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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Regulation of gibberellin signaling by E3 SUMO ligase SIZ1 in Arabidopsis thaliana

BY
Sung-Il Kim

FEBRUARY 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
COLLEGE OF AGRICULTURAL AND LIFE SCIENCES
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY
Regulation of gibberellin signaling by E3 SUMO ligase SIZ1 in *Arabidopsis thaliana*

BY

Sung-Il Kim

Gibberellins affect various plant development processes including germination, cell division and elongation, and flowering. Here, I show that the E3 SUMO ligase activity of AtSIZ1 regulates GA signaling in Arabidopsis by sumoylating SLY1. SLY1 was less abundant in *siz1-2* mutants than wild-type plants. Transgenic *sly1-13* mutants over-expressing SLY1 were phenotypically similar to wild-type plants; however, *sly1-13* plants over-expressing a mutated mSLY1 protein (K122R, a mutation at the sumoylation site) retained the mutant dwarfing phenotype. Over-expression of SLY1 in *sly1-13* mutants resulted in a return of RGA (REPRESSOR of *ga1-3*) levels to wild-type levels, but RGA accumulated to high levels in mutants over-expressing mSLY1. RGA was clearly detected in Arabidopsis co-expressing AtSIZ1 and mSLY1, but not in plants co-expressing AtSIZ1 and SLY1. In addition, sumoylated SLY1
interacted with RGA and SLY1 sumoylation was significantly increased by GA. Taken together, my results indicate that AtSIZ1 positively controls GA signaling through SLY1 sumoylation.

**Keywords:** AtSIZ1, E3 SUMO ligase, GA signaling, Gibberellin, RGA, SLY1, SUMO, sumoylation
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<th>Description</th>
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<tr>
<td>CHX</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
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<td>Abscisic acid insensitive</td>
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<tr>
<td>GA</td>
<td>Gibberellic Acid</td>
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</table>
LITERATURAL REVIEWS

1. GA signaling pathway.

The plant hormone gibberellic acids (GAs) have a pronounced impact on plant development. First identified in rice cultures infected with the fungus *Gibberella fujikuroi*, important roles in diverse processes such as seed germination, internode elongation, fruit formation, flower development and control of flowering time are known by GA signaling (Davière and Achard, 2013). For example, *Arabidopsis* plants treated with GA grow taller, display a light green color, early flowering and siliques with fewer seeds than wild type plants (Davière and Achard, 2013). This broad spectrum of action has applied GA into an important biotechnological tool both for improving field productivity and for industrial application.

Gibberellins encompass plenty of chemically related compounds, of which only amine or proportion, such as GA1, GA3, GA4 and GA7, are biologically active (Olszewski et al., 2002). GA is adapted by the GA receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1). Structural analyses have displayed conformation changes of GID1 after perceiving to bioactive GA as a key event in GA signaling (Murase et al., 2008; Shimada et al., 2008). Single gid1 mutants normally develop, and only a triple knockout mutant (gid1a·b·c) displays the dwarf phenotype characteristic of GA signaling deficiency (Griffiths et al., 2006; Willige et al., 2007), it suggests that the three receptors in
Arabidopsis have overlapping biological functions. This is in agree with the observation that the three paralogs are expressed in all tissues, but at different levels (Griffiths et al., 2006; Nakajima et al., 2006).

The basis of the GA signaling are constituted by the GA-induced conformational change in GID1a-c receptors. After binding, a pocket of GID1 locks in the bioactive GA molecule through its N-terminal region (Murase et al., 2008). The biological importance of the N-terminal extension switch covering the bioactive GA molecule relies on the interaction with the DELLA proteins, a class of repressors (Griffiths et al., 2006). Binding to bioactive GA makes a hydrophobic surface on the GID1-GA complex, which leads the interaction with the DELLA proteins. In turn, this interaction further stabilizes the GID1-GA-DELLA complex, and enhances its interaction with the F-box protein of the ubiquitin E3 ligase SCF complex. Therefore, GA perceiving to GID1 finally led to DELLA proteins ubiquitination and degradation via proteasome (Figure 1, McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004).
Figure 1. Schematic representation of activation of GA signaling in *Arabidopsis thaliana*. GA first adapted GID1s are Arabidopsis GA receptors. SLY1 or SNZ involves setting a SCF complex with DELLA proteins to plant growth. GA: gibberellic acid.
2. **SLY1, F-box protein that is involved in GA signaling.**

The SCF\textsuperscript{ Sly1} E3 ubiquitin ligase complex decreases DELLA repression by targeting DELLA for destruction by the ubiquitin-proteasome pathway. The SCF complex is composed of a Skp1 homolog termed ASK, a Cullin homolog, an Rbx1 homolog, and an F-box protein that binds a specific target. Structure of the quaternary complex contains Cul1, Rbx1, Skp1 and the F-box of Skp2, and the structure of the Cul1–Rbx1 complex is revealed (Zheng et al., 2002). There are 21 ASK family genes, five Cullins (CULs), two Rbx1 homologs, and 694 F-box proteins in Arabidopsis (Gagne et al., 2002; Gray et al., 2002; Risseeuw et al., 2003). The F-box protein binds to the Skp1 homolog of the complex through direct protein-protein interaction via the F-box domain. The F-box protein usually binds the target protein via the C-terminal domain (Dill et al., 2004; Fu et al., 2004). Steber et al. (1998) first isolate GA response sleepy1 (SLY1) mutant then presents evidence that plant brassinosteroid (BR) hormones rescues the germination phenotype of severe GA insensitive mutant sly1 as a role in promoting germination (Steber et al., 2001). Positional cloning and gene characterization of SLY1 revealed that it encodes a putative F-box protein. This result suggests that SLY1 is the F-box subunit of an SCF E3 ubiquitin ligase that regulates GA responses. Moreover, its substrates DELLA proteins also identify (McGinnis et al., 2003). Yeast two-hybrid data indicate that the SLY1 protein can bind to DELLA protein via the C-terminal LSL domain (Dill et al., 2004; Fu et al., 2004). GID2 is also identified as rice GA dependent F box
protein which is essential for GA mediated DELLA protein degradation. Moreover, GID2 specifically interacts with a phosphorylated Slender Rice 1 (SLR1, Rice DELLA Homolog) leading the ubiquitin mediated degradation (Gomi et al., 2004). Arabidopsis thaliana sleepy1gar2-1 (sly1gar2-1) mutant allele encodes a mutant E138K of subunit on C terminal LSL domain as a gain of function mutant. The DELLA target protein is more strongly interacted with sly1gar2-1 than does SLY1. In addition, in Arabidopsis thaliana also, the strength of the SCF
SLY1–DELLA interaction is increased by phosphorylated target (Fu et al., 2004). GA binding enhances the activity of the GA receptor GA-INSENSITIVEDWARF1 (GID1) to bind DELLA proteins. Based on yeast three-hybrid data, Arabidopsis GID1-GA binding to DELLA represents to increase the affinity of the F-box protein SLY1 for DELLA (Griffiths et al., 2006). Thus, GA increases SCF
SLY1 binding to DELLA proteins, thereby allowing SCF
SLY1 to facilitate the polyubiquitination of DELLA protein. Conjugation of four ubiquitin moieties to a target protein makes its recognition and proteolysis by the 26S proteasome (Smalle and Vierstra, 2004). It appears that DELLA is degraded via the ubiquitin-proteasome pathway, because both mutations in SLY1 and 26S proteasome inhibitors result in stabilization and increased level of DELLA protein in the presence of GA (Figure 2, McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004). Overexpression of the SLY1 homolog SNEZZY (SNE)/SLY2 partially rescues most of sly1 phenotypes, suggesting that SNE has similar function like a SLY1 (Ariizumi et al., 2011).
GA

+ GA

Conformational change

GA

GID1

GID1

DELLA
GRAS

- GA

GID1

DELLA
GRAS

Heterodimerization

Transcription factor

PIF, JAZ, BZR, SCL, SPL, ALC, MYC, EIN, IDD

Ubiquitination

GID1

CULLIN

Ub

E2

Rbx

26S Proteasome
Figure 2. Role of SLY1 depending on GA. In GA treatment, GA adapted GID1 has occurred conformational change to activated form then binds to DELLA protein for its ubiquitination via SCF$^\text{SLY1}$ complex. In no GA conditions, DELLA proteins are hetero-dimerized with several transcription factors involved in gibberellin, jasmonic acid, ethylene, brassinosteroid signaling to repress activity.
3. DELLA, act to transcription repressor of GA-responsive growth and development.

In Arabidopsis, DELLA proteins were first discovered in a genetic screen as a GA insensitive mutant (gai-1) (Koornneef et al., 1985; Silverstone et al., 1997). The gain-of function gai-1 mutant encodes a functional protein with a 17 amino acids deletion that deletes a conserved 5 amino acids motif (DELLA) in the N-terminal region (Peng and Harberd, 1993; Peng et al., 1997). Other GA-insensitive alleles identified in wheat (Rht), rice (SLR) and maize (D8) also encode non-functional proteins removing the DELLA domain (Fujioka et al., 1988; Ikeda et al., 2001; Pearce et al., 2011). In contrast to other species harboring a single copy gene, Arabidopsis encode 5 functionally redundant DELLA proteins: GA INSENSITIVE1 (GAI), REPRESSOR OF ga1-3 (RGA) and RGA-LIKE1, -2and -3 (RGL1, -2, -3) (Bolle, 2004). The cloning and characterization of gai-1 and other GA-insensitive DELLA mutant represented that the DELLA motif is essential for the interaction with the GID1 receptors and DELLA degradation in the presence of GA (Peng and Harberd, 1993; Peng et al., 1997; Dill et al., 2001; Willige et al., 2007). High GA levels enhance the rapid degradation of DELLA proteins via the ubiquitin-proteasome pathway; however GA-deficient mutants present increased levels of DELLA proteins (Dill et al., 2001; Silverstone et al., 2001; Griffiths et al., 2006; Willige et al., 2007). GA-insensitive DELLA mutants similar to some enhanced dark green and dwarfed gibberellin-deficient mutants but differ from them in some aspects.
First, they are genetically semi-dominant; second, they cannot be recovered by exogenous GA application; third, bioactive GA level is increased in these mutants (Koornneef et al., 1985; Dill et al., 2001). Therefore, absent of the DELLA domain are insensitive to GA because they cannot interact with GID1 receptor even in the presence of high GA level. Together, these results suggest that the high concentration of DELLA proteins in response to GA levels facilitates the biological response to this hormone. Transcriptome experiments in Arabidopsis reveal that GA controls numerous genes involved in a broad range of biological processes (Willige et al., 2007; Zentella et al., 2007). Nevertheless, the analysis of the primary protein sequence shows that the DELLA proteins do not contain a canonical DNA binding domain typical of transcription factors (Bolle, 2004). In concordance with this data, ChIP experiments failed to consistently discover binding sites, even though binding to some GA-related genes was observed (Zentella et al., 2007). These results suggest that DELLA proteins do not act as direct transcriptional regulators but instead control gene expression indirectly as co-factors. A ground breaking observation on DELLA protein function came from the study of PHYTOCHROME INTERACTING FACTOR (PIF) during photomorphogenesis. Two independent groups described the direct binding between DELLA proteins and PIF transcription factors (de Lucas et al., 2008; Feng et al., 2008). Interestingly, EMSA assay indicated that binding to DELLA proteins destroyed PIF4 binding capacity to DNA, therefore blocking its activity (de
Lucas et al., 2008). Based on these results it has been represented that DELLA proteins control effector transcription factors through direct interaction (Daviere et al., 2008). In support of this model, a little of interactions between DELLA proteins and other transcription factors have been recently discovered (Hou et al., 2010; Zhang et al., 2011; Hong et al., 2012). Recently, DELLA-dependent growth regulation can be controlled independently of GA. when a proportion of DELLAs is conjugated to the Small Ubiquitin-like Modifier (SUMO) protein, the extent of conjugation increases during stress. SUMO-conjugated DELLA binds to a SUMO interacting motif in GID1 this motif in a GA-independent manner. The consequent sequestration of GID1 by SUMO-conjugated DELLAs leads to an accumulation of non-SUMOylated DELLAs, resulting in advantageous growth attenuate during stress (Conti et al., 2014).

4. SUMO, Small Ubiquitin like Modifier.

The process of SUMOylation is contained to all eukaryotes. In animals, research has shown it to be essential in many cellular processes including human diseases, oxygen and glucose deprivation, Alzheimer’s disease, cancer (Sarge & Park-Sarge, 2009; Yang et al., 2008; Cimarosti et al., 2012; McMillan et al., 2011; Morris et al., 2009). In plants the story is similar, in that SUMOylation is necessary to be included in many ways in the cell. This involves many stress responses (Elrouby & Coupland, 2010), and SUMO paralogues have been discovered to have functions in plant development and
defense through salicylic acid-regulated defense responses (van den Burg et al., 2010). Indeed, the SUMO E3 ligase SIZ1 controls regulation on salicylic acid mediated innate immunity in *Arabidopsis*, and this the same E3 ligase also has a role in a broad range of processes such as cell growth and plant development through salicylic acid (Miura & Hasegawa, 2010), phosphate deficiency responses (Miura et al., 2005) and genetic regulation following drought stress (Catala et al., 2007). Early in Short Days 4 (ESD4), a SUMO protease contributes control of flowering time in *Arabidopsis* (Reeves et al., 2002; Murtas et al., 2003). Genetic studies of SUMO conjugation mutants has shown that SUMOylation of proteins, and with SUMO1 and SUMO2 in particular, is necessary for *Arabidopsis* viability. Indeed, where SUMO-activating and conjugating enzymes (SAE2 and SCE1) are lacking in null mutants, embryo lethality is observed (Saracco et al., 2007). Finally, increase in SUMOylation levels restrain abscisic acid (ABA)-governed inhibition of growth and enhance the induction of ABA- and stress-responsive genes like *RD29A* (Lois, Lima & Chua, 2003).

While ubiquitination and SUMOylation have many similarities, there are also some clear differences. Like ubiquitin, SUMO is existed in all eukaryotic kingdoms (Hanania et al., 1999) and well known, two systems have the same ancestry, which may date back to ancient biosynthetic pathways of prokaryotes (Hochstrasser, 2000). SUMO is highly conserved in all species from yeast to humans (Müller et al., 2001). Approximately 18% sequence identity is observed
in SUMO and ubiquitin even though they do share similar 3D structures with
the characteristic Ub-fold (Bayer et al., 1998) present in both. SUMO has
numerous paralogues in both humans and plants, with at least three in
mammalian cells (SUMO1, 2, 3) and only one in *Saccharomyces cerevisiae*
(Smt3) (Johnson, 2004; Kerscher, 2007). In humans, SUMO1 and SUMO2/3
show the separated function from each other, as they conjugate with diverse
substrates, and SUMO 2/3 have been represented to have a role in
SUMOylation in cases where proteins have become damaged, such as
environmental stress (Saitoh & Hinchev, 2000). So far 8 SUMO orthologues
have been identified in *Arabidopsis* (Kurepa et al., 2003; Colby et al., 2006),
however only four of these have been identified thus far as having post-
translational modification function, SUMO 1, 2, 3 and 5 (Colby et al., 2006;
Budhiraja et al., 2009). Studies have shown that AtSUMO1 and AtSUMO2 are
well conserved in sites on the SUMO protein governing E1-activating enzyme
recognition, and E2-conjugating enzyme and SIM (SUMO interacting-motif)
non-covalent interactions, where AtSUMO3 is less conserved, and AtSUMO5
identifies the highest degree of variation. In addition, AtSUMO1/2 are
considerate to be necessary paralogues, as they are the most efficiently
conjugated isoforms, with AtSUMO3 less efficiently conjugated, and
AtSUMO5 even less so (Castaño-Miquel, Seguí & Lois, 2011).

4.1. Consensus SUMO motif
A consensus motif for SUMOylation based on in target sequences was identified as ΨKXE, where Ψ was any large, hydrophobic amino acid, K was the target lysine, X was any amino acid, and E was a glutamic acid (Rodriguez et al., 2001). In vitro this consensus was demonstrated to enhance SUMOylation, however in vivo a nuclear localization signal was also needed for SUMOylation, suggesting that the combination of these two might direct a protein to be conjugated by SUMO (Rodriguez et al., 2001). In addition, the identification of plasma membrane proteins that are SUMOylated highly indicates that nuclear localization is not required for SUMOylation in all situations (Dai et al., 2009).

A second motif has been discovered in target proteins for SUMOylation named as Phosphorylation Dependent SUMO Modification (PDSM). The motif is set as ΨKXEXXSP in which the SUMO consensus sequence is distinguished by two amino acids from a phosphorylatable serine followed by a proline (Hietakangas et al., 2006). SUMOylation at the target lysine is blocked by serine to alanine mutations of this motif, while constitutive SUMOylation is occurred in serine to aspartic acid mutations, suggesting that phosphorylation of a PDSM directly affects the SUMOylation status of the protein (Hietakangas et al., 2006).

4.2. E3 Enzymes

Because only a single protein, UBC9, is chargeable for conjugating SUMO to all of the target proteins in a cell, E3 ligases are thought to be needed to obtain
target specificity in vivo. SUMO E3 ligases are identified by their ability to increase SUMOylation of a substrate protein in vitro, and are usually required for SUMOylation in vivo (Johnson and Gupta, 2001). The first E3 enzymes characterized for the SUMOylation pathway are homologous to Protein Inhibitor of Activated Stat (PIAS) proteins and contained a RING domain the same to known ubiquitin E3 ligases (Johnson and Gupta, 2001; Sachdev et al., 2001; Takahashi et al., 2001). SIZ proteins known as these E3 ligases in yeast and Arabidopsis (Takahashi et al., 2001; Miura et al., 2005). SUMO E3 ligases can be distinguished into two categories, the RING-type ligases and non-RING-type ligases. One such example of a non-RING domain E3 ligase is the polycomb protein Pc2, which plays to bind UBC9 and its substrate protein CtBP together in a subnuclear structure known as the PcG complex in order to increase SUMOylation (Kagey et al., 2003). In addition, the non-RING E3 ligase RanBP2 targets RanGAP1, the original SUMO target, for SUMOylation by and crystallographic data identified that these non-RING domain E3 ligases interact to a region of the UBC9 protein that is distinct from the binding domain for RING E3 ligases (Pichler et al., 2002; Tatham et al., 2005). Three different E3 ligases have been revealed to be capable of increasing in vitro SUMOylation of the protein Mdm2, including two PIAS (RING-type) E3 ligases and RanBP2 (a non-RING) E3 ligase (Miyauchi et al., 2002). This suggests that a single target can likely be played upon by multiple E3 ligases.

4.3. The SUMOylation pathway
Sumoylation is a reversible modification which covalently conjugates SUMO to a target protein followed by a cascade of enzyme reactions containing the steps of E1-activation, E2-conjugation, E3-ligation and deconjugation (Bayer et al., 1998). SUMO is matured through cleaving Gly-Gly motif in its C-terminus by the specific Ulps (ubiquitin-like proteases) and then the presence of ATP activates SUMO, heterodimeric E1 (SAE1, SAE2) catalyze the formation of cysteine (C) residue in SAE2 through high-energy thioester bond. In the next step, activated SUMO is moved to a cysteine residue of Conjugating enzyme E2 (SCE1) through transesterification played by SCE1. Subsequently, SUMO is transferred to the target through an isopeptide linkage between C-terminal glycine residue in SUMO and ε-amino group of the substrate lysine side chain in the target protein’s sumoylation consensus motif. This step is catalyzed by E3 ligase enzyme although E3-independent transfer is possible. SUMO specific proteases (ULPs) release the SUMO-substrate bonds to recycle free SUMO, as well as participated in generating mature SUMO (Figure 3).
Figure 3. SUMOylation pathway in *Arabidopsis thaliana*. Maturation, SUMO isoforms are encoded as precursor proteins and are processed by SUMO-specific cysteine proteases (ULP, ubiquitin-like protein-specific protease) with SUMO peptidase activity. Maturation consists of carboxyl terminal truncation to expose the di-glycine (GG) motif; Activation by E1, the SUMO carboxyl-terminal glycine is linked to AMP (SUMO-AMP) catalyzed by the heterodimeric E1 SUMO-activating enzymes 1 and 2 (SAE1 and SAE2) in an ATP-dependent reaction. Subsequently, the glycine of SUMO is coupled to a cysteine (C) residue in SAE2 via a high-energy thioester bond; Conjugation by E2, SUMO is transferred to a cysteine residue of the E2 SUMO-conjugating enzyme (SCE1) by transesterification catalyzed by SCE1; Ligation by E3,
SUMO is transferred to the ε-amino group of a lysine (K) side chain in the sumoylation consensus motif (ψKXE/D. ψ, a large hydrophobic residue; X, any amino acid; E/D, glutamic acid or aspartic acid) of the substrate protein, forming an isopeptide bond between the carboxyl of glycine in SUMO and the ε-amino of lysine in the substrate, a process that requires the E3 SUMO ligase in vivo; Deconjugation, SUMO-specific proteases (ULP) with isopeptidase activity cleave the isopeptide bond and SUMO is recycled through the conjugation system.
4.4. Components of the SUMO pathway in Arabidopsis

*Arabidopsis* genome analyses characterized the genes encoding eight SUMOs (AtSUMO1-AtSUMO8) and one SUMO-like pseudogene (AtSUMO9), but expression of only four paralogues (SUMO1/2, SUMO3 and 5) has been confirmed (Kurepa et al., 2003). The SUMO E1 enzyme is a heterodimer making of two small subunit (SAE1a, SAE1b) isoforms and one large subunit SAE2. SAE1 isoforms, SAE1a and SAE1b share 80% identical amino acid sequence, pronouncing functional redundancy (Saracco et al., 2007). The SUMO E2 enzyme encoded by single copy of gene although, many Ubiquitin E2 enzymes are encoded. Transcripts of SAE1a/b and SCE1 are abundantly expressed in many tissue including seedling, cotyledon, root, stem, shoot tip, leaf, flower and siliques. In addition, SCE1 and SUMO are colocalized in nucleus demonstrating that sumoylation reactions conduct in nucleus (Lois et al., 2010). Null T-DNA insertion mutants of SAE2, SCE or both SUMO1 and SUMO2 represented embryonic lethal, with restrained in early-stage embryos (globular, heart, torpedo), indicating that sumoylation is necessary in *Arabidopsis* (Saracco et al., 2007). Although there are many E3 ligases in animal, fungi and yeast (Johnson and Gupta 2001, Kahyo et al., 2001, Rose and Meier 2001, Pichler et al., 2002, Kagey et al., 2003), only two SUMO ligases [AtSIZ1 (PIAS SUMOE3 ligase) and AtMMS21/HPY2 (Mms21 SUMO E3 ligase)] are identified in *Arabidopsis*. Loss of function mutants of SIZ1 and HPY2 demonstrated pleiotropic phenotype indicating the importance of E3
ligases in sumoylation pathway (Miura et al., 2005, Catala et al., 2007, Jin et al., 2008, Huang et al., 2009, Ishida et al., 2009, Miura et al., 2010). Four of Arabidopsis SUMO specific proteases (AtULP1a, AtULP1c, AtULP1d and AtESD4) are identified so far (Murtas et al., 2003, Colby et al., 2006, Conti et al., 2008). These proteases not only have peptidase activity for cleavage of pre-SUMO at the C-terminus to release the Gly-Gly motif, but also isopeptidase activity for deconjugation of sumoylated protein to recycle pre-SUMO (Hay et al., 2007). However, these proteases have a difference of SUMO isoform discrimination and enzymatic activities (Table 1, Chosed et al., 2006, Colby et al., 2006).
<table>
<thead>
<tr>
<th>Arabidopsis</th>
<th>ATG number</th>
<th>Location</th>
<th>Description</th>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Nucleus</td>
<td>Single mutants look like wild type; double mutant is embryo lethal.</td>
</tr>
<tr>
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</tr>
<tr>
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<td></td>
<td>sae2 is embryo lethal</td>
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<tr>
<td><strong>E3 ligases</strong></td>
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</tr>
<tr>
<td>SIZ1</td>
<td>At5g60410</td>
<td>Nucleus</td>
<td>Dwarfism, elevated SA level with increased PR1 expression and resistance to bacterial pathogens, early flowering in short day, hypersensitivity to cold and phosphorus-limited condition.</td>
</tr>
<tr>
<td>MMS21/HPY2</td>
<td>At3g15150</td>
<td>Nucleus</td>
<td>Severe dwarf with short roots (fewer and shorter cells in the mature and elongation zones), premature transition from mitotic cycle to endocycle.</td>
</tr>
</tbody>
</table>

Table 1. Arabidopsis proteins of the SUMO pathway
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<th>Compartement</th>
<th>Function</th>
</tr>
</thead>
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<td>Cytosolic</td>
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</tr>
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<td>At1g10570</td>
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<td>ULP1d/OTS1</td>
<td>At1g60220</td>
<td></td>
<td>Single mutants look like wild type; double mutant is hypersensitive to salt and plants flower early.</td>
</tr>
<tr>
<td>ESD4</td>
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<td>Nucleus</td>
<td>Early flowering in short day.</td>
</tr>
<tr>
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<td>At4g33620</td>
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<td>At1g09730</td>
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</table>
5. Role of the SUMO in plants

5.1. Responses to abiotic stress

Sumoylation acts essential role in various abiotic stress responses. The accumulation of SUMO conjugates is induced by heat, cold, drought, salt, exposure to excessive copper, incubation with H$_2$O$_2$ or ethanol, suggesting that sumoylation involves in stress protection and recovery (Figure 4, Kurepa et al., 2003, Miura et al., 2005, Yoo et al., 2006, Catala et al., 2007, Saracco et al., 2007, Conti et al., 2008, Chen et al., 2011). Loss of function SIZ1 mutants represented the phenotypes of short primary roots, increased number of lateral roots, root hairs and higher anthocyanin accumulation sensitively in response to pi starvation. The expression of low-Pi transcripts of AtIPS1 and AtRNS1 is decreased in siz1-2 mutants. AtSIZ1 protein sumoylates the PHR1 (phosphate starvation response 1) which is a transcriptional regulator of AtIPS1 and AtRNS1 in vitro, implying that AtIPS1 and AtRNS1 are positively regulated through sumoylation of PHR1 by AtSIZ1 (Miura et al., 2005). SUMO conjugation is increased under drought stress. The expression of P5CS1, MYC2, COR15A and KIN1 genes induced by drought stress is decreased in siz1-3 mutants. It is suggested that SIZ1 acts a crucial role in drought stress response through the control of the genes expression (Catala et al., 2007). The siz1-3, SIZ1 null mutant, is sensitive to cold stress, and the cold-induced accumulation of sumo conjugates is decreased in the mutant, suggesting that SIZ1 acts an essential role in cold tolerance. The expression of cold-induced genes such as
COR15A, COR47 and KIN1 is controlled by transcription factor ICE1 (inducer of CBF/DREB1 expression 1). ICE1 is sumoylated by AtSIZ1 and its modification inhibited the expression of MYB15, a negative regulator of CBF3/DREB1A. This leads to the expression of CBF3/DREB1A and its downstream genes, resulted in cold tolerance. SIZ1-dependent sumoylation of ICE1 blocks its polyubiquitination and lead to increase ICE1 stability (Dong et al., 2006, Miura et al., 2007, Ulrich et al., 2008). Sumoylation of AtHsfA2, heat shock transcription factor, represses its activity and transcription of Psfl01, Hsf17.6 and Hsf17.4, HsfA2 target genes. Moreover, overexpression of SUMO in seedlings reduces tolerance on repeated heat treatment, suggesting that sumoylation might play negatively upon acquired thermo tolerance (Cohen-Peer et al., 2010). Conversely, SIZ1 is a positive regulator of Salicylic acid (SA)-independent basal thermo tolerance but not of acquired tolerance. It indicates that the SIZ1-independent pathway involves in the acquired thermo tolerance regulatory pathway (Yoo et al., 2006). Previous reports have been implied that sumoylation is involved to salt stress response in Arabidopsis. OTS1/OTS2, salt stress-related genes, double mutants represented enhanced salt sensitivity but not the single ots1 or ots2 mutant. Sumo conjugation is increased much higher in ost1ost2 double mutants compared with wild-type plants. OTS1-overexpressing transgenic plants increase salt tolerance and reduce sumoylation, but mutant overexpressing OST1 (C536S) protein disappearing sumo protease activity failed to produce a salt tolerant phenotype. These results indicate that
OST1 and OST2 conduct as sumo deconjugation and required for salt tolerance responses (Conti et al., 2008). SIZ1-mediated sumoylation also participates in copper homeostasis and tolerance. *Atsiz1* mutants represented hyposensitivity to excessive copper and accumulated more copper in shoot tissue than wild type. Excessive copper increased more sumo conjugates in wild type than in *siz1* mutant, demonstrating that sumoylation might be involved in the response on excessive copper. The expression of metal transporter genes of *YELLOW STRIPE-LIKE 1 (YSL1)* and *YSL3* is repressed under excess copper stress in the *siz1* mutant. The hyposensitivity to excess copper and abnormal distribution of copper are decreased greatly in *siz1ysl3-1* and slightly in the *siz1ysl1* double mutants. These results indicating that Cu-induced SIZ1 dependent sumoylation participated in copper distribution and tolerance through transcriptional repression of *YSL1/3* (Chen et al., 2011).

5.2. Responses to hormone signaling

Sumoylation involved in modulation of ABA responses (Figure 4). Overexpression of AtSUMO1/2 inhibits ABA-mediated root growth inhibition while co-suppression of AtSCE1a displays no significant differences. In addition, AtSUMO1- and AtSUMO2-overexpressing plants increase the expression of stress-responsive genes, *RDA29A* and *AtPLC1*, indicating that SUMO acts a role for ABA response (Lois et al., 2003). *Atsiz1* mutants show the ABA hyposensitivity phenotype that germination and seedling primary root
growth are restrained. ABI5 (ABA intensive 5), ABA-responsive transcription factor, is sumoylated on Lys-391 by AtSIZ1. Expression of ABI5 (K391/R) in abi5-4 resulted in greater sensitivity to ABA compared with ABI5 expression suggesting that SIZ1-mediated sumoylation on ABI5 negatively controls ABA signaling (Miura et al., 2009). Auxin signaling is also controlled by sumoylation. The SIZ1 mutation caused the inhibition of primary root (PR) elongation and the promotion of lateral root (LR) formation. However, similar root phenotypes appear if Pi deficient wild-type seedlings are supplemented with auxin. N-1-naphthylphthalamic acid (NPA), an auxin efflux inhibitor, reduces the Pi starvation-induced lateral root formation in wild-type and siz1 seedling. Pi starvation-induced Auxin accumulation in the PRs and LRs is faster in siz1 than in wild-type seedlings. Moreover, expression of auxin-induced genes is higher in siz1 compared to wild-type in response to Pi starvation. These results suggesting that SIZ1 negatively controls Pi starvation-induced root architecture remodeling through the regulation of auxin patterning (Miura et al., 2005, 2011). Loss of SIZ1 function leads to accumulation of Salicylic acid (SA) and constitutive Pathogenesis-related (PR) genes expression, resulting in increased disease resistance to bacterial pathogens (Lee et al., 2007). These results indicating SIZ1 regulates SA-mediated plant defense signaling.
Figure 4. The roles of SUMOylation in *Arabidopsis thaliana*. In *Arabidopsis thaliana*, SUMOylation conducts several behaviors involved in hormone signaling and abiotic responses.
INTRODUCTION

Plant hormones control essential processes of plant development, including germination, elongation growth, flowering time, floral development and senescence (Vanstraelen and Benkove, 2012). GA is an important phytohormone, regulating plant growth at various developmental stages. Mutants deficient in GA biosynthesis or signaling are non-germinating or poorly germinating, display dwarfism and are delayed in flowering or fail to flower (Daviere and Achard, 2013).

GA signaling is regulated by the ubiquitin-mediated proteolysis pathway. Target proteins are modified with ubiquitin, a process catalyzed by single polypeptide or SCF (Skp, Cullin, F-box) complex E3 ubiquitin ligases; polyubiquitinated proteins are degraded by the 26S proteosome complex. SCF complexes control multiple signal transduction cascades and developmental processes (Hua and Viestra, 2011). The F-box protein of the SCF complex plays a role in selecting specific targets for 26S proteosomal proteolysis by polyubiquitination (Zheng et al., 2002). In plants, SCF complexes also regulate a large number of regulatory processes, including GA and auxin signaling (Gray et al., 2001; Richards et al., 2001; McGinnis et al., 2003).

DELLA proteins are a subfamily of GRAS [GAI (GA INSENSITIVE), RGA (REPRESSOR OF gaI-3), and SCARECROW] proteins, which act as putative nuclear-localized transcription factors (Pysh et al., 1999). They contain a highly
conserved DELLA motif in the N-terminal that acts as a transcriptional regulator (Silverstone et al., 1998; Peng et al., 1999; Itoh et al., 2005). The Arabidopsis DELLA proteins GAI, RGA, and three RGA-LIKE proteins (RGL1, RGL2, and RGL3) negatively regulate GA signaling. DELLA proteins directly interact with SLY1 and disappear rapidly from the nucleus after ubiquitination, probably due to proteolytic degradation by the 26S proteosome complex.

The Arabidopsis SLY1 gene encodes the F-box subunit that determines the substrate specificity of the SCF complex (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004). SLY1 has three main domains (F-box, GGF, and LSL) that act as a bridge between SKP and DELLA proteins. Numerous studies demonstrate that SLY1 positively regulates growth by GA signaling; for example, sly1 mutants show increased seed dormancy and increased sensitivity to inhibition of seed germination by abscisic acid (ABA) (Strader et al., 2004).

In addition, sly1 plants are dwarfs, a phenotype that may be rescued by the absence of DELLA repressors (Olszewski et al., 2002; Dill et al., 2004; Fu et al., 2004). Moreover, average leaf cell number is decreased in sly1-10 plants and increased in the quadruple-DELLA mutant gai-t6 rga-t2 rgl1-1 rgl2-1. The cell division rate is also lower in sly1-10 and higher in quadruple-DELLA mutants than in wild-type plants (Achard et al., 2009). These observations match earlier data showing that SLY1 promotes growth by stimulating destruction of growth-repressing DELLA proteins (Peng et al., 1997; Silverstone et al., 1998; Silverstone et al., 2001; Olszewski et al., 2002). All of these results indicate that
SCF<sup>SLY1</sup> positively regulates plant growth from germination to flowering by GA signaling through the destruction of DELLA proteins.

SMALL UBIQUITIN-RELATED MODIFIER (SUMO) is a small peptide that is covalently attached to lysine residues of target proteins by E3 SUMO ligase via a reversible post-translational modification (Wilkinson et al., 2010). As a protein modifier, SUMO controls various eukaryotic cellular processes such as stress and defense responses, nitrogen metabolism, growth and the regulation of flowering (Hotson et al., 2003; Kurepa et al., 2003; Lois et al., 2003; Murtas et al., 2003; Miura et al., 2007; Catala et al., 2007; Lee et al., 2006, Conti et al., 2008; Ishida et al., 2009, 2012; Zhang et al., 2013; Conti et al., 2014). Modification of target proteins with SUMO is usually catalyzed by E3 SUMO ligases, although conjugation of SUMO to target proteins can occur in the absence of such ligases (Wilkinson et al., 2010). So far, numerous SUMO conjugates have been identified, such as the nitrate reductases NIA1 and NIA2, INDUCER OF CBF EXPRESSION 1 (ICE1), an R2R3-type transcription factor MYB30 and FLOWERING LOCUS C (FLC) (Miller et al., 2010; Elrouby and Coupland, 2010; Miura and Hasegawa, 2010; Park et al., 2011; Zheng et al., 2012; Son et al., 2014). Very recently, it was reported that DELLA RGA is also modified by SUMO, although the E3 SUMO ligase involved was not identified (Conti et al., 2014).

AtSIZ1 regulates plant responses to nutrient deficiency and environmental stress, and controls vegetative growth and development (Miura et al., 2007,
The \textit{siz1-2} mutant shows the dwarf phenotype common in plants defective in GA or in GA signaling (Catala et al., 2007) and the \textit{sly1} mutant also displays severe dark green dwarfism (McGinnis et al., 2003). On the basis of these data, I speculated that GA signaling may be impaired in the \textit{siz1-2} mutant and sumoylation might affect SLY1 activity.

Here, I show that SLY1 sumoylation by AtSIZ1 stabilizes and activates SLY1 and stimulates RGA degradation, thereby promoting SLY1-mediated plant development. My results provide the first evidence in any organism that F-box protein function can be regulated by sumoylation through the activity of E3 SUMO ligase.
MATERIALS AND METHODS

Plant materials and growth conditions
The *Arabidopsis thaliana* Columbia-0 ecotype (wild type; WT) and the T-DNA insertion knock-out mutants *siz1*-2, *sly1*-13 and *esd4* were used in this study. The *sly1*-13 mutant has 12kb of T-DNA inserted 300 bases from the 3’ end of At4g24210. This mutant was obtained from ABRC (Salk_097559) and was designated *sly1*-13 to distinguish it from other mutant alleles of *sly1*.

For plants grown on plates, seeds were surface-sterilized in commercial bleach containing 5% sodium hypochlorite and 0.1% Triton X-100 solution for 10 min, rinsed five times in sterilized water, and stratified at 4°C for 2 d in the dark. Seeds were sown on agar plates containing Murashige and Skoog (MS) medium, 2% sucrose, and 0.8% agar, buffered to pH 5.7. For plants grown in soil, seeds were directly sown in sterile vermiculite. All plants, including seedlings, were grown at 22°C under a 16 hr light/8 hr dark cycle in a growth chamber.

Effect of GA on Seed Germination and Seedling Growth
To investigate the effect of ammonium ion on germination and seedling growth, wild-type and *siz1*-2 mutant seeds were germinated on MS agar media. To assess the effects of GA on germination and vegetative growth, wild-type and *siz1*-2 mutant seeds were planted on MS agar media with or without 10 µM GA₃. One hundred seeds of each (wild-type and *siz1*-2 mutant) were examined.
Plants were grown at 22°C under a 16-h light/8-h dark cycle in a growth chamber. The experiment was repeated three times.

**Construction of recombinant plasmids**

To produce His$_6$-SLY1 and glutathione S-transferase (GST)-SLY1, the cDNA sequence encoding full-length SLY1 was amplified by PCR with gene-specific primers and inserted into the pET28a (Novagen) and pGEX4T-1 (Amersham Biosciences) vectors. For maltose binding protein (MBP)-AtSIZ1, a cDNA sequence encoding full-length AtSIZ1 was amplified by PCR with gene-specific primers and inserted into the pMALc2 vector (New England Biolabs). To produce GST-SLY1-HA, the cDNA sequence encoding full-length SLY1 was amplified by PCR with a gene-specific primer and hemagglutinin (HA) primer and inserted into the pGEX4T-1 vector. To produce the SLY1 mutant protein GST-mSLY1 (K122R; the numbers indicate the position of the lysine in SLY1 that was mutated to arginine), GST-SLY1 was subjected to site-directed mutagenesis using overlapping primers. The Arabidopsis SUMO1 full-length cDNA sequence was amplified by PCR with gene-specific primers and inserted into pET28a to produce the His$_6$-AtSUMO1-GG, containing full-length AtSUMO1 extended with GG at the 3’end.

All constructs were transformed into *Escherichia coli* BL21/DE3 (pLysS) cells. The transformed cells were treated with isopropyl-D-thiogalactoside (IPTG) to induce fusion protein expression. The sequences of the primers used in this
study are listed in Table 2. All constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced.
Table 2. List of primers used in this study.

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Purification of recombinant proteins

All recombinant proteins were expressed in *E. coli* strain BL21 and purified according to the manufacturer’s instructions. Briefly, for purification of His$_6$-AtSAE1b, His$_6$-AtSAE2, His$_6$-AtSCE1, His$_6$-AtSUMO1-GG, and His$_6$-SLY1, bacteria were lysed in 50 mM NaH$_2$PO$_4$ (pH 8.0), 300 mM NaCl, 1% Triton X-100, 1 mM imidazole, 5 mM Dithiothreitol (DTT), 2 mM phenyl methanesulfonyl fluoride (PMSF), and proteinase inhibitor cocktail (Roche), and purified on nickel-nitrilotriacetate (Ni$^{2+}$-NTA) resins (Qiagen).

For GST-SLY1, GST-mSLY1 and GST-SLY1-HA purification, bacteria were lysed in PBS buffer (pH 7.5) containing 1% Triton X-100, 2 mM PMSF, and proteinase inhibitor cocktail (Roche), and purified on glutathione resins (Pharmacia).

For MBP and MBP-AtSIZ1 purification, bacteria were lysed in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and 2 mM PMSF containing proteinase inhibitor cocktail (Roche), and purified on amylose resins (New England BioLabs). Protein concentrations were determined by the Bradford assay (Bio-Rad).

In vitro binding assay

To examine in vitro binding of MBP-AtSIZ1 to His$_6$-SLY1, 2 µg of full-length MBP-AtSIZ1 and 2 µg of full-length His$_6$-SLY1 were added to 1 ml of binding buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.2%
glycerol, 0.5 mM beta-mercaptoethanol). After incubation at 25°C for 2 hr, the reaction mixtures were incubated with an amylose resin for 2 hr before washing six times with buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Triton X-100).

Absorbed proteins were analyzed using 11% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and detected by western blotting with anti-His antibody (0.4 μg ml⁻¹; Santa Cruz Biotechnology).

For in vitro binding analysis of MBP-AtSIZ1 to GST-SLY1 or GST-GID1A, 2 µg of full-length MBP-AtSIZ1 and 2 µg of full-length His₆-SLY1 or GST-GID1A were added to 1 ml of binding buffer, as described above. After incubation at 25°C for 2 hr, reaction mixtures were incubated with glutathione resin for 1 hr before washing six times with the same buffer. Absorbed proteins were analyzed using 11% SDS-PAGE and detected by western blotting with anti-GST antibody (0.4 μg ml⁻¹; Santa Cruz Biotechnology).

Subcellular Localization of Proteins

The YFP and CFP coding sequences were fused in-frame to the 5’-end of AtSIZ1 and 3’-end of SLY1 using pEarlyGate 104 and pEarlyGate 102, respectively. Both fusion genes were expressed from the 35S promoter. The epidermis of the inner surface of onion scales was bombarded with three micrograms of each plasmid using a biolistic particle gun (Bio-Rad). Transient
expression in bombarded tissues was visualized using confocal microscopy after 14 h incubation in darkness.

**Yeast two-hybrid assays**

Yeast two-hybrid assays were performed using the GAL4-based two-hybrid system (Clontech). Full-length AtSIZ1, RGA, SLY1, mSLY1, SLY2, ASK1, ASK2, ASK3, ASK4, ASK11, ASK13 and GID1A cDNA sequences were cloned into pGAD424 containing an activating domain (AD) and pGBT8 containing a binding domain (BD) (Clontech) to generate the constructs AD-AtSIZ1, AD-RGA, AD-ASK1, AD-ASK2, AD-ASK3, AD-ASK4, AD-ASK11, AD-ASK13, BD-SLY1, BD-mSLY1, BD-SLY2 and BD-GID1A.

All constructs were transformed into yeast strain AH109 using the lithium acetate method. Yeast cells were grown on minimal medium (−Leu/−Trp). Transformants were plated onto minimal medium (−Leu/−Trp/−His/10 mM 3-AT (3-amino-1, 2, 4-triazole)) to test the interactions between AtSIZ1 and SLY1 (or mSLY1), AtSIZ1 and SLY2, and ASK proteins and SLY1 (or mSLY1).

**Sumoylation assays**

*In vitro* sumoylation assays were performed in 30 µl of reaction buffer (20 mM Hepes (pH 7.5), 5 mM MgCl2, 2 mM ATP) with 50 ng His6-AtSAE1b, 50 ng His6-AtSAE2, 50 ng His6-AtSCE1, 8 µg His6-AtSUMO1-GG and 100 ng GST-SLY (or GST-mSLY1), with or without 500 ng MBP-AtSIZ1. After incubation
for 3 hr at 30°C, the reaction mixtures were separated by 11% SDS-PAGE. Sumoylated GST-SLY1 was detected by western blotting with anti-GST antibody. To identify the sumoylation site in SLY1, GST-mSLY1 was added to the reaction mixtures instead of GST-SLY1. The reactions and subsequent steps were performed as described above.

For in vivo sumoylation assays, siz1-2 plants over-expressing HA3-AtSUMO1-GG were produced by transforming mutant plants with 35S-HA3-AtSUMO1-GG. Following this initial plant transformation, Agrobacterium transformed with 35S-SLY1-FLAG3 and 35S-Myc6-AtSIZ1 was infiltrated into siz1-2 plants over-expressing HA3-AtSUMO1-GG. After 3 d, the total proteins were extracted from each sample and immunoprecipitated with anti-HA antibody (1 μg ml⁻¹, Santa Cruz Biotechnology) in a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail (Promega). Sumoylated SLY1 was detected by western blotting with anti-FLAG antibody (0.5 μg ml⁻¹, Sigma-Aldrich) after immunoprecipitation.

To investigate the effect of GA on sumoylation of SLY1 in plant, transgenic plants over-expressing His6-strep-AtSUMO1-GG were first produced by transforming wild type Arabidopsis with 35S-His6-Strep-AtSUMO1-GG. Next, the transgenic plants were infiltrated with Agrobacterium transformed with 35S-SLY1-FLAG3 and 35S-HA3-AtSIZ1. After 2 d, the infiltrated plants were treated with 100 μM GA and then further incubated for 3 hr. Total proteins were
extracted from each sample and purified on Ni\textsuperscript{2+}-NTA resins in buffer containing 10 mM Tris (pH 8.0), 100 mM Na\textsubscript{2}HPO\textsubscript{4}, 1% triton X-100, 8M urea, 10 mM imidazole and protease inhibitor cocktail. Purified proteins were separated by 10% SDS-PAGE and sumoylated SLY1 was detected by western blotting with anti-FLAG antibody.

**Production of transgenic Arabidopsis plants and complementation tests**

To produce plants over-expressing SLY1 or mSLY1 (K122R), the corresponding full-length cDNA sequences were amplified by PCR using a forward primer and a reverse primer tagged with FLAG\textsubscript{3} and inserted into the plant expression vector pBA002. Recombinant plasmids, \textit{35S-SLY1-FLAG\textsubscript{3}} and \textit{35S-mSLY1-FLAG\textsubscript{3}}, were introduced into wild-type Arabidopsis by floral dipping, and transformants were selected on MS media containing BASTA (50 μgml\textsuperscript{-1}).

To introduce \textit{35S-SLY1-FLAG\textsubscript{3}} and \textit{35S-mSLY1-FLAG\textsubscript{3}} into \textit{sly1-13} mutants, transgenic plants were crossed with \textit{sly1-13} mutants and BASTA-resistant offspring were selected. T3 homozygote lines were used for complementation analysis. To examine SLY1-FLAG\textsubscript{3} or mSLY1-FLAG\textsubscript{3} expression, total proteins were extracted from leaves of wild-type plants, \textit{sly1-13} mutants and \textit{sly1-13} mutants over-expressing SLY1-FLAG\textsubscript{3} or mSLY1-FLAG\textsubscript{3}. SLY1-FLAG\textsubscript{3} or mSLY1-FLAG\textsubscript{3} levels were estimated by western blotting with anti-FLAG antibody.
Examination of SLY1 and RGA levels in siz1-2 and sly1-13 mutants

To examine relative levels of SLY1 in wild-type and siz1-2 plants, total proteins were extracted from the leaves of wild-type and siz1-2 plants grown for 15 d on MS media. Following 11% SDS-PAGE, levels of SLY1 were examined by western blotting with anti-SLY1 antibody. The level of SLY1 was also examined in cycloheximide-treated samples. Wild-type and siz1-2 plants grown on MS media for 15 d were treated with 100 μM cycloheximide, and the samples were collected at the indicated time points. Total proteins were extracted from each sample, and the SLY1 was detected by western blotting with anti-SLY1 antibody.

To determine levels of RGA, total proteins were extracted from the leaves of wild-type, sly1-13, siz1-2, 35S-SLY1-FLAGs/sly1-13, and 35S-mSLY1-FLAGs/sly1-13 plants grown for 15 d on MS media. Following 11% SDS-PAGE, the levels of the RGA were examined by western blotting with anti-RGA antibody.

Examination of the effect of AtSIZ1 activity and SLY1 sumoylation on RGA level

To investigate whether SLY1 sumoylation affected RGA stability in Arabidopsis, wild-type plants were infiltrated with different combinations of Agrobacterium transformed with 35S-SLY1-FLAG3, 35S-mSLY1-FLAG3 and 35S-RGA-Myc6 constructs. After 2 d, the total proteins were extracted from each
sample, and SLY1-FLAG$_3$, SLY1-mFLAG$_3$ and RGA-Myc$_6$ were detected by western blotting with anti-FLAG and anti-Myc antibodies, respectively.

To check the effect of E3 ligase activity of AtSIZ1 on RGA level through SLY1 sumoylation, wild-type and siz1-2 plants were infiltrated with different combinations of Agrobacterium transformed with 35S-HA$_3$-AtSIZ1, 35S-SLY1-FLAG$_3$, 35S-mSLY1-FLAG$_3$ and 35S-RGA-Myc$_6$. After 2 d, total proteins were extracted from each sample and separated by 11% SDS-PAGE. HA$_3$-AtSIZ1, SLY1-FLAG$_3$, SLY1-mFLAG$_3$ and RGA-Myc$_6$ were detected by western blotting with anti-HA, anti-FLAG and anti-Myc antibodies, as appropriate.

**Interaction analysis between sumoylated SLY1 and RGA by in vitro pull-down assay**

To check the effect of SUMO conjugation on the interaction between SLY1 and RGA, an *in vitro* sumoylation assay was performed as described above, and sumoylated SLY1 was then detected by western blotting with anti-GST antibody. The reaction products were mixed with purified MBP or MBP-RGA and pulled down with amylose resin. After elution, GST-SLY1 and sumoylated GST-SLY1 were detected by western blotting with anti-GST antibody.

**Examination of the effect of SUMO protease on SLY1 level**

To check the effect of SUMO protease on SLY1 stability, wild-type and *esd4* plants were grown on MS media for 15 d. After harvesting the samples, total
proteins were extracted with 2x protein loading buffer. Proteins were separated by 11% SDS-PAGE and SLY1 level was examined by western blotting with anti-SLY1 antibody.

**Measurement of transcript levels of GA-responsive genes including SLY1**

Wild-type plants and *siz1*-2 mutants were grown on plates containing MS medium for 15 d. Total RNA was extracted from the leaves of wild-type and *siz1*-2 plants, quantified and divided into equal amounts. First-strand cDNA was synthesized from 5 μg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). An equal volume of cDNA was amplified by real-time qRT-PCR (MyiQ, Bio-Rad), according to the manufacturer’s protocol.

The specific primers and template cDNA were combined with 25 μl of iQ SYBR Green Super Mix (Bio-Rad), and the reactions were performed under the following thermal conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. The C_T values obtained for target genes were normalized to the C_T value for tubulin, and the data were analyzed using iCycler IQ software (Bio-Rad). PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi), and their specificity was verified by cloning into the pGEM T-Easy vector (Promega) and sequencing using an ABI 3730xl DNA Analyzer (Applied Biosystems). The primer sets used in these studies are listed in Table 2.
Examination of *AtSIZ1* transcript and protein levels

Wild-type and *sly1-13* mutant plants were grown on plates containing MS medium for 15 d and treated with 100 μM GA₃ for 12 hr. Samples were harvested at the indicated time points, and total RNA and protein were isolated from each sample. *AtSIZ1* transcript levels were determined by real time qRT-PCR with gene-specific primers, as described above. The primer sets used in these studies are listed in Table 2.

To evaluate relative *AtSIZ1* protein levels in wild-type and *siz1-2* mutant plants, total proteins were separated by 11% SDS-PAGE and the level of *AtSIZ1* was determined by western blotting with anti-*AtSIZ1* antibody.

Hypocotyl length measurements

Seeds of wild-type, *siz1-2* and *sly1-13* plants were germinated in Petri dishes containing MS medium and grown for 7 d under long day conditions. Seedlings were harvested from each Petridish, and scanned at 600 dots per inch. Hypocotyl lengths were measured from the resulting images using Image J software. Values of hypocotyl length are the means ± SD of three independent experiments, each containing at least 20 seedlings.

Antibody production

To produce full-length or deletion mutant proteins, SLY1 and RGA cDNA sequences, encoding either the full-length or truncated gene, were amplified
using PCR with gene-specific primers and inserted into pET28a to produce His$_6$-SLY1 and His$_6$-RGA-C. His$_6$-RGA-C contained amino acids 418–587 of RGA. After transformation of *E. coli* BL21/DE3 (pLysS) cells with recombinant plasmids, the cells were treated with IPTG to induce recombinant protein production. The recombinant His$_6$-SLY1 and His$_6$-RGA-C proteins were purified using a Ni$^{2+}$-NTA affinity column, and the protein concentrations were determined. Anti-SLY1 and anti-RGA antibodies were produced by subcutaneous injection of proteins into rabbits, and used for the analysis of SLY1 and RGA levels.
RESULTS

Seed germination of the siz1-2 mutant is recovered by exogenous GA

Arabidopsis siz1-2 mutants display dwarfism, early flowering, and abnormal seed development. A recent study showed that the phenotypes of the siz1-2 mutants were recovered to wild-type phenotypes by exogenous ammonium treatment (Park et al., 2011). My further investigation revealed that siz1-2 mutants germinated unevenly and their germination ratio was relatively low compared to that of wild-type plants (Figure 5). GA positively regulates seed germination. Therefore, I investigated whether treatment with exogenous GA could recover germination and vegetative growth of the siz1-2 mutants. The results showed that germination of siz1-2 mutants was completely recovered by treatment with 10 μM GA₃ (Figure 5).
**Figure 5.** AtSIZ1 positively regulates seed germination. (A) Germination of wild-type plants and siz1-2 mutants was tested on MS media containing 10 uM GA3 for short time as indicated. Seed dormancy of siz1-2 mutants was totally overcome by treatment with GA although seed dormancy increased in siz1-2 mutant. (B) Some of the seeds shown in (A) were chosen and photographed. (C) Wild-type plants and siz1-2 mutants were also germinated on MS media
containing 10 μM GA₃ for 10 days. Vegetative growth of siz1-2 mutants was recovered to wild-type by treatment with GA₃. (D) 23% of the seeds of siz1-2 mutants germinated. Black bar, wild-type plants; white bar, siz1-2 mutants.
SLY1 is sumoylated by E3 SUMO ligase AtSIZ1

Abnormal GA signaling by the loss of SLY1 function results in defective growth, resulting in phenotypes such as severe dwarfism and low fertility (McGinnis et al., 2003). Arabidopsis siz1-2 mutants were reported to also display dwarfism and abnormal seed development, thus GA signaling may be disrupted in the siz1-2 mutant (Catala et al., 2007) due to reduced SLY1 activity or stability. To test this, I investigated whether there is an interaction between AtSIZ1 and SLY1.

Two recombinant plasmids were constructed: maltose binding protein (MBP)-tagged AtSIZ1 and histidine-tagged SLY1. Over-expression of tagged proteins in Escherichia coli was induced by IPTG treatment and proteins were purified with amylose and nickel resins, respectively (Figure 6A). After purification, I conducted in vitro pull-down assays for His6-SLY1 using MBP or MBP-AtSIZ1. As expected, I observed a strong interaction between AtSIZ1 and SLY1 (Figure 6A). A yeast two-hybrid assay was performed to confirm this interaction. The full-length cDNAs of AtSIZ1 and SLY1 were cloned into yeast expression vectors. After introducing the constructs into yeast strain AH109, I examined the protein interactions. Consistent with the results of the pull-down assay, I found that AtSIZ1 strongly interacted with SLY1 (Figure 6B).

This strong interaction between AtSIZ1 and SLY1 suggested they might co-localize in cells. I therefore examined their subcellular localization, transiently expressing SLY1 tagged with cyan fluorescent protein (CFP) and AtSIZ1 tagged
with yellow fluorescent protein (YFP) in onion epidermal cells, a model system for the processes in Arabidopsis cells. I observed localization of both proteins in the nucleus, whether expressed separately or together (Figure 7).

The direct interaction between SLY1 and AtSIZ1 suggested that AtSIZ1 may act as an E3 SUMO ligase for SLY1. To determine whether this notion is correct, I produced the recombinant proteins MBP-AtSIZ1 and GST-SLY1 (Figure 8A). *In vitro* sumoylation assays revealed that GST-SLY1 was sumoylated by AtSIZ1, and that sumoylation was dependent on E1 and E2 activities (Figure 8B).

To test whether AtSIZ1 could function as an E3 SUMO ligase for SLY1 in plants, 35S-Myc6-AtSIZ1 and 35S-SLY1-FLAG3 constructs were co-infiltrated into the leaves of siz1-2 mutants over-expressing HA3-AtSUMO1-GG. Expression of the recombinant proteins, HA3-AtSUMO1-GG, SLY1-FLAG3 and Myc6-AtSIZ1, was detected by western blotting using anti-HA, anti-FLAG and anti-Myc antibodies, respectively (Figure 9A-C). Sumoylation of SLY1 was examined by western blotting with anti-FLAG antibody following immunoprecipitation with anti-HA antibody. Notably, sumoylated SLY1 was detected only in siz1-2 mutants that had been infiltrated with 35S-Myc6-AtSIZ1 (Figure 9D). No sumoylated SLY1 was observed in mutants that had not been infiltrated.

Next, I tried to identify the SLY1 sumoylation site. The predicted amino acid sequence of SLY1 contained a putative sumoylation site (ψKXE) located at
lysine 122 (K122; Figure 10A and B). Therefore, a derivative sequence was generated containing the mutation K122R. The mutated protein was over-expressed in *E. coli* and purified with glutathione affinity columns. *In vitro* sumoylation assays using SLY1 (GST-SLY1) and mutated SLY1 (GST-mSLY1 (K122R)) showed that GST-mSLY1 was not modified with SUMO, whereas GST-SLY1 was clearly sumoylated (Figure 10C). These results indicate that K122 is the principal SUMO conjugation site in SLY1.
Figure 6. AtSIZ1 physically interacts with SLY1. (A) In vitro pull-down assay of SLY1 with full-length AtSIZ1. MBP and MBP-AtSIZ1 (bait) and His₆-SLY1 (prey) were purified using affinity chromatography (left). His₆-SLY1 absorbed with MBP or MBP-AtSIZ1 was detected by western blotting with anti-His antibody (middle). After pull-down, MBP and MBP-AtSIZ1 were also detected by western blotting with anti-MBP antibody (right). (B) Yeast two-hybrid analysis of AtSIZ1 and SLY1. Full-length AtSIZ1 and SLY1 cDNAs were fused to sequences encoding the Gal4 activation domain (AD) and the Gal4 DNA-binding domain (BD) in pGAD424 and pGBT8, respectively. Constructs were transformed into the yeast strain AH109. Numbers indicate yeast cells transformed with only pGAD424 and pGBT8 vectors or recombinant plasmids. Transformants were plated onto minimal media, -Leu/-Trp and -Leu/-Trp/-His (20 mM 3-AT), and incubated for 4 d.
Figure 10. Subcellular co-localization of AtSIZ1 and SLY1. AtSIZ1 and SLY1 co-localize in the nucleus of onion epidermal cells. YFP-AtSIZ1 and SLY1-CFP refer to YFP and CFP fusion to the N-terminal of AtSIZ1 and the C-terminal of SLY1, respectively. Dic: Differential interference contrast. Bar = 20 μm.
Figure 8. AtSIZ1 directly sumoylates SLY1 using SUMO1. (A) His$_6$-AtSAE1b, His$_6$-AtSAE2, His$_6$-AtUBC9, MBP-AtSIZ1, His$_6$-AtSUMO1-GG, and GST-SLY1 purified from E.coli were separated by 11% SDS-PAGE. (B) E3 ligase activity of AtSIZ1 for SLY1 was assayed in the presence or absence of His$_6$-AtSAE1+2, His$_6$-AtUBC9, MBP-AtSIZ1, His$_6$-AtSUMO1-GG and GST-SLY1. After the reaction, sumoylated SLY1 was detected by western blotting with anti-GST antibody.
Figure 9. AtSIZ1 sumoylates SLY1 using SUMO1 in Arabidopsis.

Sumoylation of SLY1 was also analyzed in Arabidopsis. *siz1-2* mutants over-expressing HA3-AtSUMO1-GG were infiltrated with or without 35S-SLY1-FLAG3 and 35S-Myc6-AtSIZ1, as indicated. After incubation for 3 d, SLY1-FLAG3, HA3-AtSUMO1-GG and Myc6-AtSIZ1 were detected by western blotting with anti-FLAG (A), anti-HA (B) and anti-Myc (C) antibodies, as appropriate. Asterisk indicates non-specific bands. (D) Sumoylated SLY1-FLAG3 was detected by western blotting with anti-FLAG antibody following immunoprecipitation with anti-HA antibody. IgG: immunoglobulin G. non-specific bands were indicated by asterisk.
Figure 10. Identification of the SLY1 sumoylation site. (A) Predicted amino acid sequence of the SLY1 protein. A putative sumoylation site (ψKXE), identified using the SUMOplot Analysis Program, is indicated in yellow box and F-box and LSL domains are represented in bold type and underlined, respectively. (B) Schematic diagram of the recombinant GST-SLY1 protein. The F-box, LSL and putative sumoylation site (K122) are indicated. (C) In vitro
sumoylation assays. Recombinant GST-SLY1 and GST-mSLY1 were overexpressed in *E. coli* and purified using a glutathione affinity column. The reaction mixture contained E1 (His<sub>6</sub>-AtSAE1b and His<sub>6</sub>-AtSAE2), E2 (His<sub>6</sub>-AtSCE1), His<sub>6</sub>-AtSUMO1-GG and GST-SLY1 (or GST-mSLY1), without (−) or with (+) E3 (MBP-AtSIZ1). The mutant protein mSLY1 has an amino acid substitution at the residue predicted to be the SUMO conjugation SLY1 site, as indicated. After the reaction, sumoylated SLY1 protein was detected by western blotting with anti-GST antibody.
SLY1 modification by SUMO promotes plant growth and development

The Arabidopsis thaliana T-DNA insertion knock-out mutant sly1-13 was obtained from ABRC (Salk_097559) and named it sly1-13 to distinguish it from other mutant alleles of sly1. The sly1-13 mutant has 12kb of T-DNA inserted 300 bases from the 3’ end of At4g24210 (Figure 11A). This result was confirmed by PCR using Salk_097559 line specific left, right and border primers (Data not shown). The sly1-13 mutants had the same phenotypes as other sly1 loss of function mutants, sly1-2, sly1-10 (Figure 11B). I first produced anti-SLY1 antibody and examined its specificity by western blotting, and found that SLY1 was clearly detected in wild-type but not in sly1-13 plants (Figure 11C), indicating that the antibody specifically interacts with SLY1.

One of the notable phenotypes of sly1 mutants is severe dwarfism (Steber et al., 1998). Considering the function of SLY1 in plant growth and my SLY1 sumoylation data, I therefore examined the effect of sumoylation on SLY1 activity. I produced transgenic sly1-13 mutants over-expressing SLY1-FLAG3 or mSLY1-FLAG3, and measured their growth. Growth of sly1-13 mutants over-expressing wild-type protein was restored to wild-type (Figure 12A). By contrast, growth of sly1-13 mutants over-expressing the mutated protein resembled sly1-13 mutants. To determine if these phenotypes were caused by differential expression of SLY1-FLAG3 and mSLY1-FLAG3, I analyzed levels of SLY1-FLAG3 and mSLY1-FLAG3 in transgenic sly1-13 mutants and found that SLY1-FLAG3 and mSLY1-FLAG3 were highly expressed (Figure 12B).
Figure 11. *sly1-13* mutant selection and SLY1 specific antibody production.

(A) Schematic diagram of *SLY1* (At4G24210) gene. Gain of function polymorphism (*sly1-d*) is indicated as allow and loss of function polymorphism (*sly1-10* and *sly1-13*) is represented by allow head. (B) Phenotype of wild type (Col0) and selected *sly1-13* mutant plants. (C) Determination of the specificity of anti-SLY1 antibody. Total proteins were extracted from wild-type and *sly1-13* plants grown for 15 d and SLY1 was detected by western blotting with anti-SLY1 antibody; tubulin was used as a loading control. Tubulin was used as a loading control.
Figure 12. Phenotypes of sly1-13 mutants over-expressing SLY1 or mSLY1.

Arabidopsis plants were transformed with 35S-SLY1-FLAG₃ or 35S-mSLY1-FLAG₃ constructs, and then transgenic plants were crossed with sly1-13 mutants. (A) Transgenic sly1-13 mutants over-expressing SLY1-FLAG₃ or mSLY1-FLAG₃ were selected, and their growth was examined 28 d after sowing. (B) SLY1-FLAG₃ or mSLY1-FLAG₃ expression was evaluated in transgenic sly1-13 mutants using western blotting with anti-FLAG antibody. Tubulin was used as a loading control.
siz1-2 mutant has short hypocotyls

RGA and GAI are the main repressors of hypocotyl growth and stem elongation in Arabidopsis (Peng et al., 1997; Dill et al., 2001). In addition, SLY1 positively controls the effect of GA on cell expansion and division (Achard et al., 2009). All sly1-13 mutants so far described display dwarfism, resembling the siz1-2 mutant phenotype; however, growth defects of sly1-13 mutants are more severe than those of siz1-2 plants. I found that loss of AtSIZ1 caused SLY1 degradation and RGA accumulation, suggesting that hypocotyl elongation is repressed in the siz1-2 mutant. I therefore measured hypocotyl lengths of wild-type, siz1-2 and sly1-13 plants grown in light or dark conditions. As expected, compared with sly1-13 mutants, siz1-2 mutants showed long hypocotyl phenotypes under both conditions (Figure 13A and B), although hypocotyls of siz1-2 mutants were still shorter than those of wild-type plants.
Figure 13. Hypocotyl lengths of siz1-2 mutants. Seedlings were grown for 7 d under light or dark conditions. (A) Hypocotyl phenotypes of wild-type, siz1-2 and sly1-13 plants. (B) Mean hypocotyl lengths of the seedlings grown in the growth conditions indicated in (A). Error bars indicate standard deviations.
**SLY1 localization affected by exogenous GA.**

I showed that SLY1 was SUMOylated by the E3 SUMO ligase, SIZ1. This post translational modification suggested that SLY1 might be changed its functional roles. To test this hypothesis, I first check SLY1 and mSLY1 subcellular localization, transiently expressing SLY1 and mSLY1 tagged with green fluorescent protein (GFP) with or without exogenous GA in onion epidermal cells, a model system for the processes in Arabidopsis cells. I observed localization of both proteins in the nucleus, whether expressed in the same organelles or different. Subcellular localization results showed that mSLY1 tagged with GFP localization was not changed by exogenous GA₃ (Figure 14). However, diffused form of SLY1 tagged with GFP localization was different to speckled form by exogenous GA₃.
Figure 14. Subcellular co-localization of SLY1 and mSLY1 depending on GA. SLY1 and mSLY1 localize in the nucleus of onion epidermal cells. SLY1-GFP and mSLY1-GFP refer to GFP fusion to the C-terminal of SLY1. 100 μM GA$_3$ was treated for 3 hour with MS media. Dic: Differential interference contrast. Bar = 20 μm.
SLY1 is stabilized by AtSIZ1

Many proteins are post-translationally modified by small or large molecules before becoming stable. In eukaryotes, various target proteins are stabilized by sumoylation. To investigate whether SLY1 stability is regulated by AtSIZ1 activity, I analyzed the levels of SLY1 in wild-type and $siz1$-2 plants using western blotting with anti-SLY1 antibody, and found that the level of SLY1 was significantly lower in the $siz1$-2 mutants (Figure 15A, upper panel). In addition, I examined the effect of SUMO protease on SLY1 stability. SLY1 level was evaluated in SUMO protease mutant $esd4$. As expected, the SLY amount was significantly high in $esd4$ mutants compared to wild-type plants (Figure 15A, lower panel), indicating that SLY1 is stabilized by sumoylation. I used real-time qRT-PCR to examine $SLY1$ transcript levels in the same samples and found similar levels of transcript in wild-type and $siz1$-2 plants (Figure 15B).

Many proteins are post-translationally modified by small or large molecules before becoming stable. In eukaryotes, various target proteins are stabilized by modification by SUMO. To investigate whether SLY1 stability is regulated by AtSIZ1 activity, I first performed a cell-free degradation assay using total protein extracts, prepared from leaves of wild-type and $siz1$-2 plants, and a recombinant protein, GST-SLY1-HA, over-expressed in $E.coli$ and purified on a glutathione column. Purified GST-SLY1-HA was mixed with the extracts and its degradation examined using western blotting with an anti-HA antibody. I found
GST-SLY1-HA decayed much faster in the presence of protein extracts from *siz1-2* than in extracts from wild-type plants (Figure 16A and B).

Next, the decay of SLY1 was evaluated in the presence of cycloheximide to compare the degradation rates between wild-type and *siz1-2* plants. The degradation of SLY1 was much faster in *siz1-2* plants than in wild-type plants (Figure 16C and D), indicating that SLY1 is stabilized by AtSIZ1.
Figure 15. Effect of SUMOylation on the levels of SLY1. (A) Examination of SLY1 level in siz1-2 mutant (upper panel). Total proteins were extracted from wild-type and siz1-2 plants grown for 15 d and SLY1 was detected by western blotting with anti-SLY1 antibody. Effect of Arabidopsis SUMO protease on the SLY1 level (lower panel). Total proteins were extracted from wild-type and esd4 plants grown for 15 d. SLY1 was detected by western blotting with anti-SLY1 antibody. Tubulin was used as a loading control. (B) Effect of AtSIZ1 on the transcript level of SLY1. Total RNA was isolated from the same samples and the levels of SLY1 transcript were examined by real-time qRT-PCR with gene-specific primers. Results are expressed as means ± S.D. (n = 3).
Figure 16. Effect of AtSIZ1 on the levels of SLY1. (A) cell-free degradation of SLY1. Purified GST-SLY1-HA was mixed with extracts from wild-type or *siz1-2* plants. After incubation at room temperature for 8 h, samples were collected at the times indicated and levels of GST-SLY1-HA were examined using western blotting with anti-HA antibody. Binding Protein (BiP) in the extracts is shown as loading controls. (B) GST-SLY1-HA levels during degradation depicted in graph form. Relative levels of GST-SLY1-HA shown in (C) were normalized to numerical values, based on a value of 1.0 for the protein levels at time 0. Closed and open circles indicate GST-SLY1-HA extracted from wild-type and *siz1-2* plants, respectively. (C) Total proteins were extracted
from wild-type and siz1-2 plants treated with 100 μM cycloheximide for indicated length of time and SLY1 was detected by western blotting with anti-SLY1 antibody; tubulin was used as a loading control. (F) SLY1 levels during degradation were also depicted in graph form. Relative levels of SLY1 shown in (D) were normalized to numerical values, based on a value of 1.0 for the protein levels at time 0. Closed and open circles indicate SLY1 in wild-type and siz1-2 plants, respectively.
RGA level is affected by both SLY1 and AtSIZ1

In Arabidopsis, SLY1 recognizes target DELLA proteins, including RGA, and induces their degradation by the 26S proteasome complex. Therefore, I initially examined the effect of SLY1 sumoylation on RGA levels using bacterial infiltration of Arabidopsis. To examine the effect of AtSIZ1 on RGA levels through SLY1 sumoylation, I again used bacterial infiltration of Arabidopsis. The constructs 35S-HA3-AtSIZ1, 35S-SLY1-FLAG3, 35S-mSLY1-FLAG3 and 35S-RGA-Myc6 were co-infiltrated into the leaves of wild-type and siz1-2 plants, and I used western blotting with anti-Myc antibody to detect the level of RGA-Myc6 (Figure 17A-a). RGA-Myc6 levels were decreased in both wild-type plants and siz1-2 mutant plants infiltrated with 35S-HA3-AtSIZ1 due to the presence of endogenous SLY1. As expected, co-infiltration with 35S-HA3-AtSIZ1 and 35S-SLY1-FLAG3 resulted in a significant decrease in RGA-Myc6 levels in both wild-type and siz1-2 plants; in fact, RGA-Myc6 could not be detected under this condition. However, RGA was clearly detected in both wild-type and siz1-2 plants co-infiltrated with 35S-HA3-AtSIZ1 and 35S-mSLY1-FLAG3, although the levels were relatively low compared with those of untreated plants. Western blotting also clearly detected the recombinant proteins HA3-AtSIZ1, SLY1-FLAG3 and mSLY1-FLAG3. To confirm lower level RGA-Myc6 level in SLY1-FLAG3 infiltrated plant, I check RNA level of RGA-Myc6 in the same samples, but RNA levels were not changed in all samples (Figure 17-b).
After then, I needed to clarify that mSLY1 affected by exogenous GA and 26S proteosomal degradation pathway. To check these idea, I performed similar test that co-infiltration with 35S-HA3-AtSIZ1, 35S-SLY1-FLAG3, 35S-mSLY1-FLAG3 and 35S-RGA-Myc6 into the leaves as indicated compositions with or without GA and MG132. The relative levels of RGA were much higher in non-exogenous GA treated mSLY1 and MG132 treated combinations. In contrary, RGA levels were low in non-exogenous GA treated SLY1 and both of exogenous GA treated combination even with mSLY1 (Figure 17B).

To check these results in transgenic plants, I used siz1-2 and transgenic sly1-13 mutants over-expressing SLY1 or mSLY1 to examine the effect of SLY1 sumoylation on RGA stability. The relative levels of RGA in extracts of total proteins from wild-type, sly1-13, siz1-2, 35S-SLY1-FLAG3/sly1-13, and 35S-mSLY1-FLAG3/sly1-13 plants were assessed by western blotting with anti-RGA antibody. As expected, the levels of RGA were much higher in sly1-13, siz1-2, and 35S-mSLY1-FLAG3/sly1-13 plants than in wild-type and 35S-SLY1-FLAG3/sly1-13 plants (Figure 18A). This result indicates that RGA is stabilized in siz1-2 mutants and 35S-mSLY1-FLAG3/sly1-13 plants.

The RGA levels were similar in wild-type and 35S-SLY1-FLAG3/sly1-13 plants. However, its level was relatively low in siz1-2 plants compared with sly1-13 plants (Figure 18A), which explains why the dwarfing phenotype is less severe in siz1-2 than in sly1-13.

To confirm SIZ1 direct effect on RGA level through SLY1 or mSLY1, I used
transgenic wild-type and siz1-3 mutants over-expressing SLY1 or mSLY1 to observe the RGA stability. The relative levels of RGA from Wild type, mSLY1-FLAG3/Col 0, SLY1-FLAG3/siz1-3 were low than siz1-3 and mSLY1-FLAG3/siz1-3 but SLY1-FLAG3/Col 0 was not detected (Figure 18B).
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- HA$_3$-AtSIZ1
- SLY1-FLAG$_3$
- mSLY1-FLAG$_3$
- RGA-Myc$_6$

(Immunoblot analysis)

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- HA$_3$-AtSIZ1
- SLY1-FLAG$_3$
- mSLY1-FLAG$_3$
- RGA-Myc$_6$
- GA
- MG132

(Immunoblot analysis)
Figure 17. AtSIZ1 and GA effects on RGA level depending on SLY1 or mSLY1. (A, upper panel) Arabidopsis thaliana Col0 and siz1-2 plants were infiltrated with 35S-HA3-AtSIZ1, 35S-SLY1-FLAG3, 35S-mSLY1-FLAG3 and 35S-RGA-Myc6, as indicated. After 3 days incubation, HA3-AtSIZ1, SLY1-FLAG3, mSLY1-FLAG3 and RGA-Myc6 proteins were detected using western blotting with anti-HA, anti-FLAG and anti-Myc antibodies, as appropriate. Tubulin was used as a loading control. (A-lower panel) Total RNA was extracted from the same samples and expression level of 35S-RGA-Myc6 was determined by real-time RT-PCR with gene and tag-specific primers. Tubulin was used as a loading control. (B) Arabidopsis thaliana Col0 plants were infiltrated as indicated combination. 20mM MG132 was treated for 16hr and 10uM GA3 was treated for 6 hours as indicated, respectively. Each proteins were also detected using western blotting with anti-HA, anti-FLAG and anti-Myc antibodies, as appropriate. Tubulin was used as a loading control.
(A)

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- RGA
- Tubulin

(B)

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<th>siz1-3/35S-mSLY1-FLAG3</th>
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- SLY1-FLAG3 or mSLY1-FLAG3
- RGA
- Tubulin
Figure 18. AtSIZ1 negatively regulates RGA level through sumoylation of SLY1. (A) Compared RGA level in *siz1*-2, *sly1*-13 mutants and *sly1*-13 harboring overexpressed SLY1 or mSLY1 plants. Total proteins were extracted from leaves of 15-d-old wild-type, *siz1*-2, *sly1*-13, 35S-SLY1-FLAG/sly1-13, 35S-mSLY1-FLAG/sly1-13 and *rga* (negative control) plants. Following 11% SDS-PAGE, the levels of RGA were examined by western blotting with anti-RGA antibody. Tubulin was used as a loading control. (B) RGA level in Col0 and *siz1*-3 mutants harboring overexpressed SLY1 or mSLY1 plants. Total proteins were extracted from leaves of 15-d-old plants. The levels of RGA were examined by western blotting with anti-RGA antibody. The overexpressed proteins were detected by western blotting with anti-FLAG antibody. Tubulin was used as a loading control. Non-specific bands were indicated by asterisk.
Sumoylated SLY1 interacts with RGA

Over-expression of mSLY1 in sly1-13 mutant plants does not induce RGA degradation and cannot rescue the dwarfing phenotype, which suggests that SLY1 sumoylation is important for the interaction with, and degradation of RGA. I thus investigated the interaction between sumoylated SLY1 and RGA by in vitro pull down. An in vitro sumoylation reaction was first performed with purified GST-SLY1 and sumoylation machinery. After detection of sumoylated GST-SLY1 by western blotting with anti-GST antibody (Figure 19A), the remaining reaction products were pulled down with MBP or MBP-RGA, and sumoylated SLY1 was then detected by western blotting with anti-GST antibody. The results showed that sumoylated GST-SLY1 interacted with MBP-RGA (Figure 19B).
Figure 19. RGA interacts with sumoylated SLY1. (A) In vitro sumoylation reactions were carried out as indicated, and GST-SLY1 and sumoylated GST-SLY1 levels were investigated by western blotting with anti-GST antibody. (B) Reaction mixtures in (A) were pulled down with MBP or MBP-RGA. GST-SLY1 and sumoylated GST-SLY1 were detected by western blotting with anti-GST antibody.
Interactions between SLY1 and AtSIZ1 or ASK are strong and specific

In vitro sumoylation assays showed that mSLY1 was not sumoylated by AtSIZ1. In addition, bacterial infiltration assays in Arabidopsis demonstrated that SLY1 controls the level of RGA after sumoylation. However, the possibility remains that mSLY1 has no effect on RGA stability because mSLY1 does not interact with AtSIZ1. I thus performed a yeast two-hybrid assay to examine whether mSLY1 interacts directly with AtSIZ1 and found evidence for such an interaction (Figure 20A). These results were supported by in vivo immunoprecipitation analysis with anti-FLAG and western blotting anti-HA from 35S-HA3-AtSIZ1, 35S-SLY1-FLAG3 and 35S-mSLY1-FLAG3 co infiltrated Arabidopsis leaves. In Arabidopsis result also showed that SIZ1 could interact with SLY1 and mSLY1 also (Figure 20B and C).

A previous study demonstrated that SNE/SLY2 (SNEEZY/SLEEPY2), a SLY1 homolog, is involved in degrading RGA, GAI and RGL2 (RGA-like 2) (Ariizumi and Steber, 2011). I therefore investigated whether SLY2 is activated or stabilized by sumoylation through AtSIZ1 activity, using a yeast two-hybrid assay to look for possible interactions between AtSIZ1 and SLY2. The results showed that AtSIZ1 did not interact with SLY2 (Figure 20A).

Because SLY1 interacts with Arabidopsis SKP1-like (ASK) protein to form the SCF complex required for degradation of its target DELLA proteins, I used yeast two-hybrid assays to examine further the effect of the SLY1 sumoylation site on the interaction of SLY1 with ASK proteins. I tested six ASK proteins,
ASK1, ASK2, ASK3, ASK4, ASK11 and ASK13, and found that all interacted with both SLY1 and mSLY1 (Figure 21).
Figure 20. Yeast two-hybrid analysis of interactions between mSLY1 and other proteins or between SLY2 and AtSIZ1. (A) Full-length AtSIZ1 was fused to sequences encoding the Gal4 activation domain (AD) in pGAD424. SLY1, mSLY1, and SLY2 cDNAs were fused to sequences encoding the Gal4
DNA-binding domain (BD) in pGBT8. (B) Transient expression of HA$_3$-AtSIZ1 and SLY1-FLAG$_3$ or mSLY1-FLAG$_3$ in Col0 plants. (C) HA$_3$-AtSIZ1 was detected by western blotting with anti-HA antibody following immunoprecipitation with anti-FLAG antibody. IgG: immunoglobulin G. non-specific bands were indicated by asterisk.
Figure 21. Interactions between SLY1 or mSLY1 and ASK are strong. Full-length sequences of the Arabidopsis ASK genes ASK1, ASK2, ASK3, ASK4, ASK11 and ASK13 were fused to sequences encoding the Gal4 activation domain (AD) in pGAD424. SLY1 and mSLY1 cDNAs were fused to sequences encoding the Gal4 DNA-binding domain (BD) in pGBT8. Constructs were transformed into the yeast strain AH109. Numbers indicate the yeast cells transformed with only pGAD424 and pGBT8 vectors or with recombinant plasmids. Transformants were plated onto minimal media, −Leu/−Trp and −Leu/−Trp/−His (10 mM 3-AT), and incubated for 4 d.
Expression of GA signaling-related genes was not notably affected by AtSIZ1

I have shown that AtSIZ1 is an E3 SUMO ligase for SLY1, suggesting that the defective growth of siz1-2 mutants may be caused in part by low level and activity of SLY1. However, it is also possible that dwarfism in siz1-2 mutants results from a change in gene expression. I therefore measured transcript levels of GA-responsive genes (SLY1 and SLY2), a GA-biosynthetic gene (GA3ox1), a GA-deactivating gene (GA2ox2) and DELLA protein genes (RGA and GAI) in siz1-2 mutants. Transcript levels of these genes were determined, using real-time qRT-PCR, in samples of total RNA isolated from both wild-type and siz1-2 mutants. The level of SLY1 transcript did not greatly change in siz1-2 mutants, relative to levels in wild-type plants (Figure 15B). Expression of RGA and GAI was very slightly increased in siz1-2 mutants compared with wild-type plants (Figure 22); however, there were no obvious differences in expression of GA2ox2 and GA3ox1 between wild-type and siz1-2 plants (Figure 22). Expression patterns of SLY2 were also very similar in wild-type and siz1-2 plants, although expression was somewhat higher in the siz1-2 mutants (Figure 22).
Figure 22. Transcript levels of GA-synthetic and -responsive genes in siz1-2 mutants. Total RNA was isolated from 15-d-old wild-type and siz1-2 mutant plants. Transcript levels were examined using real-time qRT-PCR with gene-specific primers. Results are expressed as means ± S.D. (n = 3). Abbreviations: RGA: Repressor of ga1-3; GAI: GA insensitive; GA2ox2: GIBBERELLIN 2-OXIDASE 2; GA3ox1: GIBBERELLIN 3-OXIDASE 1; SLY2: SLEEPY2.
**AtSIZ1 expression is regulated by GA signaling**

Our results show that SLY1-mediated GA signaling is tightly controlled by AtSIZ1. To determine the effect of GA signaling at the level of AtSIZ1, I used real-time qRT-PCR to investigate *AtSIZ1* expression in *sly1-13* mutants and found a low level of transcript compared with wild-type plants (Figure 23A). In addition, an exogenous application of GA induced *AtSIZ1* transcription in wild-type plants but not in *sly1-13* mutants (Figure 23A), although the transcript levels decreased after 3 hr. I also used western blotting with anti-AtSIZ1 antibody to analyze the level of AtSIZ1 in *sly1-13* mutants. There was a similar pattern also detected in *AtSIZ1* expression; in wild-type plants, the level of AtSIZ1 increased and then decreased after 3 hr (Figure 23B).
Figure 23. Effect of GA treatment on relative levels of \textit{AtSIZ1} transcript and protein. Wild-type and \textit{sly1-13} plants were treated with 100 uM GA\textsubscript{3} during vegetative growth and samples were collected at the indicated time points. (A) Total RNA was extracted from samples and expression level of \textit{AtSIZ1} was determined by real-time qRT-PCR with gene-specific primers. Filled circle: wild-type; Open circle: \textit{sly1-13} mutants. (B) Total proteins were
extracted from the same samples and level of AtSIZ1 was estimated by western blotting with anti-AtSIZ1 antibody. Tubulin was used as a loading control.

**Sumoylation of SLY1 is stimulated by GA supply**

The results of development assays using transgenic sly1-13 mutants (Figure 12A) strongly suggest that SLY1 must be modified by SUMO to be in an active form. The results also imply that GA can stimulate sumoylation of SLY1 to activate and stabilize this protein. I therefore examined whether SLY1 modification with SUMO is stimulated by GA. To do this, I used bacterial infiltration of Arabidopsis. The constructs 35S-HA3-AtSIZ1, 35S-SLY1-FLAG3, and 35S-His6-Strep-AtSUMO1-GG were co-infiltrated into the leaves of wild-type and siz1-2 plants. Concurrent with infiltration, the leaves were treated with 100 µM GA3. First, I examined the expression of recombinant proteins HA3-AtSIZ1, SLY1-FLAG3 and His6-Strep-AtSUMO1-GG by western blotting and found that they were clearly detected (Figure 24A). Next, I purified His6-Strep-AtSUMO1-GG-modified proteins with nickel columns and evaluated His6-Strep-AtSUMO1-GG-modified SLY1-FLAG3 by western blotting with anti-FLAG antibody. The result showed that the level of His6-Strep-AtSUMO1-GG-modified SLY1-FLAG3 was very high in GA-supplied samples whereas it was very low in non-treated samples (Figure 24B), indicating that the modification of SLY1-FLAG3 with His6-Strep-AtSUMO1-GG was stimulated by exogenously supplied GA.
**Figure 24. Effect of GA on sumoylation of SLY1.** (A) The effect of GA on sumoylation of SLY1 was examined by bacterial infiltration. Transgenic plants over-expressing His_6-strep-AtSUMO1-GG were infiltrated with Agrobacterium transformed with 35S-SLY1-FLAG_3 and 35S-HA_3-AtSIZ1, as indicated. After 2 d, 100 μM GA_3 was added as indicated. Recombinant proteins, HA_3-AtSIZ1, SLY1-FLAG_3 and His_6-Strep-AtSUMO1-GG, were detected by western blotting with anti-HA, anti-FLAG and anti-Strep antibodies, as appropriate. (B) His_6-strep-AtSUMO1-GG-conjugated proteins were purified from total proteins using Ni^{2+}-NTA resins and sumoylated SLY1 was detected by western blotting with anti-FLAG (upper panel) or anti-SUMO1 (lower panel) antibody.
**AtSIZ1 is not involved in regulation of GID1A activity**

GA signaling begins with the binding of the receptor protein GID1A (GA INSENSITIVE DWARF1A) to GA; therefore, I investigated whether AtSIZ1 can modulate the function of GID1A. I used *in vitro* pull-down and yeast two-hybrid assays to test the interaction between AtSIZ1 and GID1A because the predicted amino acid sequence of GID1A contains three putative sumoylation sites (Figure 25A). For *in vitro* pull-down assays, recombinant proteins, MBP, MBP-AtSIZ1, GST-GID1A and GST-SLY1, were prepared (Figure 25B-a), and then GST-GID1A and GST-SLY1 were pulled-down using MBP or MBP-AtSIZ1. AtSIZ1 did not interact with GID1A, but a clear interaction between AtSIZ1 and SLY1 was observed (Figure 25B-b). I also tested this interaction using a yeast two-hybrid assay; consistent with the results of the pull-down assay, AtSIZ1 did not interact with GID1A (Figure 25C).
MAASDEVNLIESRTVVPLNTOVLISNFKVAYNILRRPDGT40
FNRHLAEYLDRKVTANANPDGVFSFDVLDRRINLLSRV80
YRPAYADQEQPPSILDLEKPVGDIDVPVLFPHGGSFAHS120
SANSAIYDTRCRRLVGLCKCVVSVNYRAPPENYPYCAYD160
DGYALNWVNSRSWLSKKSSDKVHIFLAGDNSSGNIAHNV200
ALRAAGESGIDVLGNNLNLQMPGNNERTESKSLDGKYFVT240
VRDRDWWKAFLPEGEDREHPACNPFSPRGKSLGVSFFPK280
SLVVVAGDLDRQWQAYAEGLKKAGQEVKLHLEKATVG320
FYPNNNHFHIVNMDEISAFVNAEC345

Leu-/Trp-
Leu-/Trp-/His-(1mM 3-AT)
Leu-/Trp-/His-(10mM 3-AT)
**Figure 25. AtSIZ1 did not interact with GID1A.** (A) Predicted amino acid sequence of SLY1. Potential sumoylation sites (ψKXE), identified using the SUMOplot Analysis Program, are indicated as bold type and underlined. (B) *In vitro* pull-down assay of SLY1 with full-length AtSIZ1. MBP and MBP-AtSIZ1 baits and GST-SLY1 and GST-GID1A preys were purified using affinity chromatography (left). GST-SLY1 and GST-GID1A, absorbed with MBP or MBP-AtSIZ1, were detected using western blotting with an anti-GST antibody (right). (C) Yeast two-hybrid analysis of the interaction between AtSIZ1 and GID1A. Full-length AtSIZ1 was fused to a sequence encoding the Gal4 activation domain (AD) in pGAD424. GID1A cDNA was fused to a sequence encoding the Gal4 DNA-binding domain (BD) in pGBT8. The constructs were transformed into the yeast strain AH109. Numbers indicate the yeast cells transformed with only pGAD424 and pGBT8 vectors or with recombinant plasmids. Transformants were plated onto minimal media, –Leu/–Trp and –Leu/–Trp/–His (1 and 10 mM 3-AT), and incubated for 4 d.
DISCUSSION

This study highlights the notion that SLY1-mediated plant development is positively regulated by sumoylation via the E3 SUMO ligase activity of AtSIZ1. Various GA-dependent development processes (Richards et al., 2001; Hedden, 2003) are controlled by DELLA proteins, and their repressor activities are modulated by phosphorylation and ubiquitination via the E3 ligase activity of SCF<sup>SLY1</sup> (Sasaki et al., 2003; Dill et al., 2004; Gomi et al., 2004; Itoh et al., 2005). Such observations have led researchers to focus on SLY1 and DELLA proteins to elucidate GA signaling. Very recently, Conti et al. (2014) reported that DELLA protein-mediated growth can be regulated by sumoylation.

Sumoylation is an essential mechanism for regulating developmental processes in eukaryotic cells (Praefcke et al., 2012; Park and Yun, 2013) and therefore, an increasing number of studies on the function of E3 SUMO ligase have been performed since SIZ1 was first identified in (Miura et al., 2007, 2010; Yoo et al., 2006; Catala et al., 2007; Lee et al., 2007; Garcia-Dominguez et al., 2008; Jin et al., 2008; Miura and Ohta, 2010; Park et al., 2011; Son et al., 2014). One of the most prominent features of Arabidopsis <i>sis1-2</i> mutants is their dwarf phenotype (Catala et al., 2007), suggesting impairment of the GA signaling pathways responsible for elongation and growth. This hypothesis is supported by the severe, dark green dwarfism observed in <i>sly1</i> mutants (McGinnis et al., 2003). Therefore, I investigated whether an <i>AtSIZ1</i> mutation affects GA signaling by
interrupting SLY1-mediated DELLA protein degradation.

Seed germination test was the most certain way to confirm that \textit{siz1}-2 mutants whether affected on GA signaling or not. Seed germination vigorosity and radical emergency results show that \textit{siz1}-2 mutants germination rate were rescued by 10 \textmu M exogenous GA$_3$ (Figure 5). These results suggested that one of GA signaling protein could affect by SIZ1 to SUMOylate for post translational modification.

AtSIZ1 acts as an E3 SUMO ligase on various target proteins, including NIA1/NIA2, ICE1, GTE3, and FLC (Miura et al., 2007; Garcia-Dominguez et al., 2008; Park et al., 2011; Son et al., 2014). I investigated whether SLY1 is also a target of AtSIZ1 in nucleus. My various sumoylation and localization assays showed that SLY1 is directly sumoylated by AtSIZ1 \textit{in vitro} and \textit{vivo} (Figure 6, Figure 7, Figure 8 and Figure 9). It is known that sumoylation stabilizes target proteins (Praefcke et al., 2012; Park and Yun, 2013); for example, nitrate reductase decays more quickly in \textit{siz1}-2 mutants than wild-type Arabidopsis (Park et al., 2011) and RGA is more highly accumulated in the SUMO protease \textit{ots1 ots2} mutants than wild-type Arabidopsis (Conti et al., 2014). My data showed lower levels of SLY1 protein in \textit{siz1}-2 mutants than in wild-type plants (Figure 15A, upper panel), despite similar levels of transcript (Figure 15B) and faster degradation of SLY1 protein in \textit{siz1}-2 mutants than in wild-type plants (Figure 16A-D), strongly indicating that SLY1 protein is stabilized by sumoylation through AtSIZ1 activity.
The PML gene of acute promyelocytic leukemia (APL) encodes a cell growth and tumor suppressor. PML localizes to discrete nuclear bodies that is regulated by SUMO3 in human cell (Fu et al., 2005). I could hypnotize that SUMOylation of SLY1 and GA signal might affect on SLY1 localization. To reveal that SLY1 and mSLY1 tagged GFP were test with or without exogenous GA its subcellular localization. No difference in localization in mSLY1 tagged GFP but diffused form of SLY1 tagged GFP was changed speckled form by exogenous GA, which suggest that structural changes derived in SUMOylation site mutation and exogenous GA could affect SLY1 localization (Figure 14).

In addition, previous studies have shown that SUMO conjugation modulates the activity of target proteins in yeast, animals and plants (Praefcke et al., 2012; Park and Yun, 2013); for example, the activities of nitrate reductases increased significantly following sumoylation (Park et al., 2011). I thus speculated that SLY1 activity must be affected by SUMO attachment. I identified the SLY1 sumoylation site (Figure 10) and examined the effect of SUMO conjugation on SLY1 function using transgenic sly1-13 mutants over-expressing SLY1 or mSLY1. Over-expression of SLY1 restored the growth and development of sly1-13 mutants to wild-type levels but over-expression of mSLY1 did not (Figure 12A). The result suggests that SLY1 is modified by SUMO before it can function to degrade DELLA proteins and activate GA signaling. This scenario is also supported by my observation that sumoylation of SLY1 is induced by GA (Figure 24B).
GA signaling is negatively regulated by the activity of DELLA proteins, which are degraded by the 26S proteasome complex after polyubiquitination by SCF<sub>SLY1</sub> (Dill et al., 2004). My results showed that the stability and activity of SLY1 were positively regulated by sumoylation through AtSIZ1 activity (Figure 9A-D, Figure 12A, Figure 15 and Figure 16), suggesting that the levels of DELLA proteins, including RGA, would be affected. Bacterial infiltration assays in Arabidopsis revealed that over-expression of AtSIZ1 alone was sufficient to cause RGA degradation (Figure 17A). Moreover, co-expression of AtSIZ1 and SLY1 resulted in no detectable level of RGA, although RGA was clearly detected when AtSIZ1 and mSLY1 were co-expressed (Figure 17A); mSLY1 alone had no effect on RGA levels (Figure 15A). In a complete inversion of the SLY1 expression pattern, the level of RGA was much higher in siz1-2 than in wild-type plants (Figure 18A). RGA accumulated to a high level in transgenic sly1-13 mutants over-expressing mSLY1, as it did in siz1-2 mutants, but the level of RGA in transgenic sly1-13 mutants over-expressing SLY1 was as low as that observed in wild-type plants (Figure 18A). These data indicate that SLY1 is positively regulated by AtSIZ1 and SLY1-mediated RGA degradation is stimulated by SLY1 sumoylation through the E3 ligase activity of AtSIZ1 (Figure 26). In addition, the low level of RGA in the siz1-2 mutants relative to sly1-13 (Figure 18A) explains the less severe growth defects of the siz1-2 mutants.

Accumulation of DELLA repressors in the absence of SLY1 inhibits cell
elongation and division, leading to the suppression of hypocotyl growth (Peng et al., 1997; Dill et al., 2001; Achard et al., 2009). In the current study, hypocotyls of the siz1-2 mutants were shorter than those of wild-type plants but longer than those of the sly1-13 mutants (Figure 13). In addition, the level of SLY1 was much lower in siz1-2 than in wild-type plants (Figure 15A, upper panel) and RGA levels were low in siz1-2 relative to those in the sly1-13 mutants (Figure 18A). These results indicate that the relatively long hypocotyls of siz1-2, compared with sly1-13 mutants, result from the relatively low level of RGA in these mutants and that AtSIZ1 regulates SLY1- and DELLA protein-mediated hypocotyl growth.

The results of transgenic assays in which over-expression of SLY1 rescued the growth of sly1-13 mutants but over-expression of mSLY1 did not (Figure 12) suggest that SLY1 must be sumoylated to exert its function as a component of the SCF complex, which led us to examine whether there was a direct interaction between RGA and sumoylated SLY1. A pull-down assay using in vitro sumoylation reaction products demonstrated that both sumoylated and non-sumoylated SLY1 strongly interacted with RGA (Figure 19B). Moreover, a bacterial infiltration assay using Arabidopsis clearly showed that the level of sumoylated SLY1 was greatly increased by GA treatment (Figure 24B), demonstrating that GA indeed stimulates the sumoylation of SLY1 by AtSIZ1 and the function of SLY1 must be activated by sumoylation. In other words, this result demonstrates that GA positively controls plant growth and development.
through stimulation of SLY1 sumoylation by AtSIZ1. Therefore, the phenotype of sly1-13 mutants over-expressing mSLY1 must be caused by inactivation of mSLY1 due to non-sumoylation rather than the loss of RGA function.

The Arabidopsis genome contains another SLY1 homolog, SLY2. SLY2 (SNE) also directly binds to RGA protein, as well as the cullin subunit of the SCF E3 complex, suggesting that SLY2 forms a functional SCF E3 ubiquitin ligase complex that negatively regulates the subset of DELLA proteins regulated by SLY1 (Ariizumi et al., 2011). SLY2 over-expression partially rescues the germination, dwarfism and infertility of the sly1-10 mutant by reducing the accumulation of DELLA proteins RGA and GAI (Strader et al., 2004; Fu et al., 2004; Sun, 2010; Ariizumi et al., 2011). This observation suggests that the function of SLY2 may also be regulated by AtSIZ1. However, the current results showed that AtSIZ1 did not interact with SLY2 (Figure 20A), indicating that AtSIZ1-mediated GA signaling is SLY1-specific.

Another possible reason for the failure of mSLY1 over-expression to rescue the growth phenotype of sly1-13 mutant is that mSLY1 is unable to interact with ASK proteins and thereby form the SCF complex. However, yeast two-hybrid assays showed strong interactions between mSLY1 and all of the ASK proteins tested (Figure 21), indicating that mSLY1 is capable of forming the E3 ubiquitin ligase SCFmSLY1 complex.

All of these data indicate that dwarfism in the siz1-2 mutant results from the loss of AtSIZ1 activity, which leads to low SLY1 levels or activity, although the
growth defect may be caused by changes in the expression of GA synthesis and response genes. However, I found very similar expression patterns of such genes in both wild-type and siz1-2 mutant plants, although the absolute transcript levels were slightly different (Figure 22). This result suggests that AtSIZ1 mainly regulates SLY1 levels post-translationally and that the growth defect of siz1-2 mutants is caused by low activity and level of SLY1.

Finally, I investigated whether GA plays a role in regulating AtSIZ1 levels. I detected low levels of AtSIZ1 transcript in sly1-13 mutants compared with wild-type plants and found that the transcript and protein levels of AtSIZ1 increased after exogenous application of GA (Figure 23). This result indicates that the level of AtSIZ1 is controlled by GA signaling, particularly SLY1-mediated GA signaling, at both the transcriptional and translational levels.

Proteolytic turnover of GA signaling-associated proteins can be regulated by sumoylation as well as ubiquitination. Recent data (Conti et al., 2014) show that RGA is sumoylated in a GA-independent manner although its E3 SUMO ligase has not yet been identified, and SUMO-conjugated RGA directly binds to GID1A, which blocks the interaction between GID1A and the remaining non-sumoylated RGA. This interaction causes the accumulation of RGA, resulting in growth inhibition. My finding demonstrates that SLY1 is directly sumoylated by E3 SUMO ligase AtSIZ1 and subsequently induces the degradation of its target protein RGA in an AtSIZ1-dependent manner (Figure 26). In addition, exogenous GA stimulated SLY1 sumoylation (figure 24), leading to activation
of SLY1 function. On the contrary, GA induced the disappearance of sumoylated and non-sumoylated RGA, although it has not yet been revealed whether the desumoylation of RGA is primarily required for the degradation of RGA. Both studies clearly provide evidence that the function and stability of GA signaling-associated proteins can be regulated by sumoylation in the presence of GA.

It is well-known that F-box proteins are substrate-recruiting subunits of SCF-type E3 ubiquitin ligases. To date, a large number of F-box proteins have been identified in plants and human (Gray et al., 2001; Richards et al., 2001; McGinnis et al., 2003; Nelson et al., 2013). However, the regulatory mechanisms underlying the activity of F-box proteins remain unclear. My results demonstrate that SLY1 function is tightly regulated by sumoylation. This is the first report demonstrating that the activity of an F-box protein is controlled by sumoylation. I thus expect that my results will help to elucidate the mechanism by which functional SCF complexes are produced according to the requirements of the cell.

In conclusion, I found that GA stimulates the sumoylation of SLY1 through the E3 SUMO ligase activity of AtSIZ1, indicating that sumoylation is an important post-translational modification that plays a role in the regulation of GA signaling by directly modulating the stability and activity of SLY1 protein. My results provide the first biochemical evidence that sumoylation is a critical protein modification required for the regulation of SLY1 function, and that
sumoylated SLY1 induces degradation of its target proteins. My study is also the first to report that F-box protein function is regulated by sumoylation, suggesting that the regulation of plant and animal development by SCF complexes is regulated by sumoylation of F-box proteins through E3 SUMO ligase activity.
Figure 26. Schematic representation of the possible mechanism of activation of GA signaling by SLY1 sumoylation. GA and SLY1 positively regulate AtSIZ1 expression. SLY1 is sumoylated by AtSIZ1, becoming the active form. Sumoylated SLY1 forms the SCF^{SLY1-SUMO} complex, directly interacts with DELLA proteins, including RGA, and ubiquitinates them. Polyubiquitinated DELLA proteins are degraded by the 26S proteasome complex. Finally, plant cells divide and expand, which leads to growth and development. RGA is also sumoylated by E1- and E2-dependent manner and then stabilized and may be able to directly inhibit GA signaling. Sumoylated RGA can also form a complex with GID1A, which results in the accumulation of non-sumoylated RGA, leading to the blocking of GA signaling. GA: gibberellic acid; Ub: ubiquitin.
REFERENCES


Chosed R, Mukherjee S, Lois LM, Orth K. 2006. Evolution of a signalling system that incorporates both redundancy and diversity: Arabidopsis


Fu X, Richards DE, Fleck B, Xie D, Burton N, Harberd NP. 2004. The Arabidopsis mutant sleepy1gar2-1 protein promotes plant growth by increasing the affinity of the SCF^{SLY1} E3 ubiquitin ligase for DELLA protein substrates. The Plant cell 16: 1406-1418.


Garcia-Dominguez M, March-Diaz R, Reyes JC. 2008. The PHD domain of


Miura K, Hasegawa PM. 2010. Sumoylation and other ubiquitin-like post-


Reeves PH, Murtas G, Dash S, Coupland G. 2002. Early in short days 4, a mutation in Arabidopsis that causes early flowering and reduces the mRNA
abundance of the floral repressor FLC. Development 129: 5349-5361.


encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. The Plant cell 10: 155-169.


Steber CM, McCourt P. 2001. A role for brassinosteroids in germination in


Yoo CY, Miura K, Jin JB, Lee J, Park HC, Salt DE, Yun DJ, Bressan RA,


지베렐린은 종자 발아, 세포 분열과 신장 그리고 개화까지, 다양한 식물발달단계에 영향을 미치는 식물 호르몬이다. 본 연구에서는 E3 수모화 효소인 AtSIZ1 단백질이 애기장대의 지베렐린 신호전달체계를 SLY1 단백질의 수모화를 통하여 조절한다는 것을 밝혀냈다. SLY1 단백질은 애생형에 비해 siz1-2 돌연변이체에서 적은 양이 존재하였다. sly1-13 돌연변이체에 SLY1 단백질 과다발현을 시킨 형질전환체는 애생형과 비슷한 표현형을 나타냈으나 수모화 조절부위 돌연변이 형의 mSLY1 단백질이 과다 발현되는 형질전환체에서는 돌연변이체 본연의 난쟁이 표현형이 나타났다. sly1-13 돌연변이체에 SLY1 단백질 과다발현을 시킨 형질전환체에서는 RGA 단백질의 양 역시 애생형과 같은 수준으로 나타났지만 mSLY1 과다 발현체에서는 돌연변이체처럼 높은 양이 존재하였다. RGA 단백질은 mSLY1과 AtSIZ1 단백질이 함께 발현되는 조건에서도 분해되지 않았으나, SLY1과 AtSIZ1 단백질이 함께 발현되는 조건에서는 다량이 분해되었다. 게다가, 수모화된
SLY1은 RGA 단백질과 상호작용 할 수 있으며, SLY1의 수모화는 지베렐린에 의해 매우 증가하였다. 정리하자면, 본 연구는 AtSIZ1이 SLY1의 수모화를 통하여 지베렐린 신호전달체계를 조절한다는 것을 암시한다.