Running head: Interaction of SMAP1 with COP9 and AXR1

Corresponding author: Yutaka Oono

Address: Medical and Biotechnological Application Division, Japan Atomic Energy Agency (JAEA), Takasaki 370-1292, Japan

Telephone number: +81-27-346-9537

E-mail address: ohno.yutaka@jaea.go.jp

Journal research area: Signal Transduction and Hormone Action
SMALL ACIDIC PROTEIN 1 acts with RUB modification components, the COP9 signalosome and AXR1, to regulate growth and development of Arabidopsis thaliana

Akari Nakasone\textsuperscript{a,b}, Masayuki Fujiwara\textsuperscript{c}, Yoichiro Fukao\textsuperscript{c}, Kamal Kanti Biswas\textsuperscript{a}, Abidur Rahman\textsuperscript{d}, Maki Kawai-Yamada\textsuperscript{b,e}, Issay Narumi\textsuperscript{a}, Hirofumi Uchimiya\textsuperscript{e,f}, and Yutaka Oono\textsuperscript{a,*}

\textsuperscript{a} Medical and Biotechnological Application Division, Japan Atomic Energy Agency (JAEA), Takasaki 370-1292, Japan
\textsuperscript{b} Graduate School of Science and Engineering, Saitama University, Saitama 338-8570, Japan
\textsuperscript{c} Plant Science Education Unit, Nara Institute of Science and Technology (NAIST), Nara 630-0192, Japan
\textsuperscript{d} Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka, Iwate, 020-8550, Japan
\textsuperscript{e} Institute for Environmental Science and Technology (IEST), Saitama University, Saitama 338-8570, Japan
\textsuperscript{f} Iwate Biotechnology Research Center, Iwate 024-0003, Japan

Footnotes

\textsuperscript{1} This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Kakenhi; no. 22570056) to Y.O., and a Grant in-Aid for Scientific Research for Plant Graduate Students from Nara Institute Science and Technology to A.N.

\textsuperscript{*} Corresponding author; e-mail ohno.yutaka@jaea.go.jp
Abstract

Previously, a dysfunction of the *SMALL ACIDIC PROTEIN 1 (SMAP1)* gene was identified as the cause of the *antiauxin resistant 1 (aar1)* mutant of Arabidopsis. *SMAP1* is involved in the response pathway of synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), and functions upstream of the AUXIN (AUX)/INDOLE-3-ACETIC ACID (IAA)-proteins degradation step in auxin signaling. However, the exact mechanism by which SMAP1 functions in auxin signaling remains unknown. Here, we demonstrated that *SMAP1* is required for normal plant growth and development, and roots response to IAA or methyl jasmonate in the *auxin resistant 1 (axr1)* mutation background. Deletion analysis and green fluorescent protein (GFP) / glutathione S-transferase (GST) pull-down assays showed that SMAP1 physically interacts with the CONSTITUTIVE PHOTOMORPHOGENIC-9 SIGNALOSOME (CSN) via the SMAP1 F/D-region. The extremely dwarf phenotype of *aar1-1 csn5a-1* double mutant confirms the functional role of *SMAP1* in plant growth and development under limiting CSN functionality. Our findings suggest that SMAP1 is involved in the auxin response and possibly in other cullin-RING ubiquitin ligase (CRL)-regulated signaling processes *via* its interaction with components associated with related-to-ubiquitin (RUB) modification.
Introduction

The plant hormone auxin plays an indispensable role in regulating various morphogenic processes such as root growth, shoot branching, and flower bud formation (Davies, 2004). Understanding the mode of action of auxin has been a major issue in plant physiology ever since the plant hormone concept was developed (Davies, 2004). Recent studies revealed an elegant signaling model for auxin, which is centered on SCFTIR1/AFBs ubiquitin E3 ligase (Woodward and Bartel, 2005). The SCF ubiquitin E3 ligase is a multi-subunit complex that regulates ubiquitin-dependent proteolysis of many proteins. This complex consists of S-phase kinase-associated protein 1 (SKP1; ASK1 for Arabidopsis SKP1), Cullin (CUL) 1, RING H2 finger (RBX1), and substrate-recognition F-box proteins (Woodward and Bartel, 2005). In SCFTIR1/AFBs, the F-box protein TRANSPORT INHIBITOR RESISTANT 1 (TIR1) or its homologs, the AUXIN F-box BINDING (AFB) proteins, function as auxin receptors (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Binding of auxin to the TIR1/AFBs receptors facilitates ubiquitin-mediated degradation of AUX/IAA repressors, which interact with transcriptional factors, AUXIN RESPONSE FACTORs (ARFs), resulting in changes in the patterns of downstream gene expression (Kim et al., 1997; Ulmasov et al., 1997; Tiwari et al., 2001; Dharmasiri et al., 2004; Tiwari et al., 2004). Another recently discovered F-box protein, S-phase kinase-associated protein 2A (SKP2A), directly binds auxin and promotes degradation of cell cycle transcription factors (Jurado et al. 2010).

The RELATED TO UBIQUITIN (RUB, also known as NEDD8 in mammals) protein is a small conserved protein that covalently binds to several regulatory proteins. One such regulatory protein is CUL, a scaffold protein in CUL-ring ubiquitin E3 ligases (CRLs), including SCF E3 ubiquitin ligase (Dreher and Callis, 2007). This RUB/NEDD8 modification is processed though a
series of ATP-dependent steps, similar to the ubiquitin conjugation cascade (Dreher and Callis, 2007). In Arabidopsis, a heterodimer of AUXIN RESISTANT 1 (AXR1) and E1 C-TERMINAL-RELATED 1 (ECR1) proteins functions as a RUB/NEDD8-activating enzyme (E1) and catalyzes ATP-dependent formation of a thioester bond between ECR1 and RUB/NEDD8 (del Pozo et al., 1999). Activated RUB/NEDD8 is transferred from ECR1 to RUB-conjugating enzyme 1 (RCE1), which functions as a RUB E2 enzyme and directly binds to RBX1, which is thought to function as a RUB E3 ligase in the CRL complex (Dharmasiri et al., 2003). In the case of RUB modification of CUL, a $\varepsilon$-amino group of a Lys residue in CUL interacts with the thioester of the RUB-E2 conjugate, resulting in formation of an isopeptide bond between RUB and CUL (Hotton and Callis, 2008). Another protein family, defective in cullin neddylation 1 (DCN1), has also been reported as RUB E3 ligases in human and yeast (Kurz et al., 2008). Although the biochemical activity of DCN1 proteins in plants is poorly understood, the loss of a DCN1-like gene causes a 2,4-D-resistant phenotype in Arabidopsis roots (Biswas et al., 2007). A large body of experimental evidence suggests that RUB modification mechanisms are significant for plant growth and development (Dreher and Callis, 2007).

An evolutionarily conserved complex, the CONSTITUTIVE PHOTOMORPHOGENIC 9 (COP9) SIGNALOSOME (CSN), is also required for activity of CRLs (Schwechheimer et al., 2001). One of the major activities of the CSN is to de-conjugate RUB/NEDD8 from RUB/NEDD8-conjugated CULs via the metalloprotease activity of its CSN5 subunit. RUB/Nedd8-associated and -dissociated CRLs are active and inactive forms, respectively (Saha and Deshaies 2008). A recent model of the RUB/NEDD8 modification cycle suggests that both RUB/NEDD8-conjugation and deconjugation of CUL proteins are required for optimal E3 activity (Cope and Deshaies, 2003; Hotton and Callis, 2008). The stability and efficiency of CRLs depend
on the RUB/NEDD8 modification status of CULs, and this status subsequently affects their E3 activity (Schwechheimer and Isono, 2010). Indeed, mutations in either component (i.e., those promoting RUB/NEDD8 conjugation or deconjugation) cause auxin-resistant phenotypes in Arabidopsis (Gray et al., 2001; del Pozo et al., 2002; Schwechheimer et al., 2001).

To better understand auxin signaling mechanisms, we used an inhibitor of auxin action, \( p \)-chlorophenoxyisobutylic acid (PCIB), to screen Arabidopsis mutants. This resulted in isolation of several \textit{antiauxin resistant} (\textit{aar}) mutants (Oono et al., 2003; Biswas et al., 2007). Some \textit{aar} mutations were located in previously known auxin-related loci such as \textit{tir1} and \textit{cul1}, whereas other mutations were found in unknown loci (Biswas et al., 2007). One of the mutants, \textit{aar1-1}, showed 2,4-dichlorophenoxyacetic acid (2,4-D)-specific resistance without any changes in 2,4-D transport or metabolism. The causal gene of \textit{aar1} was identified by map-based cloning and designated as \textit{SMALL ACIDIC PROTEIN 1} (\textit{SMAP1}) because it encodes a 62 amino-acid-protein (6.9 kDa) with a pI of 3.4. \textit{SMAP1} confers the sensitivity of Arabidopsis roots to 2,4-D and PCIB (Rahman et al., 2006). The Arabidopsis genome has another copy of the \textit{SMAP} gene, \textit{SMAP2}, which is expressed only in siliques and anthers and potentially mediates the root response to 2,4-D as \textit{SMAP2} overexpression restores the sensitivity of \textit{aar1} to 2,4-D (Nakasone et al., 2009). Physiological and genetic analyses of \textit{aar1} mutants and the \textit{SMAP1} gene suggested that the \textit{SMAP1} protein acts upstream of the degradation step of AUX/IAA proteins in auxin signaling (Rahman et al., 2006). Although the \textit{SMAP} protein has no known functional motifs, there is a highly conserved phenylalanine (F) and aspartic acid (D)-rich 18 amino acid sequence (F/D-region) in the C-terminal region. This region is found in \textit{SMAP} genes from a wide variety of plants and animals, implying that the \textit{SMAP} genes are evolutionarily indispensable (Rahman et al., 2006; Nakasone et al., 2009).
Although SMAP1 has been shown to regulate 2,4-D sensitivity and work upstream of ubiquitin proteasome pathway, the functional significance of this gene in auxin signaling remains unclear. In the present study, we tried to elucidate SMAP1 function by examining the genetic relationships among known auxin-related mutants, and attempted to identify SMAP1-interacting proteins. Our results suggested that SMAP1 physically interacts with CSN in Arabidopsis extracts, and its function is linked to the RUB modification components AXR1 and CSN.

Results

Genetic interaction of aar1 and auxin-related mutants

To investigate the relationship between aar1 and other known auxin mutants, we crossed the aar1-1 mutant with various mutants in auxin signaling pathway: tir1-1, a mutant of an auxin receptor (Dharmasiri et al., 2005a); ecr1-1, a mutant of a subunit of the RUB activating enzyme E1 (Woodward et al., 2007); axr1-12, a mutant of another subunit of the RUB activating enzyme E1 (Leyser, et al, 1993); and aar3-2, a mutant of a DCNI-like gene (Biswas et al., 2007), and attempted to establish double mutants. aar1-1 tir1-1, aar1-1 ecr1-1, and aar1-1 aar3-2 double mutants were successfully obtained. However, the axr1-12 aar1-1 mutant showed severe morphological defects; nearly one-quarter of the offspring from the AXR1/axr1-12 aar1-1 parents did not germinate or died at the early seedling stage without developing roots (Figure 1A–F, 2A, and 2B). Genotyping with PCR markers confirmed that the root-less siblings were axr1-12 aar1-1 double mutants (data not shown). In normal embryos, the hypophysis divides asymmetrically and forms a quiescent center in the root meristem. Formation of an auxin gradient is required for this
process, which can be readily visualized by authentic auxin reporter \textit{DR5rev:Green Fluorescent Protein (GFP)} (Friml et al., 2003). However, in the abnormal embryos, neither formation of quiescent center nor GFP expression was observed at the position where descendants of hypophyseal cells should be located (Figure 1B–E), suggesting that the \textit{axr1-12 aar1-1} lacks normal auxin response. The \textit{axr1-12 aar1-1} double mutant also showed severe morphological defects in the aerial parts. For example, venation was poorly developed compared with that in \textit{axr1-12} (Figure 1F), suggesting that the effect of the \textit{aar1} mutation is substantial in the \textit{axr1-12} background. Similar abnormal seedlings or ungerminated seeds were observed in the descendants of \textit{AXR1/axr1-3 aar1-1} or \textit{AXR1-12/axr1-12 aar1-2} parental lines (Figure 2C–F), suggesting that the abnormal phenotype in double mutants is not allele-dependent.

The \textit{aar1-1} mutation has an approximately 44-kbp deletion in chromosome 4; this deleted region contains at least 10 ORFs including \textit{SMAP1} (Rahman et al., 2006). Therefore, the abnormal phenotype could be caused by the lack of other genes, rather than \textit{SMAP1}. To eliminate this possibility, we crossed \textit{axr1-12} with two independent transgenic lines harboring a 3.7-kbp \textit{BamHI/SacI} genomic DNA fragment containing the \textit{SMAP1} ORF (\textit{gSMAP1}). As shown in Figure 1A and 2G–I, the seedling populations \textit{AXR1/axr1-12 aar1-1} \textit{gSMAP1} lines showed no root-less offspring, in contrast to the line harboring the control genomic fragment (X/B). In reverse, the homozygous offspring of the \textit{AXR1/axr1-12} heterozygous parental lines harboring the \textit{SMAP1} RNAi construct were root-less (Figure 2J–N). Taken together, these results suggest that \textit{SMAP1} is necessary for root meristem formation and normal embryo development in the \textit{axr1} background.

We successfully obtained the double mutants \textit{aar1-1 tir1-1}, \textit{aar1-1 ecr1-1}, and \textit{aar1-1 aar3-2}. Most of these double mutants did not show any severe morphological defects compared with their parental lines (data not shown). However, approximately 10% of seedlings from the
aar1-1 ecr1-1 parent showed a root-less phenotype similar to that of the axr1 aar1 double mutants (Figure 2Z). The root elongation assay against 2,4-D revealed that the double mutants aar1-1 tir1-1, aar1-1 ecr1-1, and aar1-1 aar3-2 are more resistant compared with their corresponding single mutants, suggesting an additive relationship between the aar1-1 mutation and the auxin-related mutations tir1-1, ecr1-1, and aar3-2 for regulating root auxin response. (Figure 1G).

Expression of SMAP1 under control of the CaMV 35S promoter in the axr1 background partially restored wild-type phenotype

SMAP1 was fused to GFP and expressed under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter (35S:SMAP1-GFP or 35S:GFP-SMAP1) in aar1-1 plants. The expression of fusion proteins in the transgenic lines restored wild-type sensitivity to PCIB and 2,4-D in the aar1-1 roots, suggesting that the SMAP1-GFP and GFP-SMAP1 fusion proteins are functionally active (Figure S1). The mature 35S:SMAP1-GFP or 35S:GFP-SMAP1 (collectively designated as 35S:SMAP1~GFP) transgenic Arabidopsis plants were morphologically indistinguishable from non-transgenic plants in either wild-type or aar1-1 backgrounds (data not shown). When 35S:SMAP1~GFP was introduced into the axr1-12 background (35S:SMAP1-GFP/axr1-12 or 35S:GFP-SMAP1/axr1-12), morphological phenotypes of axr1-12 such as dwarfism, reduced apical dominance, protruding pistils, and low fertility (Lincoln et al., 1990) were partially alleviated (Figure 3A). In root growth assay, the axr1-12 mutant is strongly resistant to auxin and other plant hormones such as jasmonic acid (JA) (Lincoln et al., 1990; Tiryaki and Staswick, 2002). The introduction of 35S:SMAP1~GFP also partially restored 2,4-D sensitivity of axr1-12 roots (Figure 3B). Furthermore, although the aar1 mutant is reported to be a 2,4-D specific response mutant (Rahman et al., 2006), the sensitivity of 35S:SMAP1~GFP/axr1-12
to IAA (Figure 3C) and methyl jasmonate (MeJA) (Figure 3D) was intermediate between that of
the wild type and axr1-12, suggesting that in absence of functional AXR1, SMAP1 function is
required for normal IAA and JA response.

Unexpectedly, unlike the SMAP1 genomic fragment, SMAP1-GFP fusion constructs
expressed under the control of the CaMV 35S promoter did not effectively recover the root-less
phenotype of the axr1 aar1 double mutant (Figure 2O–W). Although we established homozygous
axr1-12 aar1-1 35S:SMAP1-GFP (line 1H) and axr1-12 aar1-1 35S:GFP-SMAP1 (line 2B) lines
by crossing, many seeds from these lines did not germinate, and most of those that did germinate
were root-less (Figure 2Q and S). This may be due to the low expression level of SMAP1-GFP at
the early stage of embryo development, which could not complement the loss of the SMAP1 gene
from hypophyseal initial cells. GFP expression was hardly detected in the globular embryos of the
two 35S:SMAP1-GFP lines (Figure S2). The transgene of SMAP1-GFP under the control of the
SMAP1 promoter (SMAP:SMAP1-GFP line 1D) that was strongly expressed (as observed by GFP
fluorescence) in the lower part of the globular embryo (Figure S2) effectively rescued the
morphological defects in the axr1-12 aar1-1 double mutants (Figure 2X). Another independent
line of SMAP1:SMAP1-GFP (line 1B) that showed low-level GFP expression in the globular
embryo did not recover the root development in axr1-12 aar1-1 double mutant background (Figure
2Y, Figure S2). This suggests that strong SMAP1 expression in the globular stage embryo is
required to establish root initial organization in the axr1-12 background.

Conserved F/D rich region is significant for SMAP1 function

To investigate functional aspects of the SMAP1 protein, we constructed several versions
of SMAP1 derivatives, including those with deleted versions of the conserved F/D region, fused to
GFP, under SMAP1 promoter (approximately 5 kb), (Figure 4A). These constructs were transformed into the aar1-1 mutant, and their ability to complement the aar1 mutation was investigated (Figure 4B–D). The GFP fluorescence of the fusion proteins was detected in both nuclei and the cytosol (Figure S3).

The aar1 mutant is resistant to the antiauxin PCIB and the synthetic auxin 2,4-D, and exhibits long hypocotyl when grown in the light (Rahman et al., 2006). As shown in Figure 4B to 4D, independent transgenic lines harboring SMAP1-GFP in the aar1 background recovered sensitivities to PCIB (Figure 4B) and 2,4-D (Figure 4C) and showed similar hypocotyl length to that of the wild type (Figure 4D), suggesting that the SMAP1-GFP fusion protein is functional. The construct SMAP1∆F/D-GFP, which contained a deleted version of the F/D-rich region, did not complement the aar1 mutation, although the fusion protein was expressed in the transgenic lines as confirmed by GFP fluorescence. This finding implies that the F/D region is necessary for SMAP1 function. The N-terminal deletion construct D1, in which one-third of the N-terminal amino acids were deleted, was still able to complement the aar1 phenotype. However, the D2 and D3 constructs, which consisted of 26 and 18 C-terminal amino acids, respectively, did not complement the aar1 phenotype, suggesting that the F/D-rich region alone is not sufficient for SMAP1 function.

**SMAP1 interacts with the COP9 signalosome**

Using the transgenic plants described above, we next attempted to identify SMAP1-interacting proteins in Arabidopsis extracts. Total proteins were extracted from SMAP1-GFP, SMAP1∆F/D-GFP, and GFP lines using a non-denaturing extraction buffer and subjected to pull-down assays using anti-GFP microbeads. The purified GFP- and/or SMAP1-binding proteins were separated by SDS-PAGE and subjected to silver staining and
western blotting with an anti-GFP antibody (Figure 5A). Silver staining revealed that seven distinct bands are present in SMAP1-GFP extract but absent in SMAP1ΔF/D-GFP extract, suggesting that the proteins present in these bands are possibly SMAP1 interacting proteins (Figure 5A). These proteins were digested by trypsin, eluted from the gel, and analyzed by LC-MS/MS. Mascot database search of the resulting MS/MS spectra identified the proteins as CSN1, CSN2, CSN3, CSN4, cytosolic glyceraldehyde-3-phosphate dehydrogenase 2 (GAPC2), CSN5A, and CSN8 (Figure 5A and Table I). Of all the identified proteins, six out of seven were subunits of the large CSN complex (approximately 321.3 kDa) that typically consists eight subunits (Schwechheimer et al., 2001). Taken together, these results suggest that SMAP1 physically interacts with CSN in Arabidopsis extracts and this interaction requires the presence of the F/D region.

To further elucidate the interaction between SMAP1 and CSN, we established an in vitro binding assay with GST-tagged fusion proteins expressed in *Escherichia coli* (Figure 5B). The GST-tagged SMAP1 protein and its derivatives (GST-SMAP1, GST-SMAP1ΔF/D, GST-F/D, and GST) extracted from *E. coli*. were immobilized to glutathione columns. Total proteins, extracted from the Arabidopsis *aar1-1* mutant were added to these columns. Candidate interacting proteins as well as the GST fusion proteins themselves were eluted from the columns by SDS-sample buffer, separated by SDS-PAGE, and subjected to Coomassie blue staining and western blot analysis with an anti-CSN4 antibody (Figure 5B). The results showed that GST-SMAP1 and GST-F/D but not GST-SMAP1ΔF/D or GFP pulled down CSN4 protein from the *aar1-1* extracts, suggesting that the F/D region of SMAP1 is necessary and sufficient for the interaction between SMAP1 and CSN.

**Genetic interaction of SMAP1 and CSN**
CSN was originally identified through the biochemical characterization of the COP9 protein complex (Wei et al., 1994; Chamovitz et al., 1996), which plays a significant role in regulating photomorphogenesis and post-embryo development in Arabidopsis (Kwok et al., 1996). Furthermore, auxin responses are partially impaired in csn mutants (Dohmann et al., 2008). To investigate the interaction between SMAP1 and CSN at the genetic level, we crossed the aar1-1 mutant with the weak csn mutant csn5a-1, and established a line harboring homozygous aar1-1 and heterozygous csn5a-1 (aar1-1 CSN5A/csn5a-1). In the seedling population from aar1-1 CSN5A/csn5a-1 parental line, both in light- and dark-grown conditions, we observed distinct segregation of dwarf seedlings, readily distinguishable from either aar1-1 or csn5a-1 seedlings. (70 dwarf seedlings out of 363 light-grown seedlings; \( \chi^2 = 6.33, p > 0.01 \) (Figure 6A, 6B, and S4B). Genotyping analyses confirmed that these dwarf plants are aar1-1 csn5a-1 double mutant (data not shown). The longer hypocotyl phenotype of aar1 was suppressed by csn5a mutation. In fact, the double mutant showed extreme short hypocotyl phenotype. Similarly, slower root growth phenotype was found in double mutant, although the growth of the roots of aar1-1 and csn5a-1 is comparable to wild type (Figure 6A and S4A). In addition, the double mutant showed 2,4-D-resistance for root growth like its parental lines (Figure 6C). The aar1-1 csn5a-1 plants at the rosette stage also showed an extremely small seedling phenotype (Figure 6D) and most of the plants died before producing seeds (data not shown).

When the 35S:SMAP1-GFP (line 1H) gene was introduced into the aar1-1 csn5a-1 background (35S:SMAP1-GFP/aar1-1csn5a-1) by crossing, normal hypocotyl length was restored (Figure 6A and B), suggesting that the lack of the SMAP1 gene is associated with the extreme dwarf hypocotyl phenotype of the aar1-1 csn5a-1 double mutant. When the seedlings were transferred from agar plates and further grown on soil, the morphology of the
35S:SMAP1-GFP/aar1-1csn5a-1 plant was intermediate between that of the wild type and the csn5a-1 mutant (Figure 6E and S4C), suggesting that the ectopic expression of the SMAP1 gene not only complemented the aar1-1 phenotype but also partially compensated the morphological abnormalities of the csn5a-1 mutant. Taken together, these results suggest that SMAP1 is required for normal development of seedlings under limiting CSN activity.

**RUB modification status of CUL1 in the double mutants and transgenic lines**

The results from two independent experimental approaches implied that SMAP1 interacts with the RUB modification-related factors AXR1 and CSN. Thus, we next examined the RUB modification status of the CUL1 protein, which is a core subunit of SCF E3 ligase and one of the most characterized RUB-modified proteins, in the double mutants and transgenic lines by western blotting using anti-CUL1 antibody (Figure 7). As previously published, the ratio of RUB-modified and unmodified CUL1 is lower in axr1-12 and higher in csn5a-1 compared with that in the wild type (Gray et al., 2001; del Pozo et al., 2002; Gusmaroli et al., 2007). No significant difference was observed in RUB modification depending on the presence or absence of SMAP1 gene, except a slight increase in RUB-modified CUL1 ratio was detected in the flower extracts of transgenics harboring 35S:SMAP1~GFP in axr1-12 background (Figure 7B).

**Discussion**

The functional significance of the SMAP1 protein, which confers 2,4-D sensitivity in Arabidopsis, in auxin signaling pathway remained obscure in spite of the genetic and physiological characterization of the aar1 mutants (Rahman et al., 2006; Nakasone et al., 2009). In the present study, using a combinatorial approach of genetics and biochemistry, we demonstrated that the
SMAP1 protein interacts with the RUB modification components, AXR1 and CSN, and plays an important role in regulating the growth and development of Arabidopsis seedlings under limiting AXR1 or CSN function. Both AXR1 and CSN have important roles in CRL-mediated signaling processes, including the auxin response, via RUB modification. AXR1 facilitates the RUB modification and CSN functions in deconjugating RUB from RUB modified proteins. The functional significance of these proteins in auxin signaling has been well demonstrated (Schwechheimer et al., 2001). The genetic and biochemical evidences presented here, suggest that SMAP1 associates with the RUB modification cycle, which is consistent with the previous report where we demonstrated that SMAP1 functions upstream of AUX/IAA protein degradation in Arabidopsis (Rahman et al., 2006).

Our initial characterization of SMAP1 demonstrated that plants with diminished SMAP1 function (aar1 mutants and SMAP1 RNAi lines) show altered responses to 2,4-D but not to IAA or other major plant hormones (Rahman et al., 2006). However, the results in this work suggest that under limited functionality of RUB modification components AXR1 and CSN, SMAP1 potentially regulates IAA and JA signaling pathways along with 2,4-D. The root phenotypic difference observed between axr1 aar1 and axr1 tir1 double mutants is striking. The axr1 aar1 double mutant showed severe morphological defects with no root meristem formation, while the tir1 aar1 double mutant showed no apparent root developmental phenotype, although they showed increased 2,4-D resistance compared with their respective parental lines. The tir1 axr1 double mutant mimics the tir1 aar1 root phenotype, showing no apparent change in root development and increased auxin resistance (Ruegger et al., 1998). The inability of axr1 aar1 to form root meristem suggests a strong functional interaction between AXR1 and SMAP1. This idea is further substantiated by the fact that in adult plants, the over expression of SMAP1 restored wild-type-like phenotype in axr1
mutant background, suggesting that SMAP1 functionally cooperates with AXR1 in regulating various biological processes including embryogenesis, morphology of mature plants, and hormonal responses. A similar genetic feature was reported for the AXR1 homolog gene, AXR1-LIKE (AXL). The single mutant of axl did not show any remarkable phenotype, while the axr1 axl double mutant exhibited a root-less phenotype (Dharmasiri et al., 2007). Ectopic expression of AXL under the control of CaMV 35S promoter in the axr1 background complemented the axr1 phenotype (Dharmasiri et al., 2007). Since both the AXR1 and AXL encode a subunit of the RUB E1 enzyme and promote RUB modification to regulate CRL activity, one possible explanation for SMAP function could be its involvement in RUB modification process.

In vivo GFP and in vitro GST pull-down assays revealed that SMAP1 interacts with CSN. The CSN genes were originally identified as causal genes of constitutive photomorphogenesis (cop), de-etiolated (det), or fus mutants, which show defects in photomorphogenesis or embryogenesis (Chamovitz et al., 1996). CSN influences numerous plant hormone signaling pathways including the auxin, jasmonate, and strigolactone pathways, as well as light signaling, cell cycle progression, and circadian rhythm (Schwechheimer and Isono, 2010; Somers and Fujiwara, 2009). The best-defined function of CSN is to regulate the ubiquitin/proteasome-dependent protein degradation system by removing the covalently conjugated RUB protein from CULs and by stabilizing CRL components, such as TIR1, CUL1, and ASK1 (Schwechheimer and Isono, 2010; Stuttmann et al., 2009). The fact that the functionally significant F/D region is necessary and sufficient for SMAP1 to interact with CSN implies that SMAP1 is involved in regulation of CRL activity together with CSN.

In our pull-down analysis of SMAP1-GFP, we detected six out of eight CSN subunits, but did not detect CSN6 and CSN7 (Table 1 and Figure 5A). This could be due to similar molecular
masses of CSN6, CSN7 and GFP-SMAP1, which results in an inadequate separation of these proteins in gel. Alternatively, SMAP1 may interact with a CSN subcomplex that lacks CSN6 and CSN7. A structural analysis in mammalian cells demonstrated that several CSN subcomplexes exist, for example, CSN1/2/3/8, CSN4/5/6/7, or smaller versions including CSN1/3/8 and CSN4/6/7 (Sharon et al., 2009). Two symmetrical modules, CSN1/2/3/8 and CSN4/5/6/7 are connected by interaction between CSN1 and CSN6 to form eight-subunit CSN complex. In the CSN1/2/3/8 and CSN4/5/6/7 subcomplexes, the most peripheral subunits are CSN2 and CSN5, respectively. Furthermore, the subcomplex CSN4/5/6/7 was found to be very stable (Sharon et al., 2009). Therefore, it is difficult to predict that CSN subcomplexes lacking CSN6 or CSN7 are present in plant extracts, although the binding patterns of CSN6 and CSN7 are unknown in plant cells. Further biochemical experiments are required to clarify how SMAP1 physically interacts with CSN. CSN in mammalian cells is involved in various biological responses including embryonic development, cell cycle progression, T-cell development, signal transduction, oocyte maturation, autophagy, and circadian rhythm (Seeger et al., 1998; Kato and Yoneda-Kato, 2009). Thus, a future challenge is to determine whether SMAP proteins in mammalian cells also interact with mammalian CSN and have a regulatory role in CRL-mediated biological processes.

The strong morphological defects of axr1 aar1 double mutants and increase of RUB-modified CUL1 in 35S:SMAP1–GFP/axr1-12 transgenic plants suggests that SMAP1 acts as a positive regulator for RUB modification. Because CSN activates dissociation of RUB from RUB-modified CULs, one possible explanation for SMAP1 function is that SMAP1 inhibits RUB-dissociation activity of CSN by its binding to CSN. Given the marked morphological defects in axr1 and csn mutants, small change of RUB modification status could be enough to cause the observed morphological changes in presence or absence of SMAP1. However, we cannot eliminate
the possibility that increase of RUB-modified CUL1 in flower extracts of 35S:SMAP1-GFP/axr1-12 transgenic plants might be a secondary effect resulting from the morphological changes. Even if this is the case, the morphological defects of the double mutants and physical interaction between SMAP1 and CSN imply that SMAP1 interacts with RUB cycle-related regulation of CRL activity. In a recent model in fission yeast, CSN prevents autocatalytic ubiquitin-mediated degradation of certain F-box proteins that are assembled in the CRL, resulting in maintenance of the CRL complex in absence of the substrates (Schmidt et al., 2009). Stabilization of CRL components by CSN in Arabidopsis extracts has also been reported (Stuttmann et al, 2009). Thus, it may be possible that SMAP1 affects the stability of CRL components and CRL assembly by binding with CSN. Construction of more sensitive biochemical experimental systems for monitoring RUB-modification status, CRL activity, and CRL stability in planta and in vitro would clarify how SMAP1 contributes to RUB cycle-related regulation of CRL activity.

In conclusion, two independent experimental approaches indicate that the conserved SMAP1 protein interacts with RUB modification-related components. Although the precise mode of action of SMAP1 is still unclear, further biochemical research on the functions and the relationship of SMAP1 with AXR1 and CSN might reveal its biological role in the RUB cycle, as well as in the CRL-regulated ubiquitin-proteasome system in plant cells.

**Materials and Methods**

*Plant Materials*
The Arabidopsis lines *aarl-1* and *aarl-2* (Rahman et al., 2006), *aar3-2* (Biswa et al., 2007), *axr1-3* and *axr1-12* (Leyser et al., 1993), *tir1-1* (Ruegger et al., 1998), *ecrl-1* (Woodward et al., 2007), *csn5a-1* (Gusmaroli et al., 2007), and *DR5rev:GFP* (Friml et al., 2003) were described previously. The *SMAP1* RNAi lines (520i), the transgenic lines transformed with the 3.7-kb *SMAP1* genomic fragment (B/S, described as *gSMAP1* in this report) in the *aarl-1* background, and its control line (X/B) were described in Rahman et al. (2006). 35S:SMAP1-GFP/aar1-1, 35S:GFP-SMAP1/aar1-1, SMAP1:SMAP1-GFP/aar1-1 and their derivative lines were generated in this work as described below. All transgenic and mutant lines were derived from the Columbia (Col-0) accession of *Arabidopsis thaliana* (L.) Heynh except for *aarl-2*, which is in the Wassilewskija (Ws) background. The *AAR1* locus was genotyped by PCR using primers T6G15 9538F and T6G15 53634R to detect the DNA deletion in the *aarl-1* genome, and T6G15 24488F and AT4G1 3490F2 to detect the presence of the corresponding wild-type DNA. For *aarl-2*, the mutant locus was tracked by the kanamycin-resistant phenotype resulting from the *NPTII* gene in the transgene construct (Rahman et al., 2006). The *aar3-2* mutation was genotyped using the primers K5K13 F38890 and K5K13 R39225 to detect the wild-type gene, and K5K13 F38890 and LB1 to detect the T-DNA. The *tir1-1* mutation was detected by PCR amplification using the primers TIR1gF547 and TIR1gR913, and the amplified product was digested with *DpnII*, which cuts mutant but not wild-type products. The *axr1-12* mutation was detected by PCR with the primers AXR1-12F and AXR1-12R, and the amplified product was digested with *DraI*, which cuts mutant but not wild-type products. For *csn5a-1*, the primers CSN5A-1F and CSN5A-1R2 were used to detect the wild-type locus and the primers CSN5A-1F and LBb1 were used to detect the mutation. The primer sequences are listed in Table S1. The *ecrl-1* and *axrl-3* mutations were
detected by derived cleaved amplified polymorphic sequence (dCAPS) analysis as described by Woodward et al. (2007).

Sequence data for this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *SMA1* (At4g13520), *AXR1* (At1g05180), *TIR1* (At3g62980), *AAR3* (At3g28970), *ECR1* (At5g19180), *CSN1* (At3g61140), *CSN2* (At3g26990), *CSN3* (At5g14250), *CSN4* (At5g42970), *CSN5A* (At1g22920), *CSN8* (At4g14110), *GAPC2* (At1g13440), *CUL1* (At4g02570).

*Growth analyses*

Seed sterilization and plant growth conditions were as described by Biswas et al. (2007). Briefly, seeds were plated on GM (half-strength Murashige and Skoog salts, 1% [w/v] sucrose and 0.5 gL⁻¹ MES, pH 5.8, containing 1 × B5 vitamins and 0.8% [w/v] Bacto agar) in rectangular plates, unless otherwise mentioned. For synchronous germination, the seeds were kept in the dark for 2 d at 4°C, and then the plates were transferred to a growth chamber at 23°C. For the hypocotyl and root growth assays, the plants were grown vertically on square culture plates under continuous white light (20–30 µmol m⁻² sec⁻¹). For transfer assays, seedlings were first grown on GM without plant growth regulators and then transferred after 4–5 days to fresh GM containing plant growth regulators. For the IAA root inhibition assay, seedlings were transferred onto GM 1.2 (same as GM except the agar concentration is 1.2% to prevent roots from penetrating into the agar). The hypocotyl and root lengths were analyzed using the NIH ImageJ software package (National Institution of Health, USA) after the plants were photographed with a DP50 digital camera (Olympus Corp., Tokyo, Japan). The percentage of root growth inhibition was calculated relative
to root growth on media without growth regulators. To observe morphology of plants grown on soil, the seedlings were grown in a growth chamber (BIOTRON, LH-200-RDS, NK system, Tokyo, Japan) under a 16-h light (100-130 µE m⁻²)/8-h dark photoperiod at 23°C.

**Plasmid construction and plant transformation**

All plasmids were constructed using standard recombinant DNA techniques, and their authenticity was confirmed by DNA sequencing. The SMAP1 DNA fragment was amplified from Arabidopsis BAC clone T6G15. The \( \text{SMAP1}:\text{SMAP1-GFP} \) fusion construct and its deleted versions were made by inserting appropriate DNA fragments into the binary vector pEGAD containing GFP and the \( \text{nos} \) terminator (Cutler et al., 2000). The \( \text{35S} \) promoter was removed from pEGAD by digestion with \( \text{SacI} \) and \( \text{AgeI} \), then an approximately 5-kb region of the \( \text{SMAP1} \) promoter and the full or deleted version of \( \text{SMAP1} \) coding sequences were inserted into the restriction enzyme sites. To generate \( \text{35S:SMAP1-GFP} \) and \( \text{35S:GFP-SMAP1} \), \( \text{SMAP1} \) coding sequences without or with the stop codon were amplified with the primer sets 13520ATG-topo/13520-TAA or 13520ATG-topo/13520R+TAA (Table S1), respectively, and cloned into the pENTR/D-TOPO vector (Invitrogen Corp., CA, USA). The resulting plasmids, \( \text{13520-TAA ENTR} \) or \( \text{13520+TAA ENTR} \), were assembled into the GATEWAY binary vector pK7FWG2 to generate \( \text{35S:SMAP1-GFP} \) or into pK7WGF2 to generate \( \text{35S:GFP-SMAP1} \), respectively, by the LR reaction, via a site-specific recombination reaction between \( \text{attL} \) and \( \text{attR} \) sites (Karimi et al., 2005). For the \( \text{in vitro} \) pull-down assay, GST-SMAP1 and its derivatives were constructed with the pGEX4T-2 expression vector (GE Healthcare UK Ltd., Buckinghamshire, UK). Full details of plasmid construction are available on request.
The resulting constructs in binary vectors were introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) by electroporation and used to transform Arabidopsis Columbia (Col-0) with the floral-dip method (Clough and Bent, 1998). T1 seeds obtained from infected plants were germinated and selected on GM containing the appropriate antibiotic for selection of the marker gene in the vectors. In the T2 generation, T1 lines that showed a segregation ratio of 1:3 for antibiotic-sensitive: antibiotic-resistance, were selected and antibiotic–resistant T2 seedlings were grown to harvest T3 seeds. T3 plants were expected to be offspring of T2 plants that were hetero- or homozygous for the inserted gene. Therefore, T2 lines that gave rise to only antibiotic-resistant T3 plants were selected as homozygous lines.

*Observations of embryo morphology and GFP fluorescence*

For observations of embryo morphology, siliques at different developmental stages were harvested, dissected under a stereoscope, and cleared in a derivative of Hoyer’s solution (chloral hydrate/glycerol/water, 8:1:2). Cleared ovules were removed from their siliques in a drop of the same clearing solution, mounted whole, and observed under an Olympus BX60 microscope (Olympus, Tokyo, Japan) equipped with Nomarsky optics. Digital images were captured using an Olympus DP-50 digital camera. To detect GFP accumulation, embryos were dissected in 7% glucose, mounted whole, and then fluorescent signals in roots and embryos were detected under a confocal laser-scanning microscope (Olympus Fluoview FV1000 with digital imaging processing) with a 515 ± 10-nm band pass filter. For signal localization in embryos, images of GFP and transmitted light channels were electronically overlaid and further processed with PHOTOSHOP software (Adobe Systems, Mountain View, CA, USA).
**Pull-down assay**

To detect SMAP1-GFP binding proteins, we used a µMACS GFP isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The 7-d-old plants grown under continuous light were ground in liquid nitrogen and then ground in ice-cold elution buffer [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 1% Triton X-100, protease inhibitor complete mini (Roche Diagnostics, Mannheim, Germany)]. The extract was centrifuged for 20 min at 13,000 g, and the supernatant was filtered through a syringe-driven filter unit with 0.45 µm pore size (Millipore, MA, USA). The solution containing 1 mg total protein was mixed with magnetic anti-GFP micro beads and then incubated overnight to allow GFP to bind to anti-GFP micro beads. The mixture was transferred to a pre-washed column containing magnetic beads, and then the column was washed four times with wash buffer 1 (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40), and once with wash buffer 2 (20 mM Tris-HCl pH 8.0) to flush unbound proteins and excess salt. GFP and proteins bound to it were eluted with 50 µL SDS-sample buffer. Eluted proteins were heat-denatured and separated by SDS-PAGE on a 12.5% gel. The protein spots in the silver-stained gel were cut out and digested with trypsin (Promega Corporation, WI, USA). The digested peptides were analyzed by an LTQ-Orbitrap XL mass spectrometer (LC-MS/MS; Thermo Scientific Inc., MA, USA) and MS/MS spectra were compared against TAIR8 Arabidopsis genome annotation data using the Mascot server (Matrix Science Ltd., London, UK).

For the GST pull-down assay, *E. coli* lines harboring the constructs were grown to an OD₅₉₅ of 0.5–0.6, and then expression of fusion proteins was induced by isopropyl
β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 2 h. Extraction of fusion proteins and their immobilization to the glutathione column were performed using a GST-Spin Trap Purification Module kit (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer’s protocol. The columns containing immobilized GST-SMAP1 fusion protein were washed with PBS, and then flushed with ice-cold plant GST extraction buffer [50 mM Tris-HCl pH 7.0, 10 mM MgCl₂, 150 mM NaCl, 10% glycerol, 0.01% NP-40, 0.5 mM PMSF, protease inhibitor complete mini (Roche Diagnostics)]. Total protein was prepared from 7-d-old aar1-1 plants as described above for the GFP pull-down assay, except that plant GST extraction buffer was used, and applied to the columns. The columns were incubated at room temperature for 10 min and washed five times with 600 µL plant GST extraction buffer. The GST and proteins bound to it were eluted with GST elution buffer (50 mM Tris-HCl pH 7.0 and 10 mM glutathione), and 10 µg of the eluted protein was separated by SDS-PAGE.

*Antibodies and western blotting analyses*

We used antibodies against GFP (1181446001, Roche Diagnostics) and CSN4 (PW8360, Enzo Life Sciences Inc., Farmingdale, NY, USA). For the *Arabidopsis* CUL1 antibody, the N-terminal region of CUL1 protein (380 amino acid residues of the N-terminal of CUL1) was inserted into the pET16b vector (Novagen, San Diego, CA, USA) and expressed in the *E. coli* strain BL21(DE3) (Novagen). The insoluble protein fraction was solubilized in His-binding buffer (20 mM Tris HCl pH 7.9, 0.5 M NaCl, 5 mM imidazole and 6 M guanidine HCl), bound to Ni-NTA agarose (QIAGEN K.K., Tokyo Japan), washed with washing buffer (20 mM Tris HCl pH 7.9, 0.5 M NaCl,
60 mM imidazole, and 6 M guanidine HCl) and eluted with elution buffer (20 mM Tris HCl pH 7.9, 0.5 M NaCl, 250 mM imidazole, and 6 M guanidine HCl). The eluted protein was precipitated by addition of 80% cold acetone, and then proteins were separated by SDS-PAGE and purified by electroelution from the gel. The anti-CUL1 antibody was prepared from a rabbit injected with the purified CUL1 protein. For RUB modification analysis of CUL1, Arabidopsis seedlings or flowers were ground in lysis buffer (125 mM Tris-HCl, pH 8.5, 50 mM NaS2O3, 1% SDS, 10% glycerol). The extracts were centrifuged at 13,000 g for 10 min, and the protein concentration in the supernatant diluted 1:9 with water was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA USA). Protein samples were boiled in SDS-PAGE sample buffer, run on SDS-PAGE gels, and blotted onto PVDF membranes. For detection, alkaline phosphatase conjugated anti-rabbit IgG (A3687 Sigma-Aldrich, St Louis, MO, USA) as secondary antibody, and NBT/BCIP solution (Roche Diagnostics) were used. For densitometry analyses, the digital images of the blots were acquired by scanning the blots and the peak intensity of the bands was determined by Quantity One software (Bio-Rad Laboratories). The values of the ratio of RUB-modified CUL1 to total CUL1 (RUB-modified plus unmodified CUL1) were analyzed by Fisher's Least Significant Difference (LSD) test in KaleidaGraph software (Synergy Software, Reading, PA USA).

Acknowledgments

We thank the Arabidopsis Biological Resource Center (Columbus, OH), the Salk Institute Genomic Analysis Laboratory (La Jolla, CA), and Dr. Bonnie Bartel (Rice University) for providing biological materials used in this work. We also thank Chihiro Suzuki (JAEA) for technical assistance, and Drs. Tomoki Chiba (Tsukuba University), Tomohiro Kiyosue (Gakusyuin
University), and members of Ion Beam Mutagenesis Research Group at JAEA for helpful discussions. We are grateful to Dr. William Gray (The University of Minnesota) for critical reading of the manuscript.

Literature Cited


Publishers, Dordrecht.


Arabidopsis COP/DET/FUS genes is necessary for repression of photomorphogenesis in darkness. Plant Physiol. 110: 731–742


repression domain. Plant Cell 16: 533–543


Figure legends

Figure 1. Genetic interaction between SMAP1 and auxin-related mutants

(A) Comparison of early seedling phenotype in wild type (WT), *axr1-12, aar1-1, axr1-12 aar1-1* double mutant (*axr1 aar1*) and the double mutant containing the genomic SMAP transgene (*axr1 aar1+ gSMAP1*). The seedlings were grown for 6 days in GM media and photographed. Please notice the rootless phenotype of *axr1 aar1* double mutant and complementation of the phenotype by genomic SMAP1.

(B and C) Normal (left) and abnormal (right) early globular (B) and torpedo (C) stage embryos in siliques of *AXR1/axr1-12 aar1-1* plants.

(D) Visualization of auxin response pattern in normal (left) and abnormal (right) torpedo stage embryos in siliques of *AXR1/axr1-12 aar1-1* plants containing *DR5rev: GFP*. GFP expression was absent in hypophysis of abnormal embryos.

(E) Mature seeds with normal (left) and abnormal (right) embryos obtained from *AXR1/axr1-12 aar1-1* plants.

(F) Venation patterns in cotyledons of 5-d-old wild type, *axr1-12, aar1-1*, and *axr1 aar1*.

(G) Response of single and double mutants to 2,4-D in root growth assay. Seedlings were germinated and grown on GM containing 2,4-D. Elongation of roots was measured from day 4 to 7. Data are mean ± SD (*n* = at least 10 seedlings). * The root-less seedlings (see Figure 2Z) were excluded.

Figure 2. Frequency of root-less seedlings in various genetic backgrounds.

Frequency of normal (blue), root-less (red), and non-germinated (yellow) phenotypes in offspring seedling populations from parent plants of indicated genotype. N indicates number of seedlings
observed. Vertical orange line highlights 3:1 expected ratio for normal:abnormal seedlings from parent lines. Because axr1 aar1 double mutant was postembryonic lethal, seeds harvested from AXR1/axr1 heterozygote background were tested in most of the experiments. Transgenes were introduced by crossing.

(A-F) Phenotype of axr1 aar1 double mutants. *Ws accession.

(G-I) Complementation of root-less phenotype of axr1 aar1 by genomic fragment containing SMAP1 (gSMAP1). Genomic fragment (X/B) without SMAP1 was used as control (I). Two independent transgenic lines for gSMAP1 (B/S lines 2D and 1G) and the control line X/B line 1C with the aar1-1 mutation (Rahman et al., 2006) were crossed with axr1-12 mutants to generate parental lines.

(J-N) Inactivation of SMAP1 by RNAi in axr1 background. Two independent 520i lines (line 1G and 2C) or control line (line F2) transformed with vector [pB7GWlWG2(II)] (Rahman et al., 2006) were crossed with axr1-12 mutants to generate parental lines.

(O–W) Expression of SMAP1/GFP fusion protein with under control of 35S promoter in axr1 aar1 background. Transgenic lines of 35S:SMAP1-GFP/aar1-1 (line 2G and 1H) or 35S:SMAP1-GFP/aar1-1 line 2B (Figure S1) were crossed with axr1-12 (O–S) or axr1-3 (T–W) mutants.

(X and Y) Complementation of root-less phenotype of double mutant by SMAP1:SMAP1-GFP. Two independent transgenic lines (D4 and B4) of SMAP1:SMAP1-GFP/aar1-1 were crossed with axr1-12 mutant to generate parental lines.

(Z) Root-less phenotype was observed in approximately 10% of aar1-1 ecr1-1 seedlings.

Figure 3. Phenotypic changes resulting from introduction of 35S:SMAP1~GFP fusion genes into axr1-12 mutant.
Independent transgenic lines of 35S:SMAP1-GFP (line 1H and 2G) and 35S:GFP-SMAP1 (line 2B) were crossed with axr1-12 and homozygous lines were established for axr1-12, transgene, and wild-type AAR1 locus. (A) Mature plants (27-d-old) (top), inflorescences (middle), and flowers (bottom) are shown. Bar = 1 cm (top and middle). Bar = 0.5 cm (bottom).

(B) Seeds were germinated and grown on GM containing 2,4-D at indicated concentrations. Increase in root length was measured from day 4 to 7. Data are mean ± SD (n > 9 seedlings).

(C) Seeds were germinated and grown on GM for 4 days, seedlings were then transferred onto GM containing IAA and grown for further 3 days. Root elongation after transfer was measured and plotted as a relative value compared with that on medium without chemicals. Data are mean ± SD (n = at least 21 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 1.56±0.25 (WT), 2.23±0.41 (axr1-12), 1.58±0.37 (35S:SMAP1-GFP/axr1-12 1H), 1.76±0.24 (35S:SMAP1-GFP/axr1-12 2G), and 1.64±0.47 (35S:GFP-SMAP1/axr1-12 2B).

(D) Seeds were germinated and grown on GM containing 10 µM MeJA. Root elongation was measured from day 5 to 8 and plotted as a relative value compared with that on medium without chemicals. Data are mean ± SD (n = at least 5 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 2.05±0.26 (WT), 2.63±0.19 (axr1-12), and 2.19±0.24 (35S:SMAP1-GFP/axr1-12 1H), respectively.

Figure 4. Construction and analyses of GFP fusion constructs with SMAP1 and SMAP1 deletion series.

(A) Schematic diagram of constructs. SMAP1 and GFP coding regions are shown by white and green boxes, respectively. F/D rich region in SMAP1 is shown by blue box. SMAP1 promoter is shown by black arrows. NOS terminator is not shown. Dotted lines indicate deleted region in the
constructs. **SMAP1-GFP** contains full-length **GFP**. D1, D2, and D3 are N-terminal deleted constructs. Numbers on white boxes indicate positions of amino acids within N- and C-terminal of modified SMAP1.

**(B and C)** Transgenic **aar1-1** lines, wild type (WT), and untransformed **aar1-1** were planted on GM containing 20 µM PCIB (B) or 50 nM 2,4-D (C) and root length was measured after 10 days. Data are mean ± SD (n = at least 13 seedlings).

**(D)** Hypocotyl length of 7-d-old seedlings grown on GM in the light. Data are mean ± SD (n = at least 15 seedlings).

**Figure 5. Physical interaction between SMAP1 and CSN.**

**(A)** *In vivo* pull-down assay using GFP-tagged proteins in **aar1-1** transgenic plants. Two transgenic lines, **SMAP1-GFP/aar1-1** and **SMAP1ΔF/D-GFP/aar1-1**, were used in pull-down assay. Pulled-down proteins were separated by SDS-PAGE and detected by western blotting using anti-GFP or by silver staining. Seven protein bands (marked by red circles) that bind to **SMAP1-GFP/aar1-1** but not to **SMAP1ΔF/D-GFP/aar1-1** were eluted and identified by LC-MS/MS (Table I).

**(B)** *In vitro* pull-down assay using GST-tagged protein constructs. Top: schematic diagram of constructs of GST-tagged proteins, GST control, GST-tagged full-length SMAP1 (GST-SMAP1), F/D region-deleted version of SMAP1 (GST-SMAP1ΔF/D), and the F/D region only (GST-F/D). SMAP1 and GST coding regions are shown as white and pink boxes, respectively. Blue boxes indicate F/D rich region in **SMAP1**. Black arrow shows **Ptac** promoter for expression of fusion protein in *E. coli*. Bottom: unpurified total proteins from **aar1-1** plant (left lane) and eluted proteins from glutathione columns (other lanes) were separated by SDS-PAGE and detected by Coomassie
blue (CBB) or western blot with anti-CSN4 antibody.

**Figure 6. Genetic interaction of CSN5A and SMAP1.**

We could not obtain sufficient number of seeds of aar1-1 csn5a-1 double mutant; therefore, dwarf aar1-1 csn5a-1 seedlings were selected from seed population from the aar1-1 CSN5A/csn5a-1 parental line for analysis.  

**(A and B)** Images (A) and root lengths (B) of 7-d-old seedlings grown on GM without growth regulators. Bar in (A) = 1 cm. Values in (B) are mean ± SD (n = at least 13 seedlings).  

**(C)** Seeds were germinated and grown on GM for 4 days, then transferred onto GM containing 2,4-D at indicated concentrations and grown for additional 3 days. Root elongation after transfer was measured and plotted as a relative value compared with that on medium without chemicals. Values are mean ± SD (n = at least 17 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 1.56±0.18 (WT), 1.64±0.22 (aar1-1), 1.71±0.30 (csn5a-1), and 1.21±0.22 (aar1-1 csn5a-1), respectively.  

**(D)** Photographs of plants at rosette stage. Seeds were germinated on GM and grown for 9 days, seedlings were then transferred to soil and grown for additional 11 days under 16-h light /8-h dark conditions. White arrowheads indicate aar1-1 csn5a-1 double mutants. The photograph at bottom left is an enlarged image of a aar1-1 csn5a-1 double mutant.  

**(E)** Photographs of adult plants. Seeds were germinated and grown on GM for 9 days, seedlings were then transferred to soil and further grown for 35 days under 16-h light / 8-h dark conditions. Bar = 5 cm.

**Figure 7. RUB modification of CUL1 in double mutants and transgenic lines.**
Total proteins (10 µg) extracted from 7-d-old (A) light grown seedlings or (B) flowers were separated by SDS-PAGE, then immunodetected using anti-CUL1 antibody. From left to right: wild type (WT), aar1-1, axr1-12, axr1-12 aar1-1 double mutant, 35S:SMAP1-GFP/axr1-12 1H, 35S:SMAP1-GFP/axr1-12 2G, 35S:GFP-SMAP1/axr1-12 2B, csn5a-1, aar1-1 csn5a-1 double mutant, 35S:SMAP1-GFP/aar1-1 csn5a-1 1H, and 35S:SMAP1-GFP/csn5a-1 1H are subjected to the analysis for A. We could not obtain flowers for the axr1-12 aar1-1 or aar1-1 csn5a-1 double mutant; therefore they are excluded from B.

Densitometry analyses of the ratio of RUB-modified CUL1 to total CUL1 (RUB-modified plus unmodified CUL1) are presented below the western blot images that show mean and SD derived from 4 or 3 (for A or B, respectively) independent experiments. Significant differences (P<0.05) between mean values are indicated by different letters above the bars.
Table I. LC-MS/MS identification of pull-down proteins using SMAP1-GFP protein.

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Protein Name</th>
<th>Theoretical Mass (kDa)</th>
<th>Mascot Score</th>
<th>Matched Peptides</th>
<th>Sequence Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CSN1 (At3g61140.1)</td>
<td>50.6</td>
<td>400</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>CSN2 (At2g26990.1)</td>
<td>51.1</td>
<td>142</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>CSN4 (At5g42970.1)</td>
<td>45.0</td>
<td>864</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>CSN3 (At5g14250.1)</td>
<td>47.7</td>
<td>370</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>CSN5A (At1g22920.1)</td>
<td>39.7</td>
<td>288</td>
<td>14</td>
<td>51</td>
</tr>
<tr>
<td>6</td>
<td>GAPC2 (At1g13440.1)</td>
<td>36.9</td>
<td>91</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>CSN8 (At4g14110.1)</td>
<td>22.5</td>
<td>111</td>
<td>3</td>
<td>18</td>
</tr>
</tbody>
</table>

*aThe numbers are shown in Figure 5A.
Figure 1. Genetic interaction between SMAP1 and auxin-related mutants

(A) Comparison of early seedling phenotype in wild type (WT), axr1-12, aar1-1, axr1-12 aar1-1 double mutant (axr1 aar1) and the double mutant containing the genomic SMAP transgene (axr1 aar1 + gSMAP1). The seedlings were grown for 6 days in GM media and photographed. Please notice the rootless phenotype of axr1 aar1 double mutant and complementation of the phenotype by genomic SMAP1.

(B and C) Normal (left) and abnormal (right) early globular (B) and torpedo (C) stage embryos in siliques of AXR1/axr1-12 aar1-1 plants.

(D) Visualization of auxin response pattern in normal (left) and abnormal (right) torpedo stage embryos in siliques of AXR1/axr1-12 aar1-1 plants containing DR5rev:GFP. GFP expression was absent in hypophysis of abnormal embryos.

(E) Mature seeds with normal (left) and abnormal (right) endosperm in AXR1/axr1-12 aar1-1 plants containing DR5rev:GFP.

(F) Venation patterns in cotyledons of 5-d-old wild type and mutant seedlings.

(G) Response of single and double mutants to 2,4-D in root growth assay. Seedlings were germinated and grown on GM containing 2,4-D. Elongation of roots was measured from day 4 to 7. Data are mean ± SD (n = at least 10 seedlings). *The root-less seedlings (see Figure 2Z) were excluded.
### Figure 2. Frequency of root-less seedlings in various genetic backgrounds.

Frequency of normal (blue), root-less (red), and non-germinated (yellow) phenotypes in offspring seedling populations from parent plants of indicated genotype. N indicates number of seedlings observed. Vertical orange line highlights 3:1 expected ratio for normal:abnormal seedlings from parent lines. Because *axr1 aar1* double mutant was postembryonic lethal, seeds harvested from *AXR1/axr1* heterozygote background were tested in most of the experiments. Transgenes were introduced by crossing.

(A-F) Phenotype of *axr1 aar1* double mutants. *Ws* accession.

(G-I) Complementation of root-less phenotype of *axr1 aar1* by genomic fragment containing SMAP1 (*gSMAP1*). Genomic fragment (X/B) without SMAP1 was used as control (I). Two independent transgenic lines for *gSMAP1* (B/S lines 2D and 1G) and the control line X/B line 1C with the *aar1-1* mutation (Rahman et al., 2006) were crossed with *axr1-12* mutants to generate parental lines.

(J-N) *Inactivation* of SMAP1 by RNAi in *axr1* background. Two independent 520i lines (line 1G and 2C) or control line (line F2) transformed with vector [pB7GW1WG2(II)] (Rahman et al., 2006) were crossed with *axr1-12* mutants to generate parental lines.

(O-W) Expression of SMAP1/GFP fusion protein with under control of 35S promoter in *axr1 aar1* background. Transgenic lines of 35S::SMAP1-GFP*aa1-1* (line 2G and 1H) or 35S::SMAP1-GFP*aa1-1* line 2B (Figure S1) were crossed with *axr1-12* (O-S) or *axr1-3* (T-W) mutants.

(X and Y) Complementation of root-less phenotype of double mutant by 35S::SMAP1-GFP*aa1-1* transgenic lines (D4 and B4) of SMAP1::SMAP1-GFP*aa1-1* were crossed with *axr1-12* mutant to generate parental lines.

(Z) Root-less phenotype was observed in approximately 10% of *aa1-1 ecr1-1* seedlings.
Figure 3. Phenotypic changes resulting from introduction of 35S::SMAPI–GFP fusion genes into axr1-12 mutant.

Independent transgenic lines of 35S::SMAPI–GFP (line 1H and 2G) and 35S::GFP–SMAPI (line 2B) were crossed with axr1-12 and homozygous lines were established for axr1-12, transgene, and wild-type AAR1 locus. (A) Mature plants (27-d-old) (top), inflorescences (middle), and flowers (bottom) are shown. Bar = 1 cm (top and middle). Bar = 0.5 cm (bottom).

(B) Seeds were germinated and grown on GM containing 2,4-D at indicated concentrations. Increase in root length was measured from day 4 to 7. Data are mean ± SD (n > 9 seedlings).

(C) Seeds were germinated and grown on GM for 4 days, seedlings were then transferred onto GM containing IAA and grown for further 3 days. Root elongation after transfer was measured and plotted as a relative value compared with that on medium without chemicals. Data are mean ± SD (n = at least 21 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 1.56±0.25 (WT), 2.23±0.41 (axr1-12), 1.58±0.37 (35S::SMAPI–GFP/axr1-12 1H), 1.76±0.24 (35S::SMAPI–GFP/axr1-12 2G), and 1.64±0.47 (35S::GFP–SMAPI/axr1-12 2B).

(D) Seeds were germinated and grown on GM containing the indicated chemicals for 4 days, then transferred to GM containing the same chemicals and grown for 3 days with JA-Me and IAA. Root elongation after transfer was measured and plotted as a relative value compared with that on medium without chemicals. Data are mean ± SD (n = at least 5 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 2.05±0.26 (WT), 2.63±0.19 (axr1-12), and 2.19±0.24 (35S::SMAPI–GFP/axr1-12 1H), respectively.
Figure 4. Construction and analyses of GFP fusion constructs with SMAP1 and SMAP1 deletion series.

(A) Schematic diagram of constructs. SMAP1 and GFP coding regions are shown by white and green boxes, respectively. F/D rich region in SMAP1 is shown by blue box. SMAP1 promoter is shown by black arrows. NOS terminator is not shown. Dotted lines indicate deleted region in the constructs. SMAP1-GFP contains full-length GFP. D1, D2, and D3 are N-terminal deleted constructs. Numbers on white boxes indicate positions of amino acids within N- and C-terminal of modified SMAP1.

(B and C) Transgenic aar1-1 lines, wild type (WT), and untransformed aar1-1 were planted on GM containing 20 μM PCIB (B) or 50 nM 2,4-D (C) and root length was measured after 10 days. Data are mean ± SD (n = at least 13 seedlings).

(D) Hypocotyl length of 7-d-old seedlings was measured on GM in the light. Data are mean ± SD (n = at least 15 seedlings).
Figure 5. Physical interaction between SMAP1 and CSN.

(A) In vivo pull-down assay using GFP-tagged proteins in aar1-1 transgenic plants. Two transgenic lines, SMAP1-GFP/aar1-1 and SMAP1ΔF/D-GFP/aar1-1, were used in pull-down assay. Pulled-down proteins were separated by SDS-PAGE and detected by western blotting using anti-GFP or by silver staining. Seven protein bands (marked by red circles) that bind to SMAP1-GFP/aar1-1 but not to SMAP1ΔF/D-GFP/aar1-1 were eluted and identified by LC-MS/MS (Table 1).

(B) In vitro pull-down assay using GST-tagged protein constructs. Top: schematic diagram of constructs of GST-tagged proteins. GST control, GST-tagged full-length SMAP1 (GST-SMAP1), F/D region-deleted version of SMAP1 (GST-SMAP1ΔF/D), and the F/D region only (GST-F/D), SMAP1 and GST coding regions are shown as white and pink boxes, respectively. Blue boxes indicate F/D rich region in SMAP1. Black arrow shows Pte promoter for expression of fusion protein in E. coli. Bottom: unpurified total proteins from aar1-1 plant (left lane) and eluted proteins from glutathione columns (other lanes) were separated by SDS-PAGE and stained with Coomassie blue (CBB) or western blot with anti-CSN4 antibody.
Figure 6. Genetic interaction of CSN5A and SMAP1.

We could not obtain sufficient number of seeds of aar1-1 csn5a-1 double mutant; therefore, dwarf aar1-1 csn5a-1 seedlings were selected from seed population from the aar1-1 CSN5A/csn5a-1 parental line for analysis.

(A and B) Images (A) and root lengths (B) of 7-d-old seedlings grown on GM without growth regulators. Bar in (A) = 1 cm. Values in (B) are mean ± SD (n = at least 13 seedlings).

(C) Seeds were germinated and grown on GM for 4 days, then transferred onto GM containing 2,4-D at indicated concentrations and grown for additional 3 days. Root elongation after transfer was measured and plotted as a relative value compared with that on medium without chemicals. Values are mean ± SD (n = at least 17 seedlings). Mean values (cm) ± SD in the absence of chemicals controlling root elongation were as follows: 1.56 ± 0.18 (WT), 1.64 ± 0.22 (aar1-1), 1.71 ± 0.30 (csn5a-1), and 1.21 ± 0.22 (aar1-1 csn5a-1), respectively.

(D) Photographs of plants at rosette stage. Seeds were germinated on GM and grown for 11 days. Seedlings were then transferred to soil and grown for additional 11 days under 16-h light/8-h dark conditions. White arrowheads indicate aar1-1 csn5a-1 double mutants. The photograph at bottom left is an enlarged image of a csn5a-1 aar1-1 double mutant.

(E) Photographs of adult plants. Seeds were germinated and grown on GM for 9 days, seedlings were then transferred to soil and further grown for 35 days under 16-h light/8-h dark conditions. Bar = 5 cm.
Figure 7. RUB modification of CUL1 in double mutants and transgenic lines.

Total proteins (10 μg) extracted from 7-d-old (A) light grown seedlings or (B) flowers were separated by SDS-PAGE, then immunodetected using anti-CUL1 antibody. From left to right: wild type (WT), aar1-1, aar1-12, aar1-12 aar1-1 double mutant, 35S::SMAP1-GFP/aar1-12 1H, 35S::SMAP1-GFP/aar1-12 2G, 35S::GFP-SMAP1/aar1-12 2B, csn5a-1, aar1-1 csn5a-1 double mutant, 35S::SMAP1-GFP/aar1-1 csn5a-1 1H, and 35S::SMAP1-GFP/csn5a-1 1H are subjected to the analysis for A. We could not obtain flowers for the aar1-12 aar1-1 or aar1-1 csn5a-1 double mutant; therefore they are excluded from B. Densitometry analyses of the ratio of RUB-modified CUL1 to total CUL1 (RUB-modified plus unmodified CUL1) are presented below the western blot images that show mean and SD derived from 4 or 3 (for A or B, respectively) independent experiments. Significant differences (P<0.05) between mean values are indicated by different letters above the bars.