gold particle diameter, 1.0 μm; helium pressure, 1.100 p.s.i.; target distance, ±9 cm; and chamber vacuum pressure, 550 mm Hg. Samples were incubated in the dark, 24-48 h before observation using Olympus DP71 camera on Olympus BX51 microscope. Nucleus position was confirmed with DAPI (4,6'-diamidino-2-phenylindole).

RESULTS AND DISCUSSION

Identification of *Arabidopsis* SUMO E2 enzyme interacting proteins

Recognition of SUMO targets is partly mediated by the E2 enzyme. *A. thaliana* has one pseudogene and one active gene (AtSCE1a; At3g57870) for SUMO E2 enzyme (Novatchkova et al., 2012). To identify potential target proteins for SUMOylation in *A. thaliana*, a yeast two-hybrid screening using an *Arabidopsis* SUMO E2 enzyme, AtSCE1a, as the bait was conducted. After the correct expression of GBD-HA-AtSCE1a had been confirmed (data not shown), ~2x10^6 clones have been screened and 16 positive AtSCE1a-interacting clones were obtained. Out of 16 positive clones, 7 clones were found to be derived from the same *Arabidopsis* gene (AtSIZ1; At5g60410) that encodes an SUMO E3 ligase and 4 clones were from the At2g20310 gene that encodes a RIN13 protein of 430 amino acids. The identification of SUMO E3 ligase, AtSIZ1, as an AtSCE1a-interacting protein seems to be quite reasonable. To eliminate the possibility that RIN13 is promiscuous in protein interaction, a specificity test in which its interaction with AtSCE1a was compared with control vector was performed. As shown in Figure 1 (line 1-2, 4-5, last row), the Interaction between RIN13 to AtSCE1a seemed to be specific and failed in interaction with control vector.

Interaction between RIN13 and RIL1 with AtSUMOs

Comparison to the *Arabidopsis* genome revealed that At2g20310 is a single-copy gene with 2 analogs, 448 amino acids hypothetical protein of At4g28690 (36% identity, 48% similarity) and 182 amino acids hypothetical protein of At4g27660 gene (43% identity, 61% similarity) (Figure 2). Amino acids similarity was also found in other organisms, such as with hypothetical protein ACG43586.1 constituting of 570 amino acids from *Zea mays* (33% identity, 48% similarity), 557 amino acids hypothetical protein product of *Os10g0466000* from *Oryza sativa Japonica group* (41% identity, 54% similarity) and 511 amino acids unnamed protein product of CAO40534.1 from *Vitis vinifera* (41% identity, 61% similarity). Due to the high degree of similarity between At2g20310, At4g28690, and At4g27660, the possibility of interaction of both analogs with AtSCE1a were tested. As shown in Figure 1 (line 3 and 6, last row), At4g28690 could also interact with AtSCE1a in the two-hybrid assay, while At4g27660 could not (data not shown).

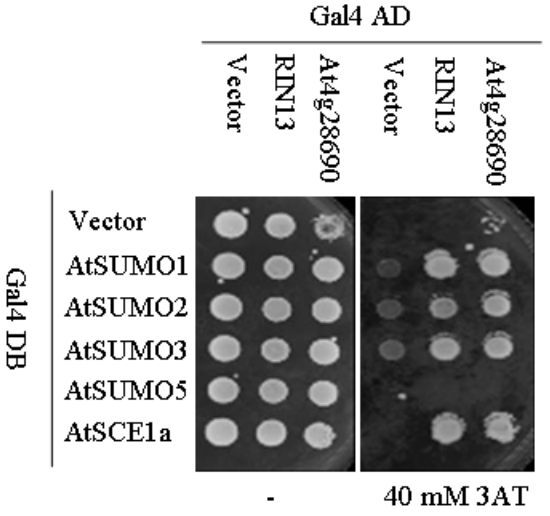

Figure 1. RIN13 and At4g28690 interacted with AtSCE1a and SUMO1, 2, and 3 in yeast two-hybrid. The AtSCE1a-interacting clones were screened on the synthetic medium containing 40 mM 3-amino-1,2,4-triazol (3-AT).

Several proteins that interact directly with SUMO by the yeast two-hybrid system have been demonstrated to be direct targets for SUMOylation (Elrouby & Coupland, 2010). Hence, the interaction of RIN13 and At4g28690 with isoforms of *Arabidopsis* SUMO proteins (AtSUMOs; AtSUMO1, AtSUMO2, AtSUMO3, AtSUMO5) whose transcripts have been detected (Kurepa et al., 2003)location, and/or half-life. Here we show that the SUMO conjugation system operates in plants through a characterization of the Arabidopsis SUMO pathway. An eight-gene family encoding the SUMO tag was discovered as were genes encoding the various enzymes required for SUMO processing, ligation, and release. A diverse array of conjugates could be detected, some of which appear to be SUMO isoform-specific. The levels of SUMO1 and -2 conjugates but not SUMO3 conjugates increased substantially following exposure of seedlings to stress conditions, including heat shock, H(2 were also tested.

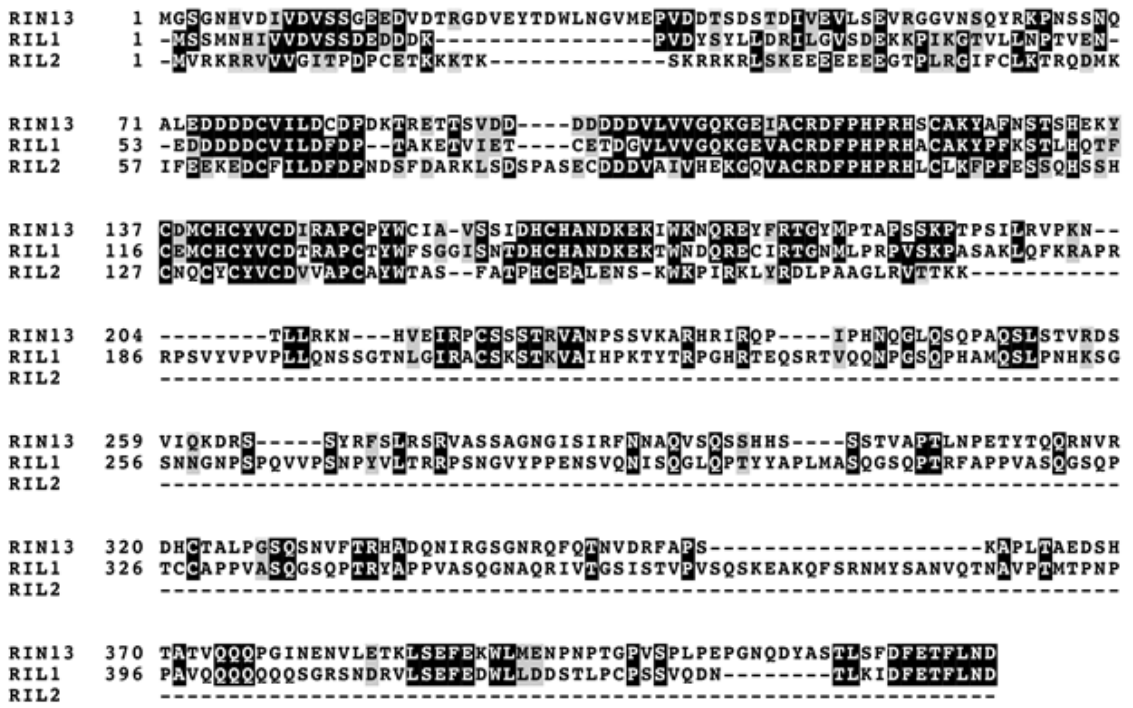

Figure 2. RIN13 Gene Family of *Arabidopsis thaliana***.** Amino acids comparison between RIN13, RIL1, and RIL2. RIN13: *At2g20310*; RIL1:*At4g28690*; RIL2:*At4g27660*. RIL : RIN13-Like.

Interestingly, both RIN13 and At4g28690 could interact with AtSUMO1, 2, and 3, but not with AtSUMO5 (Figure 1), suggesting that RIN13 and At4g28690 are potential targets of AtSUMO1, 2, and 3 modification. At this initial stage, this study focused only to RIN13.

RIN13 was modified by SUMO *in vitro*

Many studies had developed *in vitro* and *in vivo* methods to search for potential targets for SUMOylation (Elrouby, 2015; Elrouby & Coupland, 2010; Vethantham & Manley, 2008)inactivation of genes encoding SUMO or SUMO-conjugation enzymes is lethal, emphasizing the importance of SUMOylation in plant development. Despite this, little is known about SUMO targets in plants. Here we identified 238 Arabidopsis proteins as potential SUMO substrates because they interacted with SUMO-conjugating enzyme and/or SUMO protease (ESD4. To confirm whether RIN13 is a potential target for SUMOylation, *in vitro* modification assay with the purified components were used. Purified components sufficient for Pmt3 modification in fission yeast (Fub2/Rad31 (E1), Hus5 (E2), Pli1 (E3), and the mature form of Pmt3 (Pmt3-GG, SUMO) was employed. The T7-His_6-RIN13 produced by *E.coli* were incubated with the purified components of the fission yeast Pmt3 modification machinery in the presence of ATP and Pmt3-GG.

As shown in Figure 3A, lane 4, *in vitro* modification of T7-His_6- RIN13 gave rise to the appearance of larger forms of T7-His_6- RIN13. These sizes were consistent with those of T7-His_6-RIN13 modified by one or two molecules of Pmt3, respectively. The identities of these modifications of RIN13 was further supported by the fact that it did not occur in the absence of Pmt3-GG or ATP (Figure 3A, lane 1 and 2).

RIN13 was SUMOylated by SUMO1, 2, 3 and 5 in *E. coli* SUMOylation system

To further confirm RIN13 SUMOylation and its isoform specificity, the reconstituted *Arabidopsis* SUMOylation system in *E. coli* (Okada et al., 2009) was used. SUMOAA for each SUMOs was employed as negative control. These SUMOAAs have modified carboxy terminal processing pattern, from Gly-Gly to Ala-Ala (SUMOGG to SUMOAA). This modification eliminates the capability of SUMO conjugation to any substrates.

As shown in Figure 3B, the substrate bands of T7-His-RIN13 were detected at approx. 80 kDa. Conjugation of His-SUMO (detected at ~20 kDa) gave rise to SUMO-T7-His-RIN13 at around 110 kDa. The *in vitro* results are well consistent with the *in vivo* observation (Figure 3B), and thus eliminated the possibility of two-hybrid false positive. Combined, the results suggested

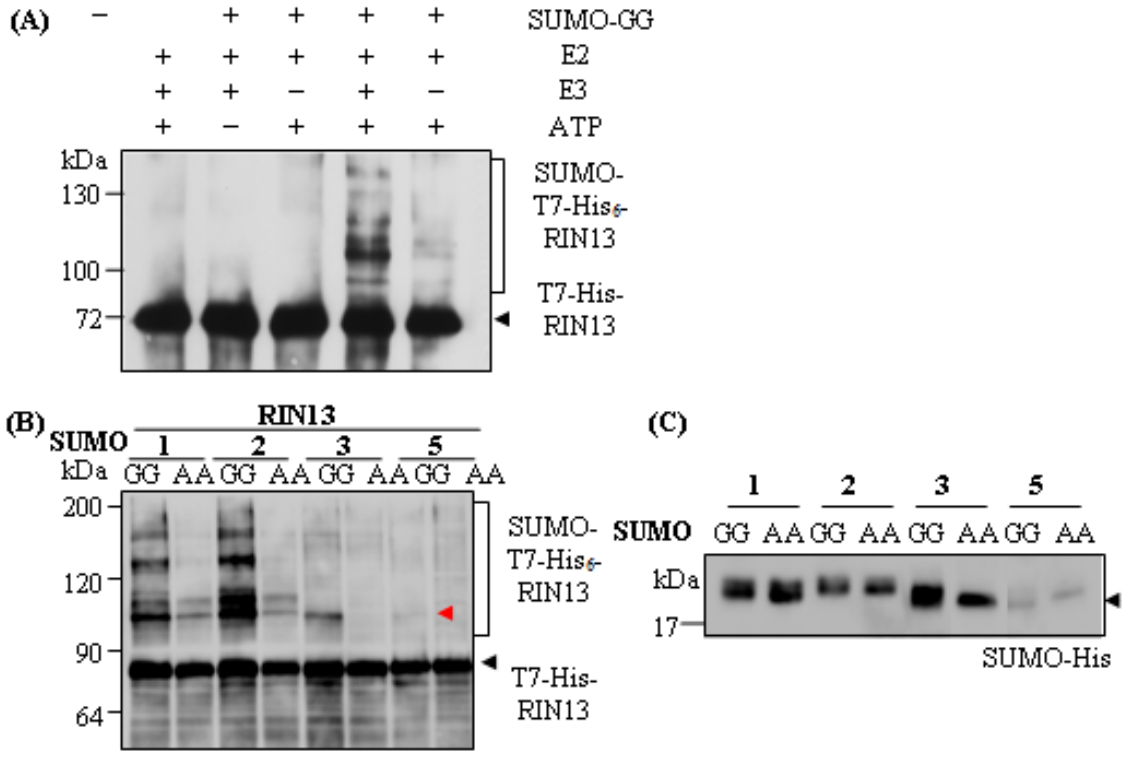

Figure 3. RIN13 was SUMOylated *in vitro* and *in vivo*. (A) T7-His_6-RIN13 (100 ng) were incubated in *in vitro* SUMOylation system for 90 min. The reaction was terminated by SDS buffer containing 1 mM dithiothreitol (DTT) prior separation and detection. (B) T7-His_6-RIN13 and His-SUMO + E2 were coexpressed in *E.coli* containing E1 enzyme. The expression of T7- His_6-RIN13 was induced by 0.1 mM IPTG addition. Fusion proteins were then detected using appropriate antibodies. SUMOAAs served as negative control. (C) SUMO expression of each SUMO.

that RIN13 is a subject of SUMOylation.

Isoform specific SUMOylation of RIN13 and poly-SUMO chain formation

Using *in vivo* SUMOylation system, RIN13 was conjugated to SUMO1, SUMO2, SUMO3, and SUMO5. However, SUMO5 did not interact with RIN13 (Figure 1) in yeast two-hybrid. Considering the possibility of false negative result of yeast two-hybrid, the emphasis was put to SUMOylation assay *in vivo*. Thus far, it is assumed that the low expression level of SUMO5 caused insufficient expression of histidine to encounter the effect of 3-AT used for sensitivity control.

Poly-SUMO chain formation was observed clearly in SUMO1 and SUMO2 modification of RIN13, at >120 kDa (Figure 3A). Most of SUMO target proteins were known to conjugate to monomeric SUMO. However, similar to ubiquitin, certain SUMO isoforms were able to multimerize and construct poly-SUMO chain (Vertegaal, 2007). In *vitro* experiment showed that yeast SUMO and human SUMO1 was able to form chains using Lys7, Lys16 and Lys 17 (Colby, 2006; Eckhoff & Dohmen, 2015; Park et al., 2011; Yang, Hsu, Ting, Liu, & Hwang, 2006). In addition, human SUMO2 and SUMO3 formed chains in vitro and in vivo, primarily through a conserved acceptor, Lys11 (Park et al., 2011; Tatham et al., 2001). The SUMO polymerization was assisted by conjugating enzyme and SUMO E3 ligase, and diassembled by SUMO proteases. Poly-SUMO chain for SUMO3 was not observed clearly in the experiment, perhaps due to the low level of poly-SUMO chains.

Unfortunately, these isoform specific SUMOylation could not be tested in the complete series of *Arabidopsis* SUMO due to the unavailable EST (expression sequence tag) for the other SUMOs. Thus far, SUMOylation by SUMO1 and SUMO2 were predictable due to their high similarity. SUMO5 was the most different among all isoforms. Moreover, SUMO isoforms are likely to have redundant function and often have overlapping substrates specificity (Chosed et al., 2006). Considering that there is only one gene for the larger subunit of SUMO-E1 (SAE2) and two genes for its smaller subunit (SAE1a/SAE1b) and

one active gene for SUMO-E2 (SCE) in *A. thaliana*, substrate specificity was usually determined by specific E3 ligase or a certain motif within the target proteins. Covalent modification by SUMO requires consensus motif of ψKXD/E (ψ, hydrophobic amino acid; K, lysine; X, any amino acid, D, aspartic acid; E, glutamic acid), while non-covalent binding of SUMO usually determined by SIM (SUMO-interacting Motif) or SBM (SUMO-binding Motif) (Novatchkova et al., 2012; Park et al., 2011).

Gene expression pattern of *RIN13 and RIL1*

RIN13 gene expression was ubiquitous in *A. thaliana*, during all of the developmental stage (Figure 4). The expression was considerably high during germination, boulting, flowering and silique development. This ubiquitous and constitutive expression of RIN13 promotes its binding to SUMO protein, especially AtSUMO1 and AtSUMO2, which also have constitutive expression (Park et al., 2011). Interestingly, the gene expression in etiolated seedlings was considerably lower than in normal seedlings; implying that *RIN13* was partially down-regulated in the dark. It is feasible that ubiquitous expression of *RIN13* might suggest its fundamental roles in the development of *A. thaliana*, rather than served location-specific function. As SUMO has been shown to play crucial roles in a variety of process, it is also possible that RIN13 has broader function and is involved in more than single mechanism.

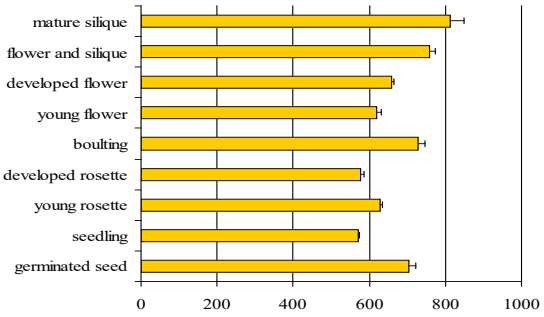

Figure 4. Gene expression profile of *RIN13***.** The data was obtained from www.genevestigator.com.

Subcellular Localization of RIN13

To understand the subcellular localization of RIN13, an expression plasmid containing the native *RIN13* promoter was used to drive the expression of the RIN13::GFP (green fluorescent protein) fusion proteins. The plasmid was transiently expressed in *Allium* stem cell. As clearly shown in Figure 5A(3), RIN13 was recruited into nuclear body.

Although many novel plant nuclear bodies, along with their nuclear domains, had been discovered, their functions are not clear yet. Interestingly, the formation and disruption of nuclear body in many organisms often required PML (pro-myeloid leukemia) SUMOylation. Moreover, the formation of poly-SUMO5 mediates the life cycle of nuclear bodies in human (Liang, Lee, Yao, Lai, & Schmitz, 2016; Percherancier et al., 2009; Shen, Lin, Scaglioni, Yung, & Pandolfi, 2006). However, PML itself does not evolutionarily conserved in *A. thaliana*. PML-nuclear bodies are shown to be involved in various mechanism, such as transcription, defense response, DNA repair, apoptosis, and senescence (Liang et al., 2016). RIN13 localization to nuclear bodies/domains may reflect the interaction sites, the sequestration location, and the site of function or modification, such as splicing (Shaw & Brown, 2004). Alternatively, nuclear bodies may serve as the assembly location prior to transport to the protein functional site (Liang et al., 2016).

To further study RIN13 SUMOylation, RFP fusion proteins of AtSCE1a and AtSCE1a_C94S were employed. This mutant possessed altered amino acid in its catalytic site, Cys-94, and therefore became inactive (Lois et al., 2003). There was no difference between the signal of AtSCE1a and AtSCE1a_C94S. Both proteins

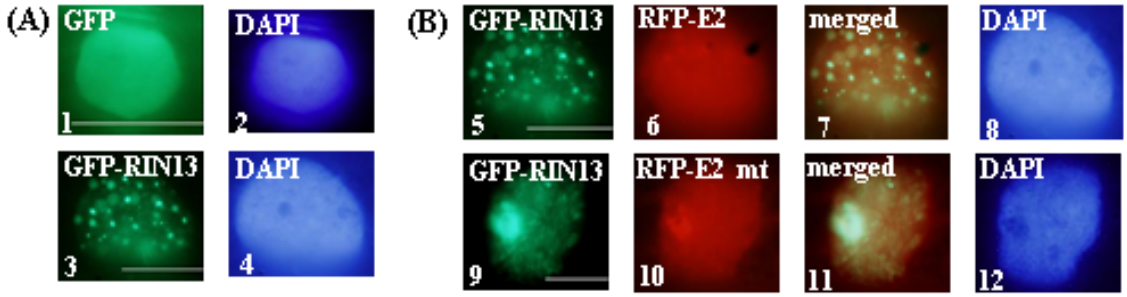

Figure 5. RIN13 localized in nuclear body. (A)-(B) Transformed *Allium* stem cells with transient expression of desired protein(s). For (B), merged figures of first and 2nd respective rows were shown on 3rd rows. DAPI staining confirmed the nucleus position. Bars = 20 μm.

showed uniform signals within the nucleus (Figure 5B(6,10)). Interestingly, when coexpressed with RIN13, this mutation caused striking differences to RIN13 signal. The recruitment of RIN13 to nuclear body became less clear and RIN13 signal were distributed evenly within the nucleus (Figure 5B(9)). Thus, SUMOylation changed the RIN13 localization or disrupt the formation of nuclear body. However, it has not been determined yet whether the direct or the indirect SUMOylation is the cause for this change.

Protein localization correlates directly to its putative function. RIN13 was reported to take part in pathogenic resistance through its interaction to RPM1. Similar to RIN13, RPM1 is a nuclear protein and both proteins may be able to interact due to specific location placement. Thus, the localization change due to the lost SUMOylation may cause a hindrance for RIN13 to function.

Collectively, the results above enhanced the understanding of SUMO processes specifically in plants and eukaryotes in general. The results showed that SUMO proteins functions in the regulation of other protein function. It has been shown that SUMO proteins influence the function of a partially conserved protein in plants. Thus, it is feasible that SUMO proteins are involved in the basal mechanism of eukaryotes. Therefore, this study may serve as reference to other studies focusing on the molecular approach of post-translational modification by SUMO proteins.

CONCLUSIONS

The study showed that RIN13 is a target of SUMOylation, and was shown to interact to SUMO1, SUMO2, SUMO3, and SUMO5. Interaction to other isoforms remains to be confirmed. RIN13 was found to be expressed ubiquitously in *A. thaliana* and localizes subcellularly in nuclear body. This localization to nuclear body is SUMOylation dependent, as the complete abolishment of SUMOylation caused an aberrant localization of RIN13 within the nucleus. It has been known that SUMOylation is involved in stress responses, which may include pathogenic resistance. It is feasible that the interaction and modification of RIN13 by SUMO proteins is involved in the putative role of RIN13 as a positive regulator of RPM1. However, further study is still needed.

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