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修士論文

Analysis of *SMAPs* that mediate response of the *Arabidopsis* root to synthetic auxin, 2,4-D
(シロイヌナズナの根における2,4-D応答に関与する *SMAPs* の解析)

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Abstract

Plant hormone auxin is a vital factor for plant development, growth, and senescence. A *Small Acidic Protein 1* (*SMAP1*) gene is recently identified as a factor that mediates synthetic auxin, 2,4-dichrolophenoxy acetic acid, response in *Arabidopsis* root. It encodes a 62 amino acid-long protein that has conserved C-terminal region among SMAP orthologues in plants and animals. However, any previously known functional domains are not present in SMAP1 amino acid sequence. To elucidate biological function of SMAP1 and its homologoue SMAP2 in *Arabidopsis*, I performed several lines of experiments. The expression analysis and RNA interference experiments for *SMAP1* and *SMAP2* indicated that *SMAP1* is expressed in young and elongating tissues. SMAP1 protein is present at functionally saturated level in wild-type plant, and SMAP2 has similar biological activity with SMAP1 in 2,4-D response. GFP fusion analysis of SMAP1 suggested that SMAP1 is localized in nucleus and its C-terminal region is functionally important. The results suggest that SMAP1 is novel nuclear protein that functions in cell elongation and 2,4-D response.

Introduction

Auxin is one of the plant hormones and is essential to control growth in various parts and life stages in plant (Davies, 1995; Bennett et al., 1996). Even though this substance is known for a long time, details of the functions of auxin remain secret. However, in recent year, notable progress has been made in this area: a F-box protein, transport inhibitor response protein 1 (TIR1), which binds to short-lived nuclear protein Aux/IAAs, was recognized as an auxin receptor (Gray et al., 2001; Leyser, 2002; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). TIR1 is one of the subunits of E3 ubiquitin ligase, SCF^{TIR1}, which consists of SKP (ASK1 in *Arabidopsis*), CUL (AtCUL1), and F-BOX (TIR1) proteins. The SCF^{TIR1} is well recognized for its function in auxin- and ubiquitin-mediated protein degradation. Once auxin binds to TIR1, SCF^{TIR1} attaches ubiquitin to Aux/IAA proteins, which interact and inhibit DNA binding proteins, Auxin Response Factors (ARFs), in absence of auxin (Kim et al., 1997; Ulmasov et al., 1997; Tiwari et al., 2001; Dharmasiri et al., 2003). The ubiquitinated Aux/IAA proteins are quickly degraded by the 26S proteasome. Once ARFs are released from Aux/IAA proteins, ARFs promote or inhibit expression of the down stream genes (Ulmasov et al., 1997). Most of components involved in the ubiquitin-mediated Aux/IAA degradation such as SCF^{TIR1}, COP9 signalosome (CSN), and Aux/IAA proteins form nuclear protein bodies (NPB), a protein complex in nucleus, upon the application of auxin. The NPB is observed soon after external auxin application, but NPB gradually decomposes in about one hour because the complex is proteolytically active (Tao et al., 2005). The

Aux/IAA protein degradation pathway is controlled further by another mechanism with ubiquitin-like polypeptide RUBs (also known as Nedd8 in mammals), CSN, auxin-resistant 1 (AXR1), ECR1, and RUB-conjugating enzyme 1 (RCE1) proteins. In nucleus, a heterodimer of AXR1 and ECR1 that form RUB activating enzyme generates a thioester-linked conjugate of RUB and RCE1, then RCE1 transfer RUB to AtCUL1 in SCF complex (del Pozo and Estelle, 1999; del Pozo et al., 2002). When CUL1 is modified with RUB, it is degraded like ubiquitinated proteins, and subunits of SCF cannot form the complex. CSN promotes RUB deconjugation from AtCUL1, stabilizing SCF complex (Lyapina et al., 2001; Wu et al., 2005).

Although TIR1 was identified as an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), *tir1* mutants do not exhibit severe morphological phenotype. This is different from *cul1* mutant, which exhibit serve morphological phenotype and is homozygous lethal (Shen et al., 2002; Moon et al., 2006). This difference may imply that TIR1 is only a limited contributor in the auxin signaling transduction. In *Arabidopsis*, there are at least three F-Box proteins (AFB) that are closely related to TIR1 function in auxin signaling other than TIR1 (Dharmasiri et al., 2005). All three AFBs could binds to Aux/IAA proteins in the presence of auxin, and this result insisted that these AFBs are also auxin receptors (Dharmasiri et al., 2005). Plants that are deficient in all three AFBs and TIR1 proteins exhibited severe morphologically altered phenotype (Dharmasiri et al., 2005). However, the fact that even quadruple mutant deficient in *TIR1* and three *AFB*s still can be alive suggests that other unknown

pathway might exist for auxin signaling. Furthermore, it was reported that TIR1 shows weaker conjugation with Aux/IAA proteins in presence of synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) than in presence of natural auxin indole-3-acetic acid (IAA) (Kepinski and Leyser, 2005). This fact is contrary to the very strong auxin activity of 2,4-D and leads some speculation that plants might have other responding pathway(s) (Kepinski and Leyser, 2005). Indeed, some reports supported this possibility that IAA and 2,4-D function differently in plants (Rahman et al., 2006; Walsh et al., 2006), but the detail of this speculation is yet to be elucidated.

To investigate new perspective of auxin signaling, obtaining and analyzing novel auxin mutants has been used as a powerful method. Although many auxin mutants have been already found and analyzed (Woodward and Bartel, 2005), only limited information has been obtained from the available auxin-related mutants in consequence of the limited variation of screening methods for auxin mutants. Since auxin signaling is very complicated, more number of auxin mutants that have mutation in different genes are required to elucidate the cascade of auxin signaling in details. To obtain novel auxin-related mutants, our group mutagenized *Arabidopsis* thaliana with ion-beam (Hase et al., 2000; Rahman et al., 2006), and screened mutants with an anti-auxin, *p*-chlorophenoxyisobutyric acid (PCIB). The isolated mutants were named as *anti-auxin resistant* (*aar*) mutants, which included the mutants that deficient in TIR1 and AtCUL1 as well as a novel mutation *aar1-1*. Because *aar1-1* was found with the auxin signaling mutants, *tir1* and *cul1*, by the same screening method,

aar1 is expected to be also deficient in auxin signaling.

The *aar1-1* plant is less sensitive to 2,4-D but not to indole-3-acetic acid (IAA), compared to wild-type plant (Fig. 1b). Furthermore, *aar1-1* shows wild-type sensitivity toward auxin efflux inhibitors NPA and TIBA, and its 2,4-D metabolism was not different from wild-type. These results indicated that *aar1-1* is a unique auxin signaling mutant that exhibits 2,4-D specific resistance without any changes in auxin transport and 2,4-D metabolism.

Another interesting feature of *aar1-1* is that *aar1-1* has an elongated hypocotyl, unlike other auxin mutants, which usually have shortened hypocotyl. Even though a few mutants such as *hy5* and *hyh* exhibit altered auxin response and elongated hypocotyls (REF), the detailed relationship between these two phenotypes have not been investigated.

The At4g13520 gene, which encodes a small unknown protein (62 amino acid, pK =3.3, molecular weight 6.9kDa) and is named as *small acidic protein 1* (*SMAP1*), is responsible for the *aar1* phenotypes (Rahman et al., 2006). The evidence that *SMAP1* exhibits altered sensitivity toward 2,4-D but not to IAA indicates that 2,4-D and IAA might be perceived by different mechanism in plants. In fact, their molecular structures and biological efficiencies are significantly different even though both of them were designated as same auxin, and it is understandable if they were perceived in different mechanism. Functional analysis of *SMAP1* may become a clue to clarify this speculation as well as general auxin signaling cascade.

In *Arabidopsis*, *At3g24280* gene is present as a homologous gene for

SMAP1, and many orthologue genes are present in plants and animals. Although there are no previously known domain in SMAP1 protein, SMAP1 has highly conserved C-terminal phenylalanine and aspartic acid (F/D) rich domain, which is also highly conserved among orthologous genes in other species. Therefore, SMAP proteins may constitute a novel protein family and may have common function(s) among plants and animals. The results from previous works suggest that *SMAP1* works in early stage of auxin signaling pathway. Since *SMAP1* is very different form previously known proteins functioning in auxin signaling, elucidation of the function of *SMAP1* might help to clarify new aspects of the cascade of auxin signaling.

In this thesis, to enquire into functions of *SMAP*s, I have decided to investigate the phenotype in knockout and overexpressed (O/E) transgenic plants of *SMAP1* and *SMAP2*. I also tried to discuss the SMAP function from its promoter activity and protein localization. Furthermore, I investigated the relationship of hypocotyl elongation of *aar1* mutant and light responses. The results indicate: 1) SMAP2 is a functional protein that works for 2,4-D perception but has limited activity under its authentic promoter, 2) SMAP1 promoter is expressed at elongating cells, especially in young tissues, 3) SMAP1 localizes at the peripheral of nuclei, and 4) C-terminal conserved F/D domain of SMAP is important for 2,4-D responses.

Result and Discussion

1. Functional analysis of *SMAP2*

Overexpression of *SMAP2*

Although previous study suggested that *SMAP1* seems to have very important role in the auxin signaling, *SMAP1* RNAi lines and *aar1-1* plants, in which S*MAP1* is deleted, do not have distinct phenotype other than long hypocotyl and reduced sensitivity towards 2,4-D and PCIB (Rahman et al., 2006). The simple explanation of this weak phenotype in *SMAP1*-deficient plants could be that the *SMAP1* homologue *SMAP2* gene is present in *Arabidopsis* genome. Because amino acid sequence of SMAP2 is quite similar to SMAP1 especially at the C-terminal region (Fig. 1c), *SMAP2* might work as a complementary of *SMAP1*. Northern hybridization of *SMAP1* and *SMAP2* suggested that *SMAP2* RNA is expressed at only low level in most of organs except siliques in *Arabidopsis* (Fig. 2). However, it is still possible that the low amounts of *SMAP2* RNA are sufficient to complement the deficiency of *SMAP1*. Therefore, I made several transgenic lines to investigate functional significance of *SMAP2* in plant growth and development as well as auxin and anti-auxin responses.

At first, to examine if *SMAP2* has similar biological activity to *SMAP1*, *SMAP2* was overexpressed (O/E) under the cauliflower mosaic virus (CAMV) *35S* promoter in the *aar1-1* plants. *Arabidopsis* seeds of wild-type, *aar1-1*, and two independent lines of *aar1-1* containing *35S::SMAP2* (SMAP2 O/E) were plated

on 40 nM 2,4-D or 20 μM anti-auxin PCIB, and then, root length of these lines were measured (Fig. 4b and c). On 2,4-D medium, wild-type root growth was significantly inhibited, whereas *aar1-1* roots showed resistance to 2,4-D (Fig. 4b), suggesting that the SMAP2 O/E lines partially restored 2,4-D sensitivity in *aar1-1* (Fig. 4b). On 20 μM PCIB medium, the root of wild-type was significantly inhibited while *aar1-1* root showed minor inhibition. SMAP2 O/E lines also showed shorter root than *aar1-1* and restored PCIB sensitivity (Fig. 5 b). SMAP2 O/E/*aar1-1* did not restore the hypocotyl length to the wild-type level (Fig. 5a), but the hypocotyl length was partially inhibited on GM medium. These results shows that SMAP2 O/E partially complemented wild-type phenotype for 2,4-D and PCIB sensitivity and hypocotyl length in *aar1-1* plants, implying that function of *SMAP2* is partially overlapped to *SMAP1*.

Gene knockout analysis of *SMAP2*

Next, I tried to investigate the phenotype changes in the loss of *SMAP2* plants. Because no T-DNA insertion or Enhancer Trap (ET) line of *SMAP2* was found in seed resources bank, RNA interference (RNAi) experiment was performed. Root growth and hypocotyl length of two homozygous *SMAP2* RNAi lines (*SMAP2*i) were analyzed, and compared to wild-type. As expected from its low RNA content in plant, the *SMAP2*i lines did not show any distinct morphological changes compared to wild-type. Root growth in the *SMAP2*i lines on 2,4-D medium were inhibited less severely than in wild-type but more strongly than in *aar1-1* or the *SMAP1*i lines, suggesting that *SMAP2* contributes to 2,4-D

response in some extent but less effectively than *SMAP1* (Fig. 6c). On the other hand, the *SMAP2*i lines did not show resistance against 20 μM PCIB (Fig. 6d). Hypocotyl length of the *SMAP2*i lines was almost as same as wild-type while the *SMAP1*i plants showed elongated hypocotyl (Fig. 6a and b). These results suggest that SMAP2 functions only 2,4-D response in *Arabidopsis* when it is not over-expressed.

Analysis of *SMAP1*i and *SMAP2*i double knockout lines

The results that *SMAP2* have similar biological activity to *SMAP1* and contribute to 2,4-D response in some extent imply the possibility that S*MAP2* acts with *SMAP1* for plant growth and development redundantly. Thus, I speculated the plants inactivated both *SMAP1* and *SMAP2* genes might exhibit more distinct phenotypes than *aar1-1* or RNAi lines for single *SMAP* gene. To generate seeds of double knockout lines, the *SMAP1*i and *SMAP2*i transgenic plants were crossed. F1 plants, which are supposed to be knocked out both *SMAP1* and *SMAP2*, were placed on 40 nM 2,4-D or 20 μM PCIB plates, and root length was measured on day 10 after germination. The roots of double knockout lines showed almost the same length as the roots of *aar1-1* and *SMAP1*i on both 2,4-D (Fig. 6b and 7b) and PCIB medium (Fig. 6c and 7c). Any noticeable phenotypes other than elongated hypocotyl (Fig. 7a) were observed in double knockout lines as far as I observed. The hypocotyl length in double knockout lines was shorter than *aar1-1* hypocotyl (Fig. 7a). The very weak phenotype in the double knockout lines implies that both *SMAPs* seem to have only minor function in normal growth condition.

2. Expression analysis of *SMAP1*

From the result of *SMAP2* O/E and RNAi experiments, it was insisted that *SMAP2* does not have significant function in plant growth. Even though *SMAP2* showed partial 2,4-D resistance, it was weak, and other phenotype change was not observed. Therefore, *SMAP1* must have main role in 2,4-D perception and regulation of hypocotyl length. To investigate *SMAP1* function in more details in plant, the expression pattern of *SMAP1* was investigated.

Histochemical analysis of *SMAP1* expression using β-glucuronidase

At first, to analyze expression patterns of *SMAP1* gene, β-glucuronidase (GUS) assay was performed. Bacterial *GUS* gene, which converts 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-Gluc) to indigo-blue and is often used as an expression reporter in plant, was placed at down stream of *SMAP1* promoter, (P*SMAP1*::*GUS*). The transgenic plant containing P*SMAP1*::*GUS* were treated in GUS buffer containing X-Gluc, incubated at 37 °C for over night, and observed under microscopes (Fig. 8). In general, as it was expected from the Northern analysis, *SMAP1* promoter showed strong activity in young leaves, roots, and flowers. The strongest GUS activity was observed in root-stem junction and root tips except root meristem (Fig. 8). The time course analysis of GUS expression pattern revealed dynamic change of *SMAP 1* promoter activity during seedling development. On day 1, GUS activity was observed at young cotyledons and tip of the root. There was unstained gap at the upper part of the roots (Fig.

8a). However, on day 2, GUS activity was observed at the whole parts of seedling except in the root meristem region (Fig. 8b and c). On day 10, no longer GUS activity was detected in the upper part of the cotyledon petioles that are supposed to be composed of mature cells (Fig. 8d and i). Strong GUS activity was also observed at the sepals of immature flower, anther of blooming flower, junctions of silique and pedicle, tip of siliques, funicle, and placenta (Fig. 8j, k, l, and m). Over all, *SMAP1* was strongly expressed in young organs and roots, especially in the elongating cells.

Physiology of overexpression of *SMAP1* in wild-type plant

The GUS assay of *SMAP1* promoter insisted that *SMAP1* promoter is expressed strongly in elongating tissue. Thus, *SMAP1* may have functions in elongating tissues. Therefore, we expected if *SMAP1* were made expresse ectopically in non-elongating cells, the plants would show additional phenotype change(s) that may help us to understand *SMAP1* function. For this purpose, *SMAP1* was placed at down stream of CAMV *35S* promoter, and *SMAP1* over expression (SMAP1 O/E) lines were generated. The picture of *SMAP1* O/E lines in wild-type plant on day 7 is shown in Figure 9a. The response against 50 nM 2,4-D and 20 μM PCIB in the roots of SMAP1 O/E was not different from the one of wild-type (Fig. 9c). Root growth on 50 nM and 250 nM indole-3-acetic acid (IAA) was also tested. However, as expected from the previous study, wild-type, *aar1-1*, and SMAP1 O/E did not show obvious differences in root growth and morphological phenotype (Fig. 9d). Hypocotyl length of the SMAP1 O/E lines

was not different from that of wild-type (Fig. 9b).

Because SMAP1 O/E lines did not show any noticeable phenotype, I suspected that changing promoter from its authentic *SMAP1* promoter to *CAMV 35S* promoter might not effectively increase the amount of the SMAP1 protein in transgenic lines. To estimate the differences of the SMAP1 protein production level between *CAMV 35S* and *SMAP1* promoters, the amount of green fluorescent (GFP) tagged SMAP1 expressed under *35S* and *SMAP1* authentic promoters in transgenic lines was compared by western blotting analysis using monoclonal anti-GFP antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). More amount of the GFP-SMAP1 protein was produced when it is expressed at the down stream of *35S* promoter compared to *SMAP1* promoter. The GFP-SMAP1 fusion protein was functional because it complemented *aar1-1* mutant phenotype (Fig. 10a and b). However, SMAP1 derived from *35S* promoter did not influence the wild-type phenotype (Fig. 9b and c). The experiments using CAMV *35S* promoter indicated that over-expression of *SMAP1* does not show any phenotype, suggesting that SMAP1 protein is already present at functionally saturated level in wild-type plants.

3. Analysis of SMAP1 protein

The *aar1-1* mutant defective in *SMAP1* was identified in the screening of PCIB resistant mutants. Because auxin signaling mutants such as *tir1* and *cul1* were also obtained by the same screening, I have expected that SMAP1 protein

might also work in early auxin signaling. However, because SMAP1 protein did not have any known functional domain even though it has highly conserved domain among plants and animals, I could not speculate function of SMAP1 from its amino acid sequence. Therefore, I tried to investigate SMAP1 functions from its localization and domain analysis, using GFP-fusion protein. To start series of GFP-fusion experiments, we first observed the transgenic plants expressing *35S::SMAP1-GFP* (SMAP1-GFP) and *35S::GFP-SMAP1* (GFP-SMAP1) in *aar1-1*, which were generated to estimate the effect of GFP protein fusion to the activity of SMAP1. The size of GFP is estimated to be about 30 kDa from its amino acid (Cubitt et al., 1998), and SMAP1 protein is estimated to be only 6.9 kDa (Rahman et al., 2006). Because the size of GFP is much larger than SMAP1, it was suspected that GFP might interfere with SMAP1 activity. The root sensitivity against 40 nM 2,4-D in the SMAP1-GFP and GFP-SMAP1 transgenic lines were recovered to the wild-type level (Fig. 10b). The result of hypocotyl complementation analysis in Figure 10a also insisted that SMAP1-GFP and GFP-SMAP1 fusion proteins are able to shorten hypocotyl length. Although there were minor variations between individual tested lines, no significant differences in growth between SMAP1-GFP and GFP-SMAP1 transgenic lines were observed (Fig. 10b). Therefore, it was suggested that both N and C-terminal fusions of GFP seem not to affect activities of the SMAP1 protein.

Analysis of SMAP1 functional domain

The F/D region of SMAP1 protein at C-terminal is highly conserved in

many organisms (Fig. 1c). Therefore, it was suspected that F/D region is the functionally important domain for SMAP1. To prove this speculation, modified *SMAP1* varieties were fused with *GFP*, placed at the down stream of *SMAP1* promoter (P*SMAP1*::*SMAP1-GFPs*), and transformed into *aar1-1*. The construction of P*SMAP1*::*SMAP1-GFPs* are shown in Figure 3c, and its amino acid sequences are shown in Figure 3d. P*SMAP1*::*SMAP1-GFP1* and P*SMAP1*::*SMAP1-GFP2* have same sequence except that P*SMAP1*::*SMAP1-GFP2* has *Nde* I endonuclease restriction site before the start codon of SMAP1. The *Nde* I site is also present in deleted constructs (D1, D2, and D3) (Fig. 3c). P*SMAP1*::*SMAP1-GFP* 1 and 2 encode whole SMAP1 protein while del. F/D, D1, D2, and D3 encode partially deleted SMAP1. In del. F/D, C-terminal F/D domain except the first two amino acids was deleted. D1 to D3 deletion constructs that lack a part of N-terminal amino acids were created to determine how the non-conserved regions contribute to the function of SMAP1 protein.

SMAP1 and SMAP1 deletion constructs were transformed into *aar1-1* plant via Agrobacterium-mediated transformation. To verify whether modified SMAP1s are functional or not, response to the 50 nM 2,4-D and 20 μM PCIB and hypocotyl length in the transgenic plants were analyzed (Fig. 11, 12, and 13). The P*SMAP1*::*SMAP1-GFP*1, P*SMAP1*::*SMAP1-GFP*2, and D1 transgenic plants showed same sensitivity to 2,4-D and PCIB as wild type (Fig. 12b and 13b). On the other hand, del. F/D, D3, and control transgenic plants exhibited same root length as *aar1-1* on both 2,4-D and PCIB medium (Fig. 12b and 13b). Most of D2 plants partially recovered sensitivity against 2,4-D and PCIB (Fig. 12b and 13b).

From these experiments, it was insisted that SMAP1 protein does not function without the conserved F/D domain, and at the same time, SMAP1 protein cannot function only with F/D domain since the D3 construct did not complement *aar1-1* response on both 2,4-D and PCIB media. The F/D region of SMAP1 protein was suspected to have important role in wide-variety of species, including plants and animals (Rahman et al., 2006).

SMAP1-GFP localization

In P*SMAP1*::*SMAP1-GFP* 1 and P*SMAP1*::*SMAP1-GFP* 2 plants, strong GFP signal were detected in peripheral region of nucleus (Fig. 14). Similar GFP localization pattern was detected in D1 and D2 lines. Whereas in del F/D and control plants, GFP signal were detected mainly in cytosol (Fig. 14). (Note that GFP signal seems to localize in nuclear-like structure in some elongated cells. However, this seems to be due to limited cytosol pushed by enlarged vacuole in the cells). D3 lines also showed the GFP localization in nucleus, although the localization was slightly leaky, and weak GFP signal was observed in cytosol, compared to full-length SMAP1 lines (Fig. 15). Same localization pattern of GFP was detected in mature cells located in upper part of the root or in the root of older plants (10-d-old), suggesting SMAP1 localization does not change by aging of cells.

SMAP1 was initially expected to be a cytosolic protein because no obvious nuclear or any localization signal sequence was detected in SMAP sequences. My observation showed that SMAP1 was localized at nucleus, and was not scattered in the cytosol. This finding suggested that SMAP1 may have novel

nuclear localization signal (NLS). The deletion analysis indicated F/D rich region is strong candidate of the NLS. The F/D rich region is also required for SMAP1 function, indicating that SMAP1 works in nucleus.

In general, the protein that participate in early auxin signaling like CSN, SCF^{TIR1}, Aux/IAA, AXR1, and RUB1 in ubiquitin-mediated Aux/IAAs proteolysis localizes at the nuclei; therefore, putative nuclear protein SMAP1 that supposes to work in early auxin signaling might also work with these proteins. Recently, it was reported that the ubiquitin and RUB1-mediated proteolysis takes place at nuclear protein bodies (NPB), which was observed in nuclear when ubiquitin-mediated Aux/IAA degradation is processed in the presence of externally applied auxin (Tao et al., 2005). If SMAP1 functions together with these proteins, which are related to auxin-induced ubiquitin-mediated proteolysis, SMAP1-GFP signal might also localize in NBP after the application of auxin. To observe the influence of auxin to the SMAP1-GFP localization, P*SMAP1*::*SMAP1-GFP* 1, del. F/D, and control plants were dipped in GM media that contain 20 μM NAA or DMSO. However, 30 minutes incubation did not alter the localization of GFP in P*SMAP1*::*SMAP1-GFP* 1 plant (Fig. 15). After over night incubation, the GFP localized mainly at the same position, although signal located outside of the nucleus was increased (Fig. 15). The over night incubation did not alter the localization of GFP in del. F/D plant, either (Fig. 15). In both case, total intensity of the GFP signal has slightly increased. It was reported that the NPB is proteolytically active, and the GFP-tagged protein in NPB is appeared as strong signal for 15 to 30 minutes after application of NAA

but discomposes about one hour duration (Tao et al., 2005). GFP-fusion SMAP1 did not show any change in its localization, indicating that SMAP1 does not work in NPB.

The GFP-fused SMAP1 localization is similar to the Suppressor of Auxin Resistance1 (SAR1) and SAR3 protein, which are the repressors of *axr1* and *rce1* (Cernac et al., 1997; Parry et al., 2006). SAR1 and SAR3 are components of putative Nuclear Porin Complexes (NPCs) and exert on mRNA export. Therefore, *sar1* or *sar3* mutation leads to accumulation of poly-adenylated mRNAs in nuclei (Parry et al., 2006). The relationship SMAP1 and NPCs should be elucidated in future research.

Detection of fusion protein with western blotting

To determine the size and quantity of produced fusion protein in transgenic lines, Western blotting was performed, using anti-GFP antibody. Del. F/D and D3 bands are appeared clearly in the western blot; whereas, P*SMAP1*::*SMAP1-GFP* 1, D1 and D3 bands did not show strong signal. The result indicates that P*SMAP1*::*SMAP1-GFP* 1 and its active variants decomposed in certain extent. Because un-fused GFP that was extracted from 35S::*GFP* transgenic plant was appeared as single band (data not shown), this decomposition was considered to be due to SMAP1 protein, probably F/D rich region. The results indicate that SMAP1 protein, which possesses the C-terminal conserved region, may be unstable.

4. Hypocotyl physiology of wild-type and *aar1-1*

The elongated hypocotyl is one of the most distinct phenotype of *aar1-1*. On the other hand, one of *aar* mutants, *tir1*, exhibits shorter hypocotyl (Ruegger et al., 1998). Other auxin mutants such as *axr1* and *cul1-6* also have shorter hypocotyls (stems) (Lincoln et al., 1990; Ruegger et al., 1998; Moon et al., 2006). Many light mutants such as *hy* and *cry* mutants have elongated hypocotyl, and some of them were reported that they also have altered auxin signaling in recent study. For example in the *hy5* mutants, the *AXR2/IAA7* and *Solitary Root (SLR)/IAA14* genes, the negative regulators of auxin signaling, are under-expressed (Cluis et al., 2004). HY5 were known to regulate auxin signaling under the control of COP1, and it reported to inhibit hypocotyl elongation in dark (Osterlund et al., 2000; Sibout et al., 2006). Therefore, *aar1-1* was also suspected to be deficient in not only auxin but also light signaling. However, the relationship between light response in hypocotyl and auxin response in root is still not well understood. Therefore, to investigate if *aar1-1* has an altered response against light might help to understand interaction between light and auxin responses.

Hypocotyl elongation on IAA, 2,4-D and PCIB medium

First, I examined effect of auxin to hypocotyl length in wild-type and *aar1-1*. As demonstrated before, *aar1-1* showed longer hypocotyl than wild-type in the absence of auxin. Interestingly, I noticed this long hypocotyl phenotype in

aar1-1 was canceled by application of external auxin and PCIB (Fig. 17a-c). To explore further relationship between hypocotyls and the *SMAP* genes, the hypocotyl length of SMAPs double knockout lines were compared with wild-type and *aar1-1* on the DMSO, 40 nM 2,4-D, and 20 μM PCIB in 10-day-old seedlings (Fig. 17 d). The result showed the double knockouts lines also exhibited showed shortened hypocotyls in the presence of 2,4-D or PCIB even though length of their hypocotyls are intermediate between those of wild-type and *aar1-1*, suggesting inactivation of *SMAPs* is also involved in auxin hyper-sensitivity in *aar1-1* (Fig. 17a). Contrasting to *aar1-1*, auxin resistant mutants, *axr1-12* and *axr3-1*, and auxin overproducing line 35S-iaaL are reported that they are more resistant to exogenous auxin than wild-type (Collett et al., 2000). Although the reason why *aar1-1* hypocotyl growth is hypersensitive to externally applied auxin and PCIB is unknown, further investigation would provide us valuable information regarding to relationship between auxin and hypocotyl growth.

Light and dark treatment

The difference in hypocotyl length between wild-type and *aar1-1* is visible in early stage after germination (Fig. 18a). The elongation rate of hypocotyl is the largest between day 3 and 5, and the difference in hypocotyl length is the most significant on day 7 (Fig. 18a). Figure 18b shows the hypocotyl length of light or dark-grown wild-type and *aar1-1* plants on 7 day. It is noticeable that the difference in elongation is larger in light than in dark. In the Figure 19c, P*SMAP1*::*GUS* plants were grown in light or dark for 3 days and

incubated in GUS buffer for 16 h. It was observed that the dark-grown plant showed stronger GUS activity in wider area of hypocotyl than light-grown plant. Therefore, it was insisted that *SMAP1* promoter is more active in dark, but the putative function of SMAP1, inhibition of hypocotyl elongation, is not likely functioning in dark.

Hypocotyl elongation under blue, red, and far-red light

Because long hypocotyl phenotype in *aar1-1* was significant in light grown seedlings, it was suspected that *SMAP1* might inhibit hypocotyl elongation under light condition. It is known that several light signaling pathway regulates hypocotyl length (Hsieh et al., 2000; Schwechheimer et al., 2002; Moon et al., 2006; Sibout et al., 2006). To investigate which light pathway is involved in *aar1-1* long hypocotyl phenotype, the effect of different wavelengths of the light on the hypocotyl elongation in wild-type and *aar1-1* was examined. Under the blue and red light, wild-type and *aar1-1* hypocotyls elongated differently, and *aar1-1* had longer hypocotyl as usually observed under white light (Fig. 19a, b, c, and d). However, under far-red light, the hypocotyl elongation of wild-type and *aar1-1* were indifferent (Fig. 19e and f). The *SMAP1*i lines show clear long hypocotyl phenotype under blue light implies that *SMAP1* is involved in blue-light dependent hypocotyls growth inhibition. It is known that auxin sensitivity may decrease in blue light and cause slow hypocotyl growth (Folta et al., 2003). Therefore, SMAP1 might be act in the cross point of the blue light and auxin signaling pathways. Further investigation on SMAPs may help to elucidate an

interaction of both cascades.

Conclusion:

In this thesis, to elucidate mechanism of auxin action I have analyzed biological effect of SMAP1 protein that is required for normal 2,4-D response in root and hypocotyl growth by generating and analyzing transgenic *Arabidopsis* expressing many variations of SMAP1 and its homologue SMAP2. The results obtained in this research suggested that SMAP1 is a nuclear protein that functions in cell elongation, 2,4-D response, and light signaling. It also suggested the SMAP1 homologue, SMAP2, potentially functions in 2,4-D perception even though its expression is very weak. In addition, I have revealed that the *SMAP1* gene is expressed in young and elongating tissues, SMAP1 protein is present at functionally saturated level in wild-type plant, and it may not function in ubiquitin and RUB1-mediated proteolysis. Finally, SMAP1 protein might be related to both light and auxin signaling in hypocotyls growth. I believe the thesis would provide novel and important findings to all the researchers who are interested in the mechanism of auxin action, blue light signal transduction and plant growth regulation.

Material and Methods

Growth Conditions

Seeds were sterilized in the 10% (v/v) disodium hypochloride and 0.1% (v/v) Tween 20 solution for 20 minutes with continuous agitation, using stirring-bar, and washed 3 times in sterilized dH_2O. Then, seeds were plated on 0.8% germination medium (GM) agar. GM medium contains half-strength Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 1% (w/v) sucrose, 0.1% (w/v) MES, and pH was equilibrated to pH 5.8 with 1M KOH.

Plant Materials

Arabidopsis thaliana (L.) Heynh was used, and all plant materials were Columbia background, including *aar1-1*. *aar1-1* were obtained from the ion beam-mutagenized population (Hase et al., 2000; Rahman et al., 2006). *35S::SMAP1-GFP*, *35S::GFP-SMAP1*, *35S::SMAP2*, and P*SMAP1*::*SMAP1-GFP* were transformed into *aar1-1* (Table 1). *SMAP1*i, *SMAP2*i, P*SMAP1*::*GUS*, and *35S::SMAP1* transgenic plants were made in wild-type background.

The detailed processes of the vector constructions of SMAP1-GFP and its derivatives are described in Fig. 1. For *SMAP1* promoter (P*SMAP1*)::*SMAP1-GFP* and P*SMAP1*::*SMAP1 del. F/D-GFP* (del. F/D) was constructed, using pEGAD vector (Cutler et al., 2000). Four kbp DNA fragment of P*SMAP1* region, which is located between *Sac* I and *Bgl* II site was obtained with endonuclease digestion and following gel extraction with QIAquick Gel Extraction Kit (QIAGEN K.K.,

Japan). The fragment between *Bgl* II and whole sequence of *SMAP1* with *Age* I was amplified by PCR using the forward primer (5'-GATTAAAATTAACATGGGCCACA-3'), the reverse primer (5'-TGGCGACCGGTAAGTTGATACGGTGTCATCG-3') and high fidelity polymerase Pfu-Turbo (Stratagene, CA, USA). Another *SMAP1* fragment, del. F/D, was PCR amplified between the same forward primer and the reverse primer (5'-TGGCGACCGGTAGGCGAGCTTGTTATCG-3'). Both PCR-amplified products were digested with endonulaease *Bgl* II and *Age* I, and purified with gel extraction kit (BIO RAD, USA). The *SMAP1* fragment and P*SMAP1* products were ligated with pEGAD fragment that was digested with *Sac* I and *Age* I. The ligated product was transformed into *Escherichia coli* DH5 α . After the transformation, the antibiotic resistant colonies were checked by colony PCR with Ex-Taq polymerase (TaKaRa BIO Inc., Tokyo, Japan), following endonuclease digestion of whole plasmids, and electrophoresis to verify insertion of the collect fragments in the vector. After the collect fragments were confirmed, DNA sequence of the PCR-amplified regions and ligated sites were obtained with ABI Prism™ 377 DNA Sequencer (PERKIN ELMER), using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). The other information regarding to making of transgenic plants are shown in Table 1, and the primers used in vector construction are listed in Table 2.

For *Arabidopsis* transformation, Agrobacterium GV3101 (MP90) was used with flower dip protocol (Steven J. Clough and Bent, 1998). After T1 seeds were obtained from infected plants, transformed seedlings were selected with

antibiotic suitable for selection marker of transformed vectors. In the selection against antibiotics, round culture plates, 90 x 20mm, were used (TERUMO® Corporation, Japan), and all antibiotics were obtained from Sigma Chemical Co. (St Louis and MO, USA). T2 seeds were supposed to segregate into 1 antibiotic sensitive to 3 antibiotic resistant plants if the insertion of transgene occurs in one locus. Thus, the T1 lines that showed 1:3 segregation in T2 generation were selected. From the selected T1 lines, antibiotic resistant T2 plants were grown to harvest T3 seeds. T3 plants were expected as offsprings of hetero- or homozygous T2 plants for the inserted gene. Therefore, T2 lines that show all antibiotic resistant T3 plants were selected as homozygous lines.

Physiological Experiments

For the hypocotyl and root growth assays, the plants were grown under continuous white light, 20-30 μ mol $m^{-2}sec^{-1}$, at 23℃ otherwise noted. The plants were grown on vertical square culture plates 100 x 100 x 15mm (Simport, Quebec, Canada), except for light experiments. In light experiments, the plants were grown in a pot that is horizontally placed under diode illuminator MIL-C1000T (Sanyo Biomedical, Oosaka, Japan). Plant images were pictured with digital camera (Olympus Corporation, Tokyo, Japan), and hypocotyl and root lengths were analyzed, using the software NIH Image 1.63 (National Institution of Health, USA).

β -Glucuronidase (GUS) Assay

Twelve T2 PSMAP1::GUS::TSMAP1 transgenic lines that show 1:3 segregation was isolated, and 6 lines of which showed average GUS staining were selected and grown to harvest homozygous seeds. To observe SMAP1 promoter activity of day 1 to day 10 plants, T3 homozygous plants was grown on GM media. The plants were transferred to soil after 14 days for observation of flowers and siliques. GUS staining buffer is consists of 100 mM NaPO4 (pH 7.0), 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, and 0.1% (v/v) Triton X-100. 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-Gluc) 20 mg was dissolved in 1ml DMSO, and this solution was used as X-Gluc stock. For staining the plants, 20 μl X-Gluc stock was dissolved in 1 ml GUS staining buffer, and the plants were dipped in the solution for over night at 37 °C.

Western Blotting

Western blotting were performed with monoclonal anti-GFP antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), and anti-mouse IgG antibody conjugated with horseradish peroxidase (GE Healthcare, UK) as the secondly antibody. The protein extraction buffer is consists of 62.5 mM Tris-HCl pH6.8, 5% SDS, and complete protein inhibitor cocktail mini (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Seven 15-day-old plants were grinded in 1.5 ml centrifuge tube with 100 μl extraction buffer on ice. PAGE was performed for 50 min with constant 160 V, using 15% acrylamide gel with 12 lanes (BIO-RAD). PVDF membrane with 0.45 μm pore size (Millipore, MA, USA) was used for electro-transfer of proteins with 25V for 1 hour.

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Table 1. List of constructs made in this study

Name	Vector	Promoter	Host Plant	Discription
P*SMAP1*::SMAP1-GFP*	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* with authentic promoter (a.p.)
del F/D	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* N-end deletion with a.p.
P*SMAP1*::SMAP1-GFP2*	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* with Authentic promoter
D1	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* C-end deletion with a.p.
D2	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* C-end deletion with a.p.
D3	pEGAD	*SMAP1*	*aar1-1*	GFP fusion *SMAP1* C-end deletion with a.p.
SMAP2 O/E	pB7WG2	*35S*	*aar1-1*	35S::SMAP2 in aar1-1
SMAP1 O/E	pB7WG2	*35S*	wild-type	35S::SMAP1 in wild-type
SMAP2i	pH7GWIWG2(II)	*35S*	wild-type	RNAi for *SMAP2*
SMAP1i	pB7GWIWG2(II)	*35S*	wild-type	RNAi for *SMAP1*
35S::SMAP1-GFP	pK7FWG2	*35S*	*aar1-1*	N-end GFP fusion *SMAP1* with *35S* promoter
35S::GFP-SMAP1	pK7WGF2	*35S*	*aar1-1*	C-end GFP fusion *SMAP1* with *35S* promoter

*P*SMAP1*::SMAP1-GFP1and P*SMAP1*::SMAP1-GFP2 are the same construction except #2 has a *Nde* I site.

Table 2. Primers used in the construction for GFP-fusions of SMAP1 and its deleted variety.

Primer's Name	5'-sequence-3'	Construct Generated
Primer 1	gattaaaattaacatgggccaca	PSMAP1::SMAP1-GFP1 and del. F/D
Primer 2	tggcgaccggtaagttgatatcggtgtcatcg	PSMAP1::SMAP1-GFP1
Primer 3	tggcgaccggtaggcgagcttgttatctg	del. F/D
Primer 4	ggaattccatatgaggccgatgcagctgga	PSMAP1::SMAP1-GFP-2
Primer 5	ggaattccatatggacgttgatgacctgga	Del. SMAP1GFP (D1)
Primer 6	ggaattccatatgcttgtctcagataacaag	Del. SMAP1GFP (D2)
Primer 7	ggaattccatatggacgccgatttcttcaa	Del. SMAP1GFP (D3)
Primer 8	gaattccatatgttctctcttcgtctcttctt	PSMAP1::SMAP1-GFP-2, D1, D2 and D3

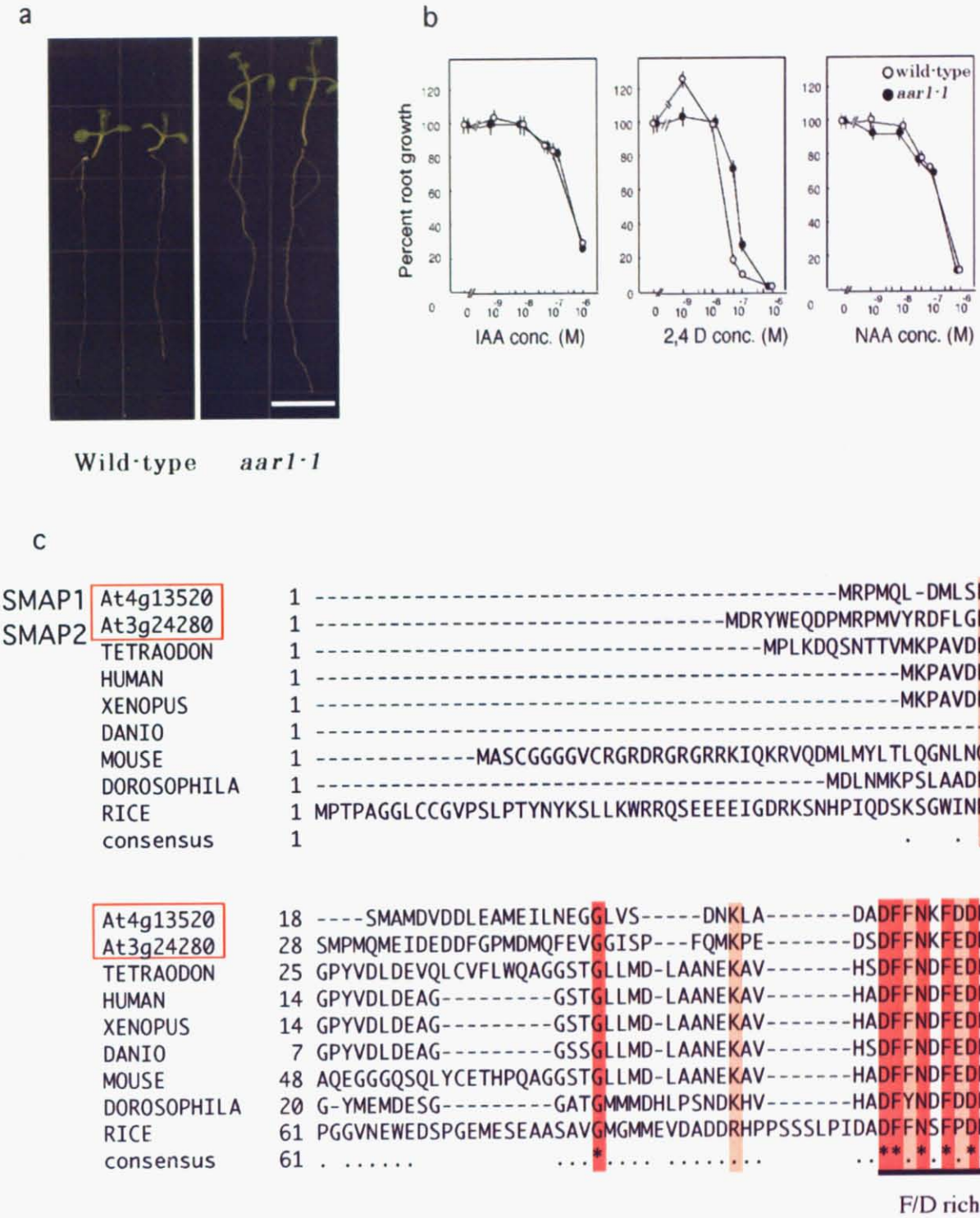


Figure 1. Phenotype of *aar1-1* and sequence of SMAP proteins.

a. Eleven-day-old wild-type (left) and *aar1-1* (right) plants. The bar indicates 1cm. b. Relative root growth of wild-type (open circle) and *aar1-1* (closed circle) plants in various concentrations of IAA, 2,4-D, and NAA. Symbols show mean ± SE. c. Alignment of putative SMAP1 homologues. The *A. thaliana* SMAP1 sequence is shown on the top and identical amino acids are shown in red and similar ones in orange. F/D rich region is indicated with underline.

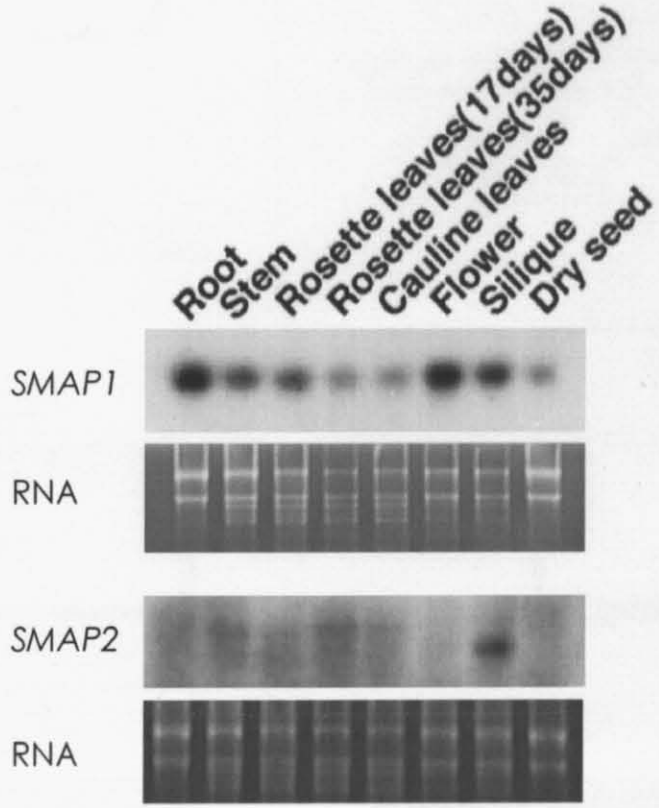


Figure 2. Northern hybridization for *SMAP1* and *SMAP2*.

Total RNA 2 μ g was applied for electrophoresis. DNA fragments covering whole length *SMAP1* or *SMAP2* genes were used as probes. Root and 17-day-old rosette leaves were sampled from 17-day–old plants grown on MS media after 6-day dark treatment. The other parts were sampled from 32-day–old plants except for the rosette leaves that sampled from 35-day–old plants. Loading equal amount of total RNA was shown by ethidium bromide staining at the bottom.

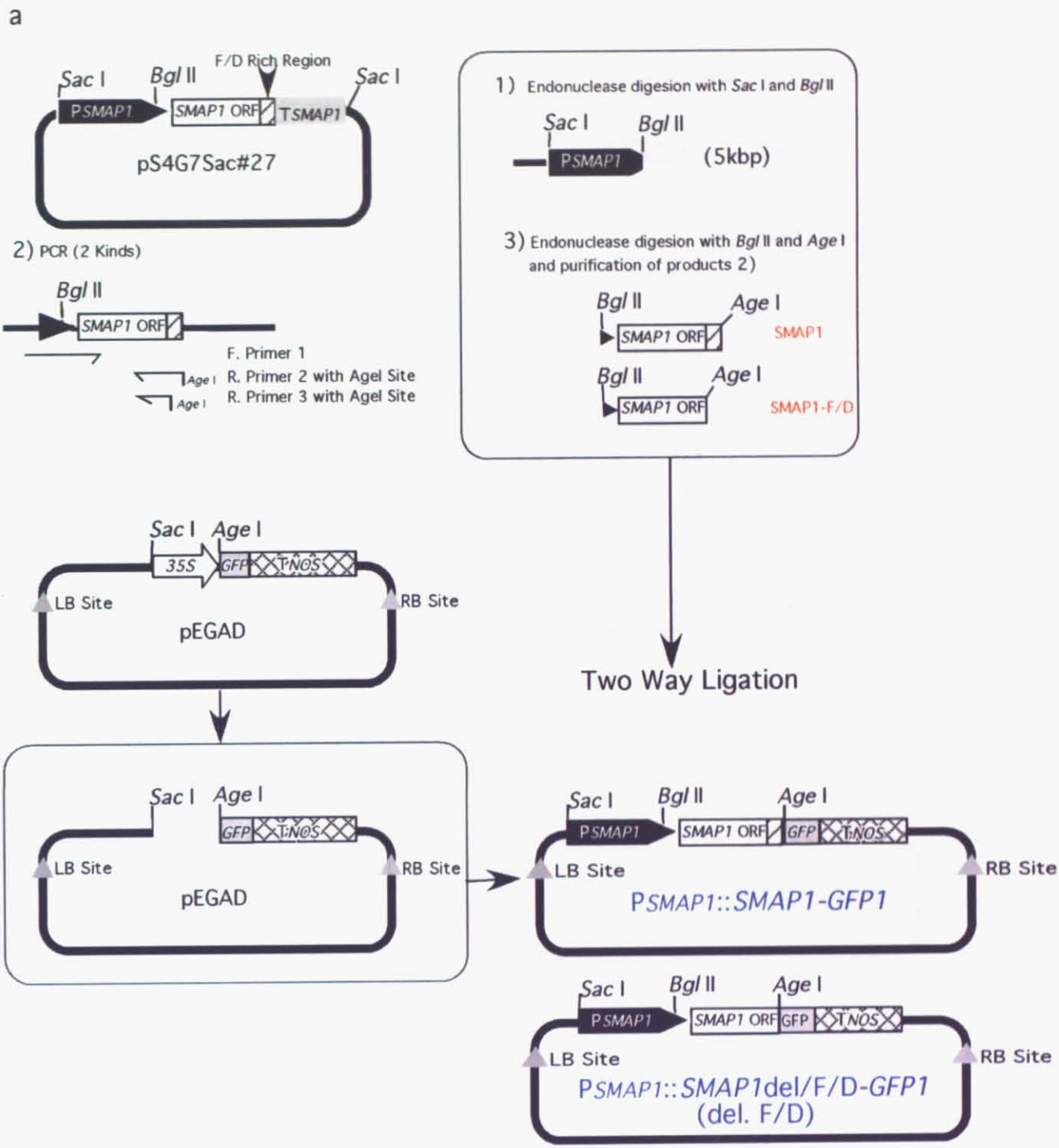
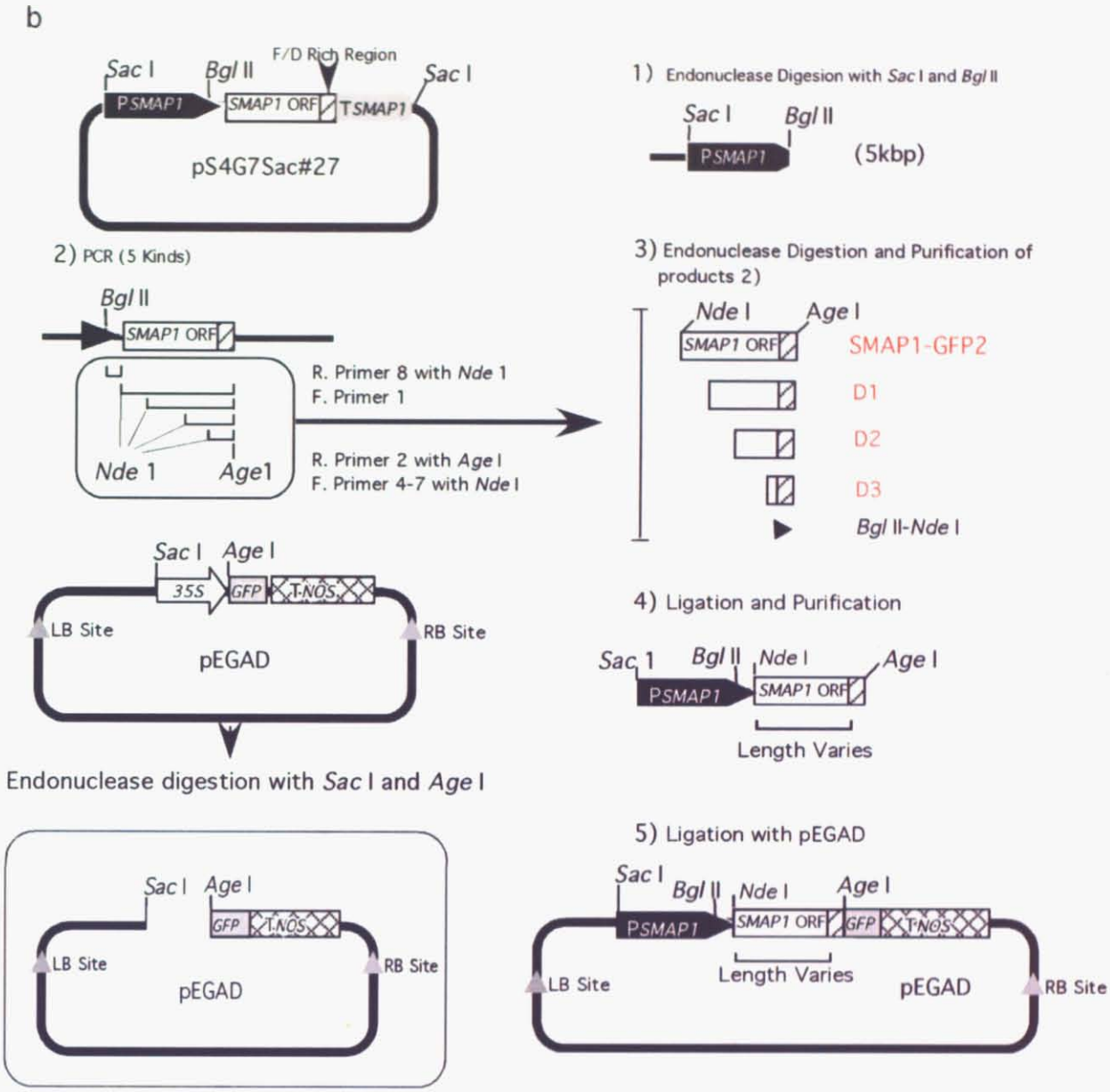


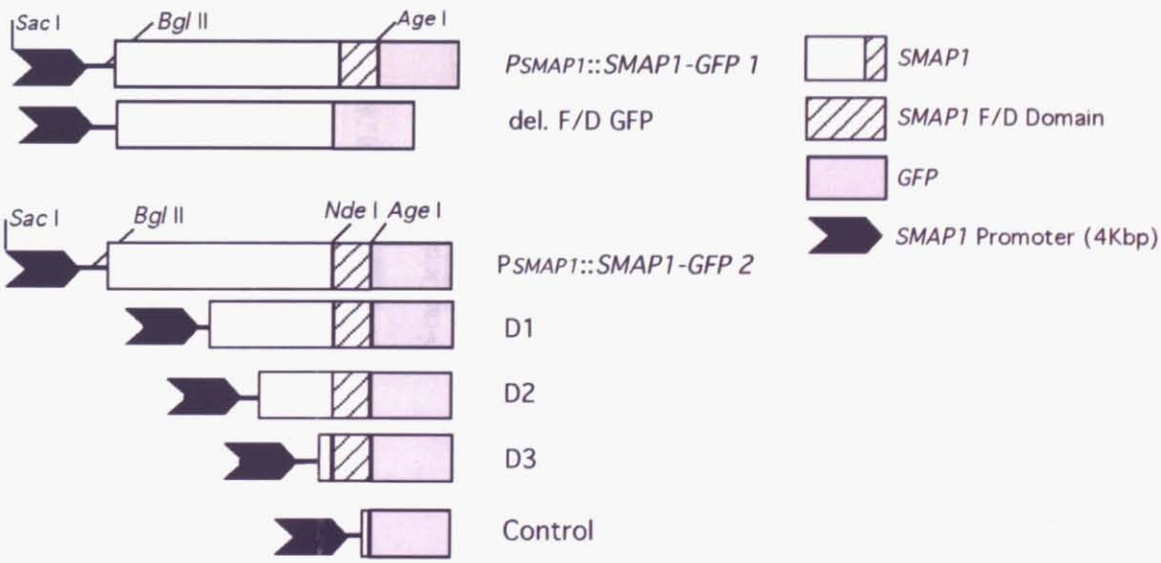
Figure 3. Construction of the P*SMAP1*::*SMAP1-GFP* and its variants.

a. Construction of SMAP-GFP1 and del. F/D, b. construction of SMAP1-GFP2 and D2 thorough D3, and c. the schemes of final products of a. and b. are shown. d. The amino acid sequences of SMAP1-deleted constructs are shown. del. F/D lacks the C-terminal F/D region, and D1 to D3 lack a part of N-terminal of SMAP1. For the control, *GFP* derived under *SMAP1* promoter was also constructed.

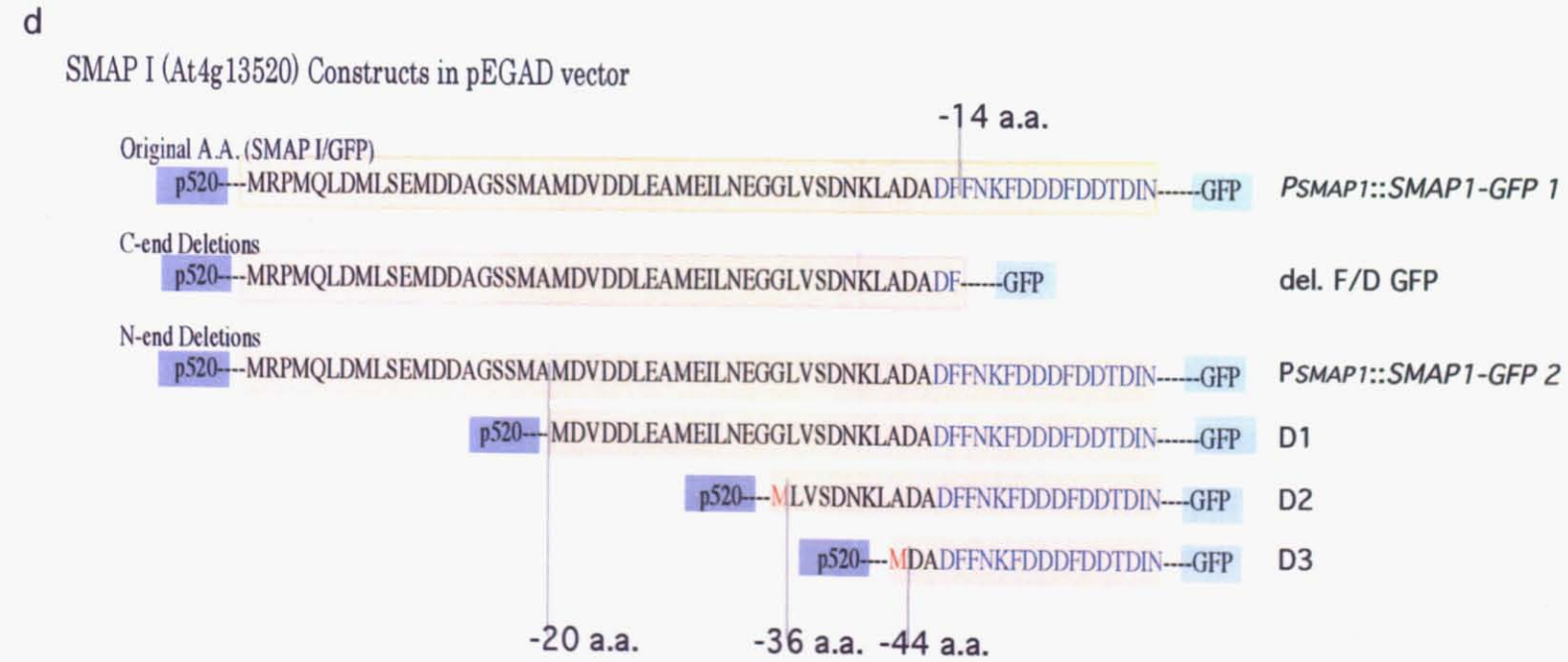
b


Sac I
Bgl II
F/D Rich Region
Sac I
PSMAP1
SMAP1 ORF
TSMAP1
pS4G7Sac#27
1) Endonuclease Digesion with Sac I and Bgl II
Sac I
Bgl II
PSMAP1
(5kbp)
2) PCR (5 Kinds)
Bgl II
SMAP1 ORF
R. Primer 8 with Nde 1
F. Primer 1
R. Primer 2 with Age I
F. Primer 4-7 with Nde I
Nde 1
Age1
3) Endonuclease Digestion and Purification of products 2)
Nde I
Age I
SMAP1 ORF
SMAP1-GFP2
D1
D2
D3
Bgl II-Nde I
Sac I
Age I
35S
GFP
T-NOS
LB Site
RB Site
pEGAD
4) Ligation and Purification
Sac I
Bgl II
Nde I
Age I
PSMAP1
SMAP1 ORF
Length Varies
Endonuclease digestion with Sac I and Age I
Sac I
Age I
GFP
T-NOS
LB Site
RB Site
pEGAD
5) Ligation with pEGAD
Sac I
Bgl II
Nde I
Age I
PSMAP1
SMAP1 ORF
GFP
T-NOS
Length Varies
LB Site
pEGAD
RB Site

c


Sac I
Bgl II
Age I
PSMAP1::SMAP1-GFP 1
del. F/D GFP
Sac I
Bgl II
Nde I
Age I
PSMAP1::SMAP1-GFP 2
D1
D2
D3
Control
SMAP1
SMAP1 F/D Domain
GFP
SMAP1 Promoter (4Kbp)

d


SMAP I (At4g13520) Constructs in pEGAD vector
-14 a.a.
Original A.A. (SMAP I/GFP)
p520----MRPMQLDMLSEMDDAGSSMAMDVDDLEAMEILNEGGLVSDNKLADADFFNKFDDDFDDTDIN------GFP
PSMAP1::SMAP1-GFP 1
C-end Deletions
p520----MRPMQLDMLSEMDDAGSSMAMDVDDLEAMEILNEGGLVSDNKLADADF------GFP
del. F/D GFP
N-end Deletions
p520----MRPMQLDMLSEMDDAGSSMAMDVDDLEAMEILNEGGLVSDNKLADADFFNKFDDDFDDTDIN------GFP
PSMAP1::SMAP1-GFP 2
p520----MDVDDLEAMEILNEGGLVSDNKLADADFFNKFDDDFDDTDIN------GFP
D1
p520----MLVSDNKLADADFFNKFDDDFDDTDIN----GFP
D2
p520----MDADFFNKFDDDFDDTDIN----GFP
D3
-20 a.a.
-36 a.a.
-44 a.a.

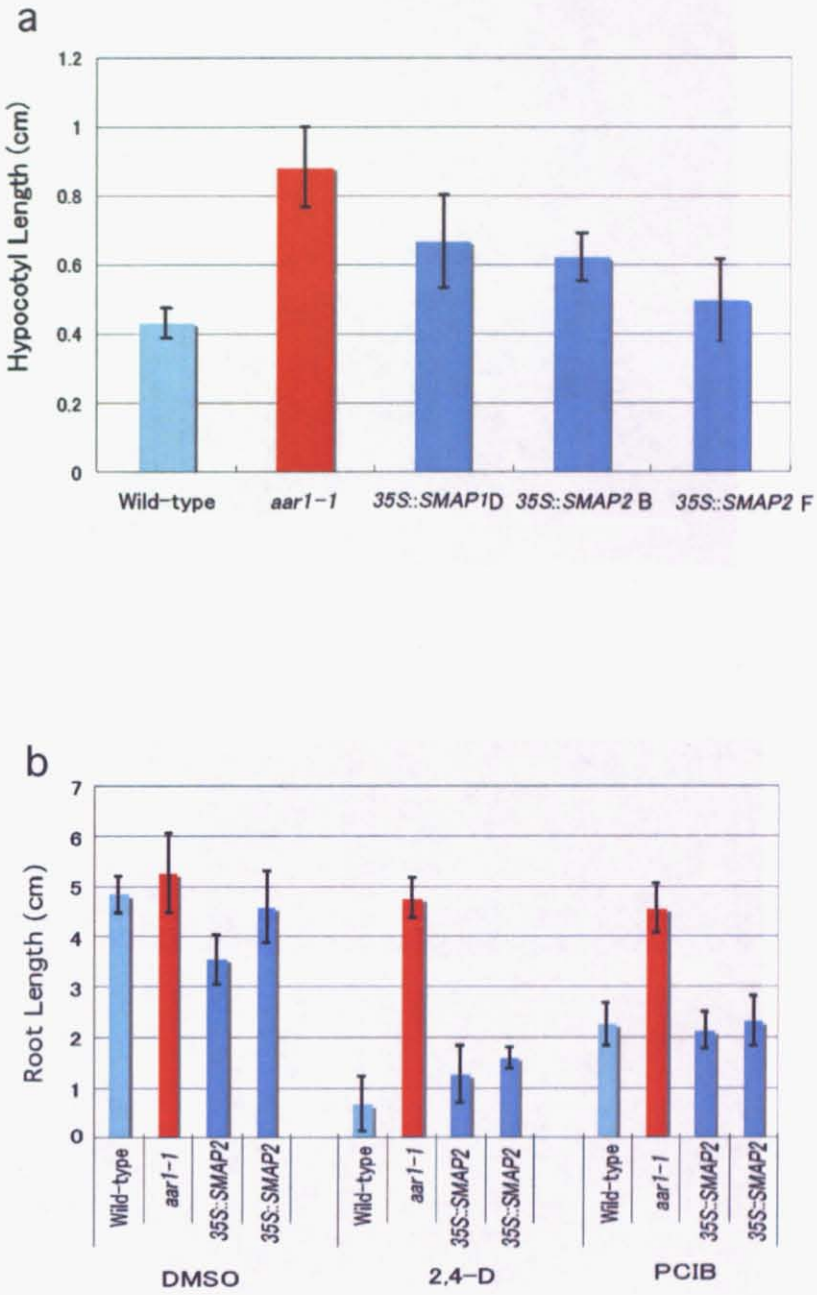


Figure 4. Hypocotyl elongation and root growth of SMAP2 O/E.

Wild-type, aar1-1, and SMAP2 O/E transgenic plants were compared. a. Hypocotyl elongation of SMAP2 O/E on 7 day was graphed. b. Root length of 10-day-old SMP2 O/E plants on DMSO, 40 nM 2,4-D, and 20 μM PCIB media were graphed.

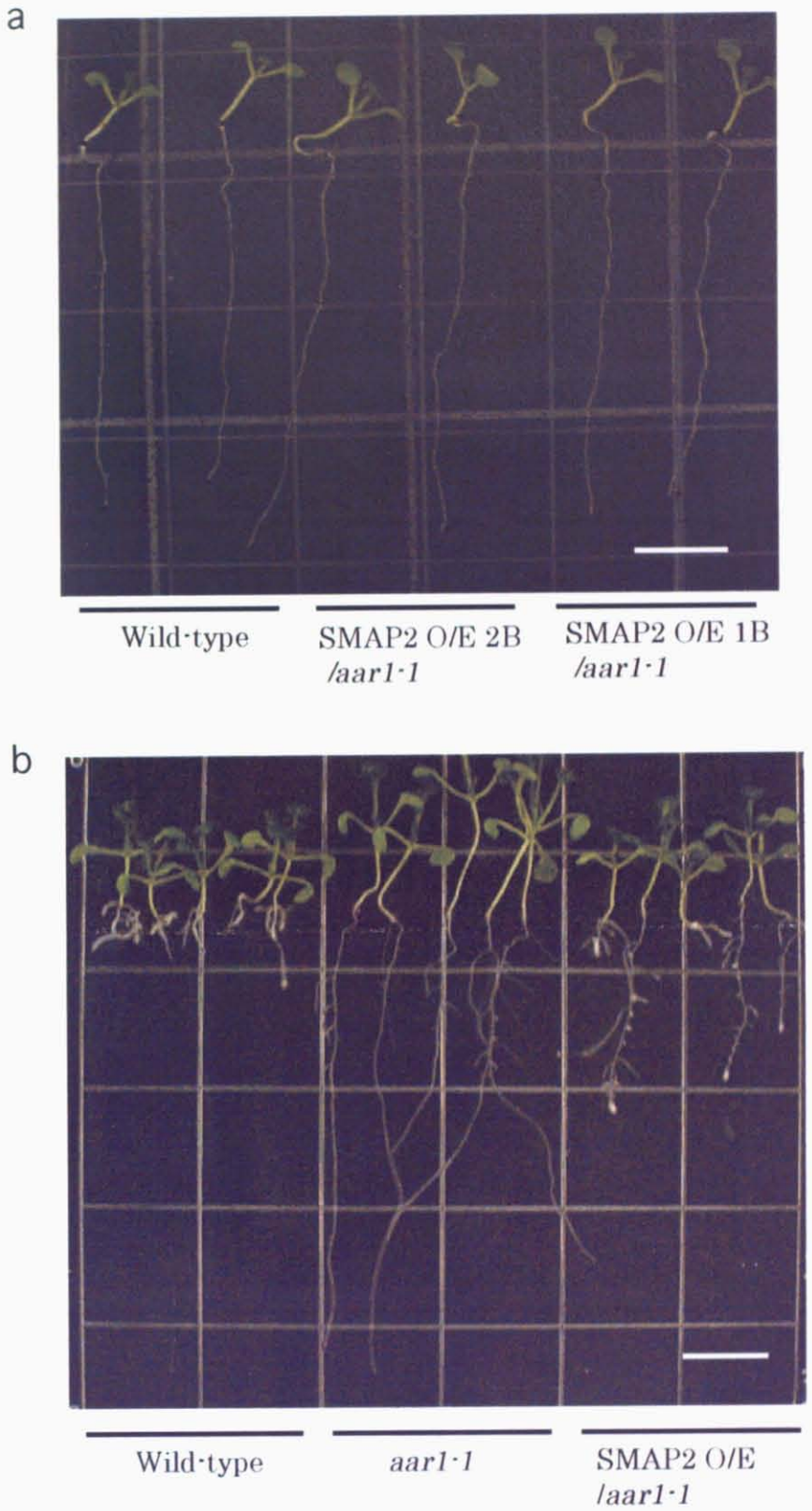


Figure 5. Photographs of wild type and SMAP2 O/E lines.

a. Seven-day-old plants grown on MS media without auxin. b. Ten-day-old plants grown on 40 nM 2,4-D. The white bars represent 1 cm.

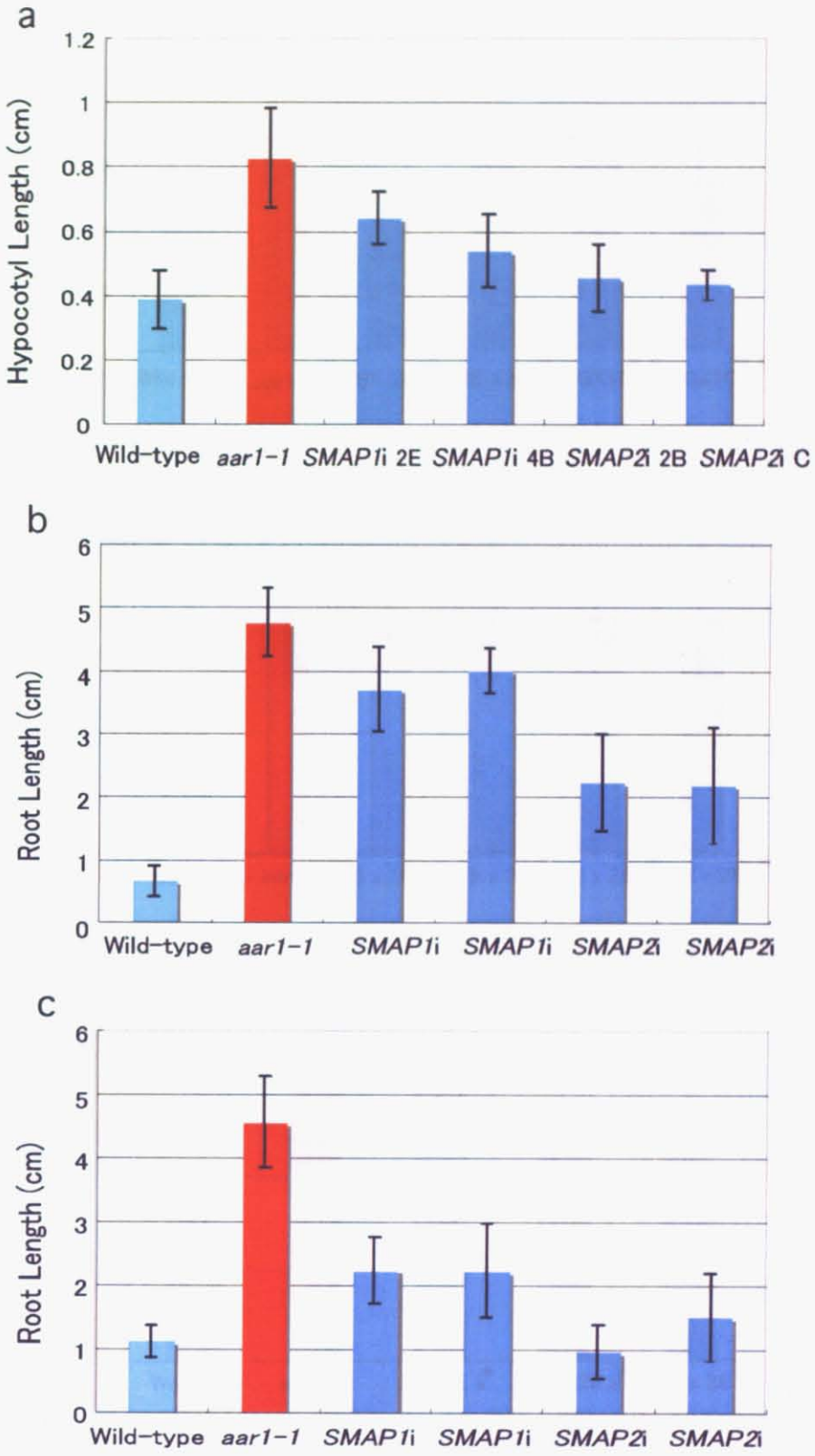


Figure 6. Hypocotyl length and root growth of RNAi lines.

a, Hypocotyl length at day 7. b. and c. The root length of 10-day-old plants grown on 40 nM 2,4-D (b) or on 20 μ M PCIB (c).

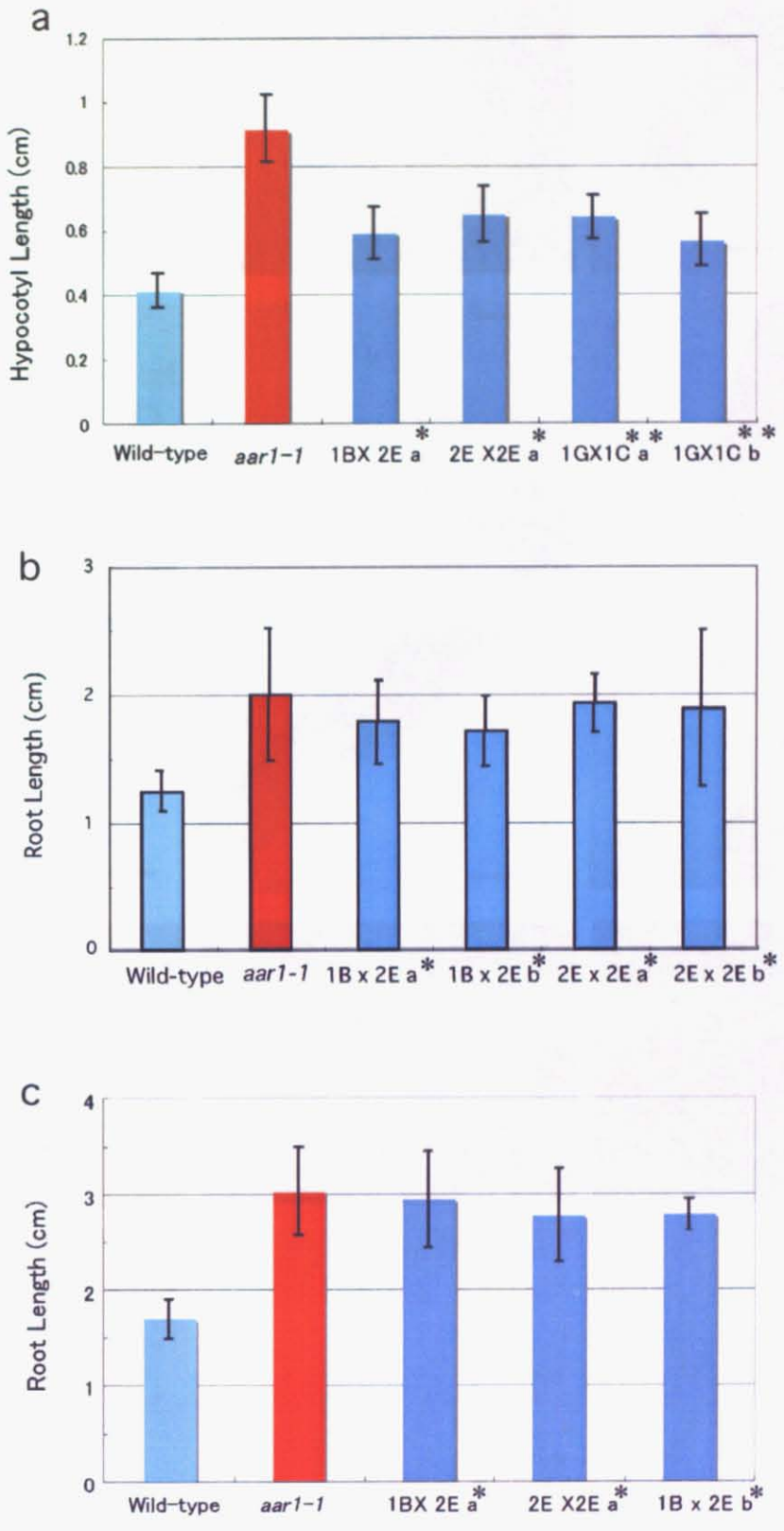


Figure 7. Hypocotyl length and root length of double knockout lines.

*SMAP1*i (line 2E or 1C) and *SMAP2*i (line 1G, 2E, or 1B) were crossed, and F1 seeds were used. a. Hypocotyl length on 7-day-old seedlings. b. and c. Root growth of 10-d-old seedlings on 40 nM 2,4-D medium (b) or 20 μM PCIB (c). The symbol '*' indicates *SMAP2*i x *SMAP1*i, and '**' indicates *SMAP1*i x *SMAP2*i. The 'a' and 'b' indicate independent crosses.

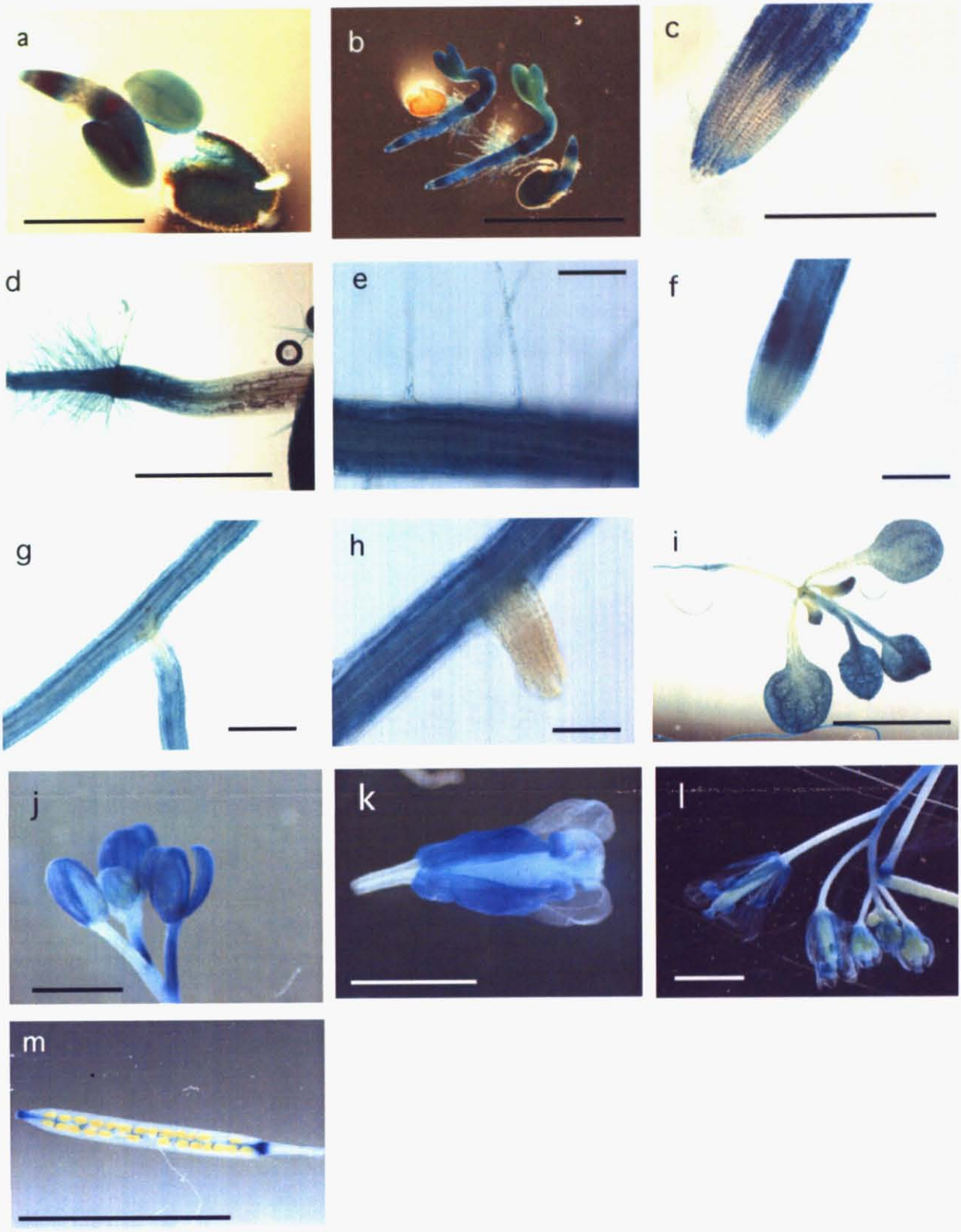


Figure 8. GUS staining of P*SMAP1*::*GUS*.

P*SMAP1*::*GUS* transgenic plants were incubated with GUS buffer containing X-gluc for 16 h. a. The 1-day-old plants. b and c. The two-day-old. d. through i. 10-day-old plants. j. A cluster of buds. k. A flower. l. A cluster of flowers. m. A silique. The bars indicate 0.5 mm (a), 2 mm (b), 50 mm (c), 1mm (d), 100 μm (e) and (f), 0.2mm (g), 100 μm (h), 1 cm (i), 1mm (j thorough l), and 1cm (m).

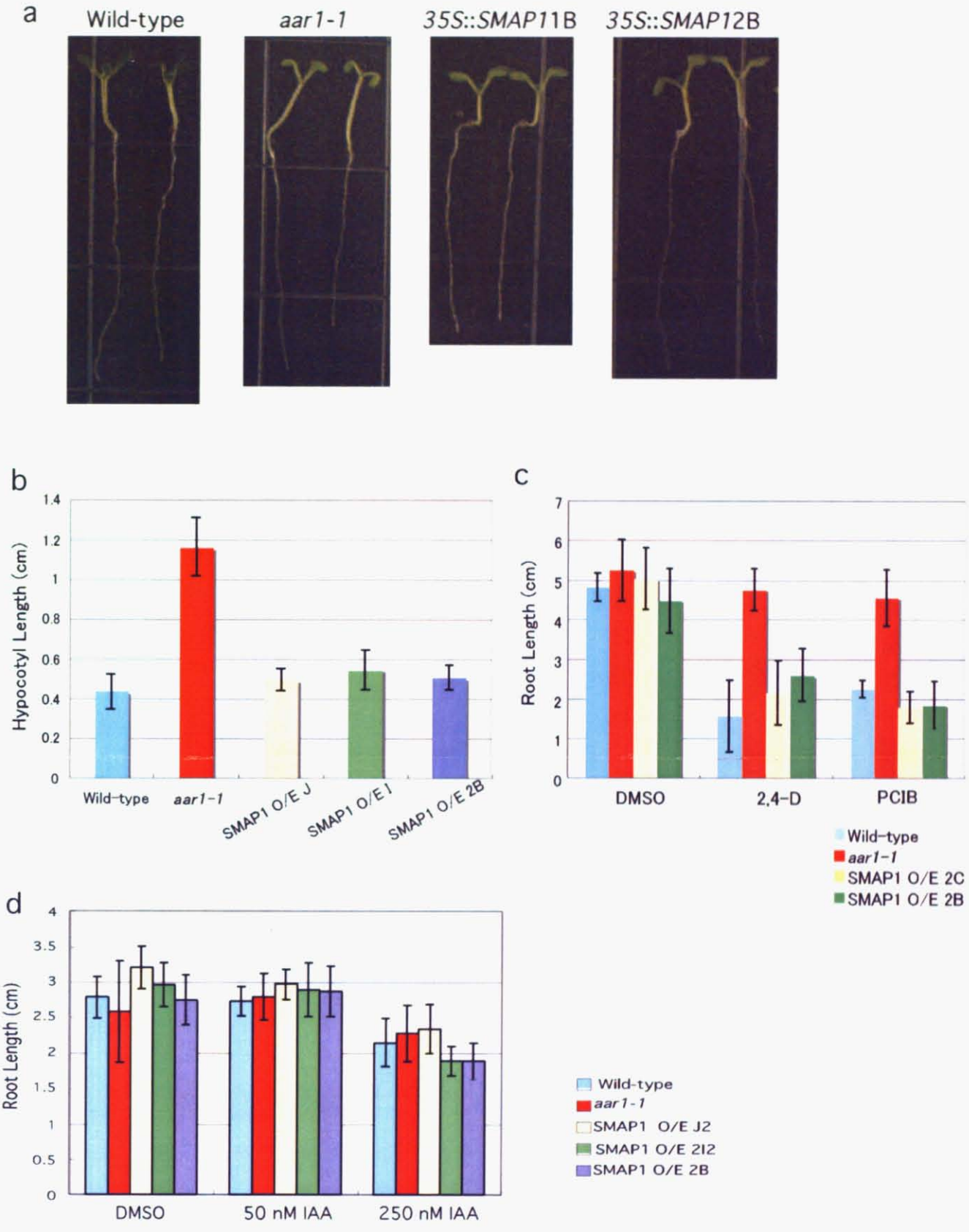


Figure 9. Characterization of *35S::SMAP1* (SMAP1 O/E).

Two or three independent lines of SMAP1 O/E were compared with wild-type and *aar1-1*. a. Photographs of 7-day-old grown on GM without auxin. b. Hypocotyl length of 7-day-old plants. c. Root length of 10-day-old seedling grown on DMSO, 40 nM 2,4-D, and 20 μM PCIB. d. Root length of 10-day-old seedling grown on DMSO, 50 nM IAA, and 250 nM IAA.

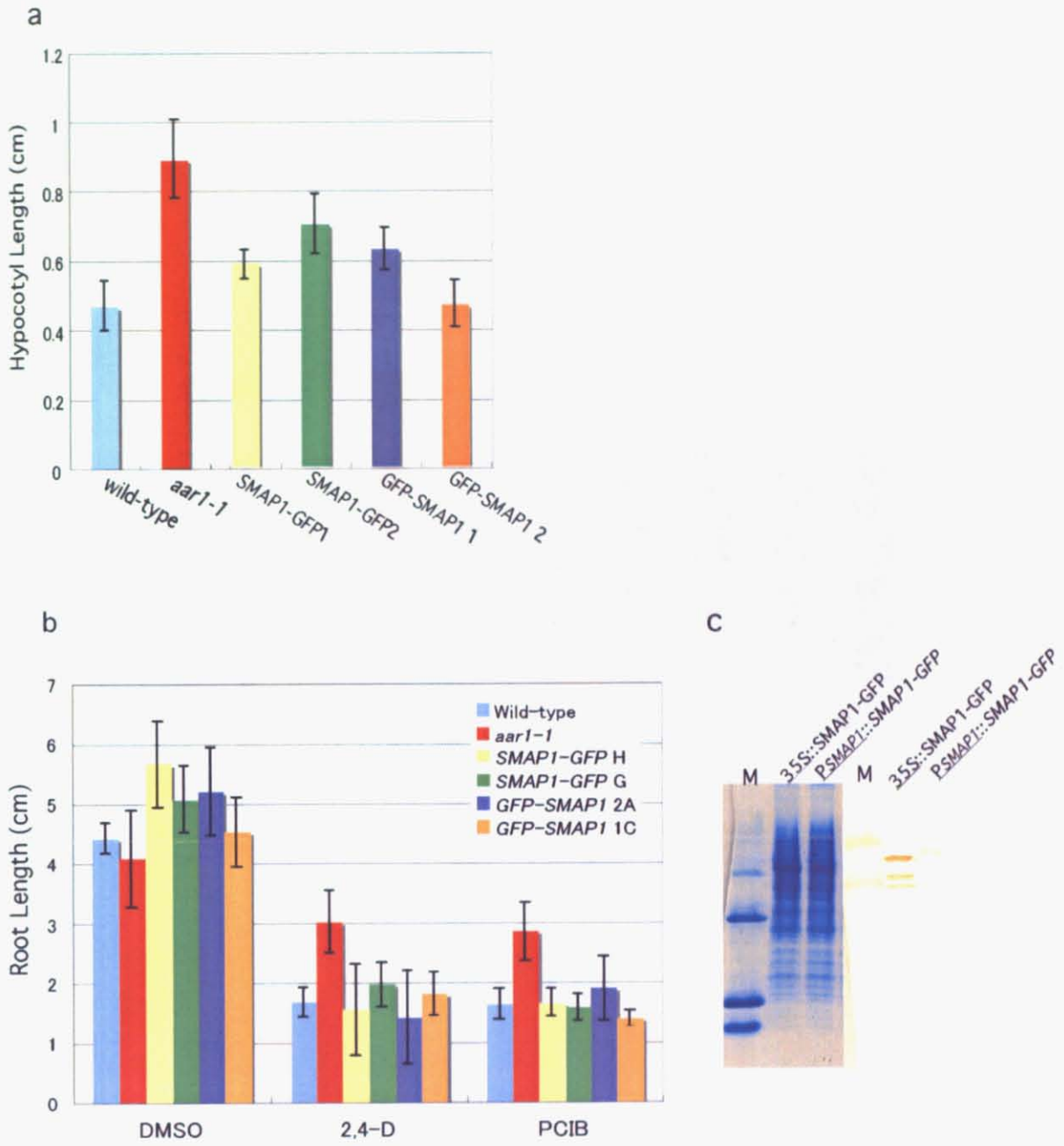


Figure 10. Effects of overexpression of fusion protein of SMAP1 and GFP in the *aar1-1* mutant.

The transgenic plants, *35S::SMAP1-GFP* (SMAP1-GFP) and *35S::GFP-SMAP1* (GFP-SMAP1) in *aar1-1*, were generated to determine the influence of GFP fusion on SMAP1 protein and were compared with wild-type and *aar1-1*. a. Hypocotyl elongation of 7-day-old plants grown on GM. b. Root length of 7-day-old plants grown on GM containing DMSO, 40 nM 2,4-D, or 20 μ M PCIB. c. Expression of SMAP1-GFP protein derived by *35S* or *SMAP1* promoter. Western blotting was performed with anti-GFP antibody. The symbol M in c. indicates molecular weight marker.

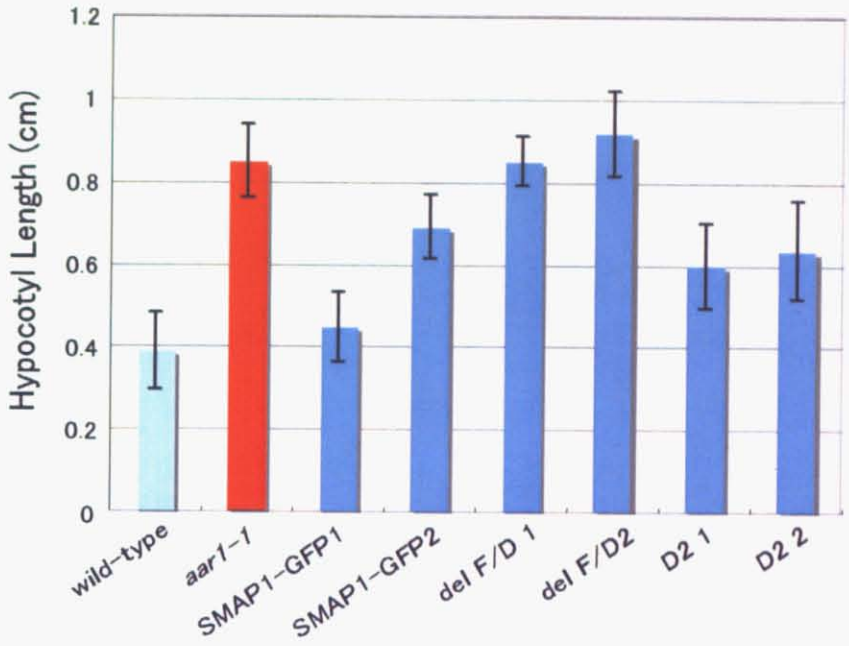


Figure 11. Hypocotyl length of P*SMAP1*::*SMAP1-GFP* transgenic plants.
Hypocotyl length of 7-day-old P*SMAP1*::*SMAP1-GFP* transgenic plants were compared with wild-type and *aar1-1*. The plants were grown on GM. Bars show mean ± SD.

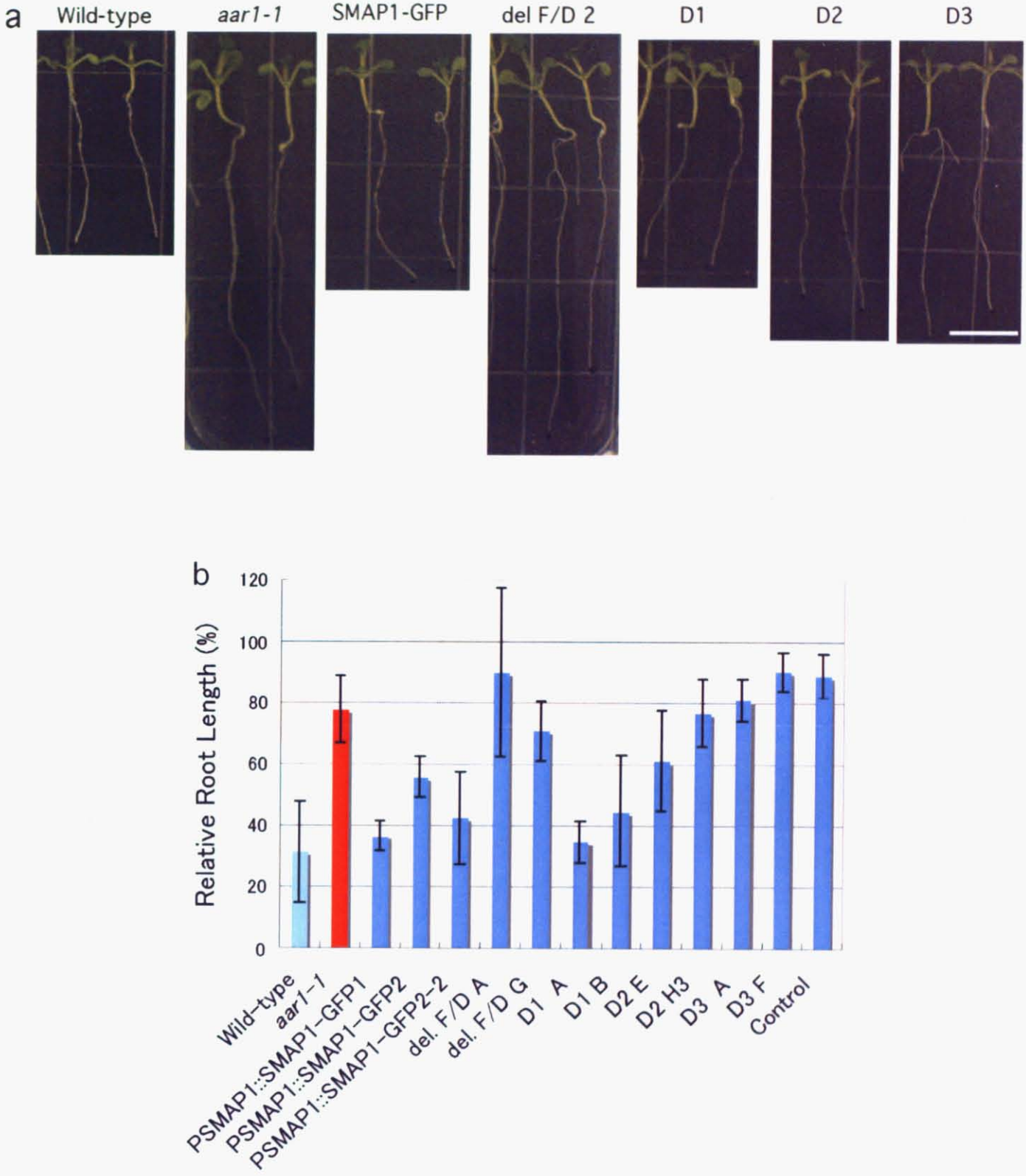


Figure 12. Root growth of the *aar1-1* plants expressing SMAP1 GFP-fusion and its deleted derivatives on 40 nM 2,4-D.

a. Photographs of 10-day-old plants. The bar indicates 1 cm. b. Root length of 10-day-old plants was measured and expressed relative to controls grown on GM without 2,4-D. Bars show mean ± SD.

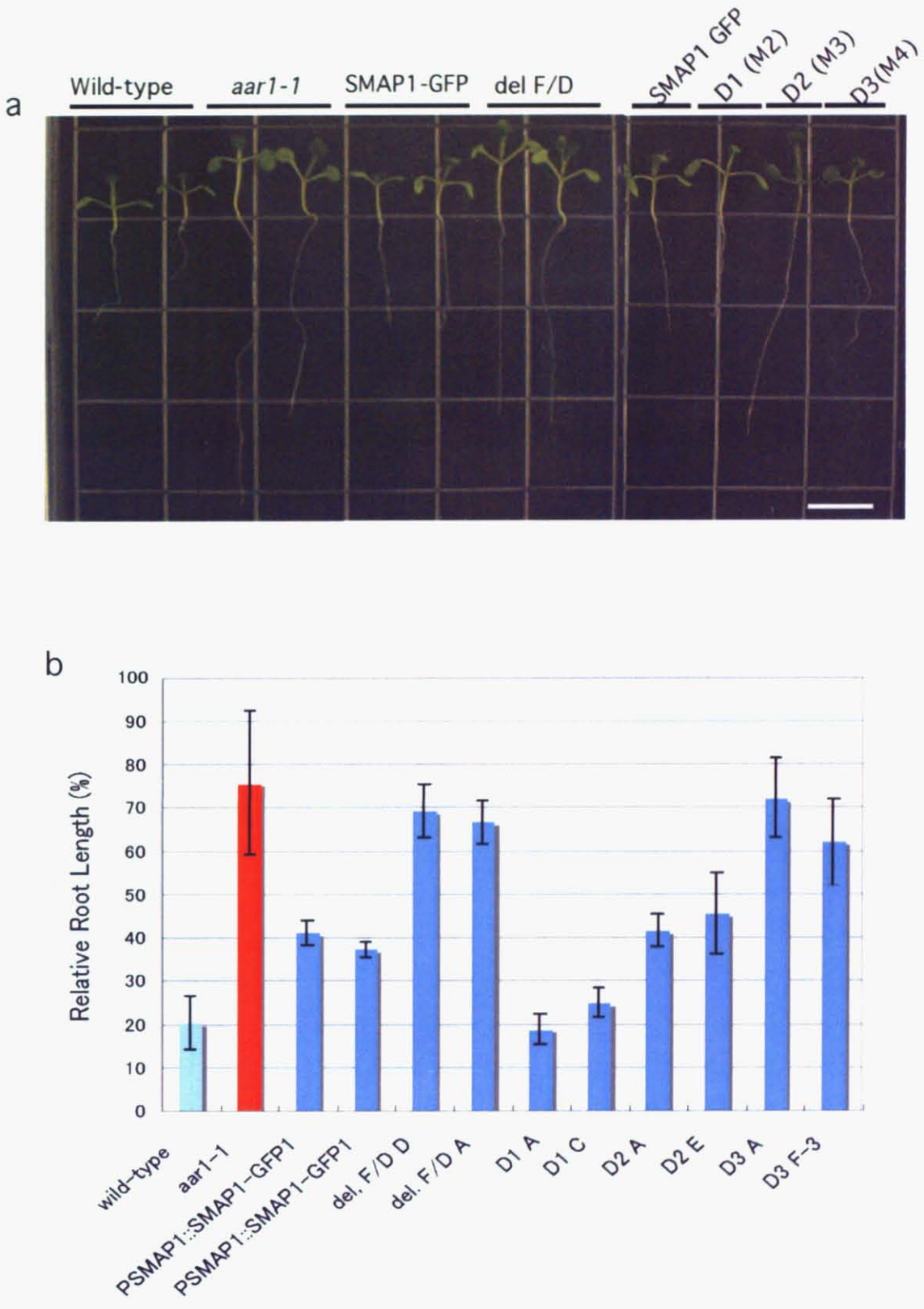


Figure 13. Root growth of the *aar1-1* plants expressing SMAP1 GFP-fusion and its deleted derivatives on 20 μM PCIB.

a. Photographs of 10-day-old plants were photographed. The white bar indicates 1 cm. b. Root length of 10-day-old plants was measured and expressed relative to controls grown on GM without 2,4-D. Bars show mean ± SD.

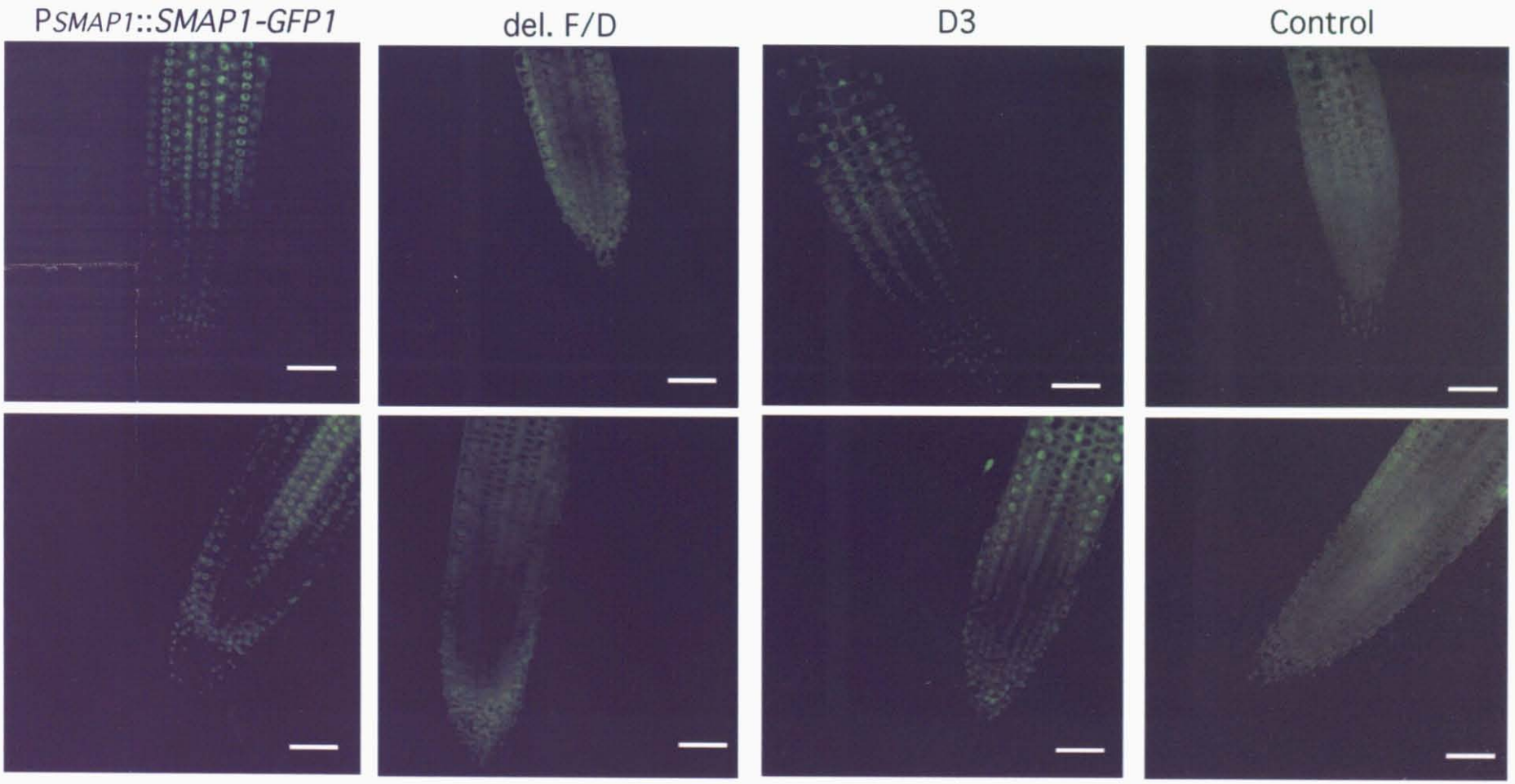


Figure 14. SMAP1 protein localization at root
P*SMAP1*::*SMAP1-GFP* and its variants were observed on day 5 and day10.
Control is P*SMAP1*::*GFP*. The bars indicate 50 μm.

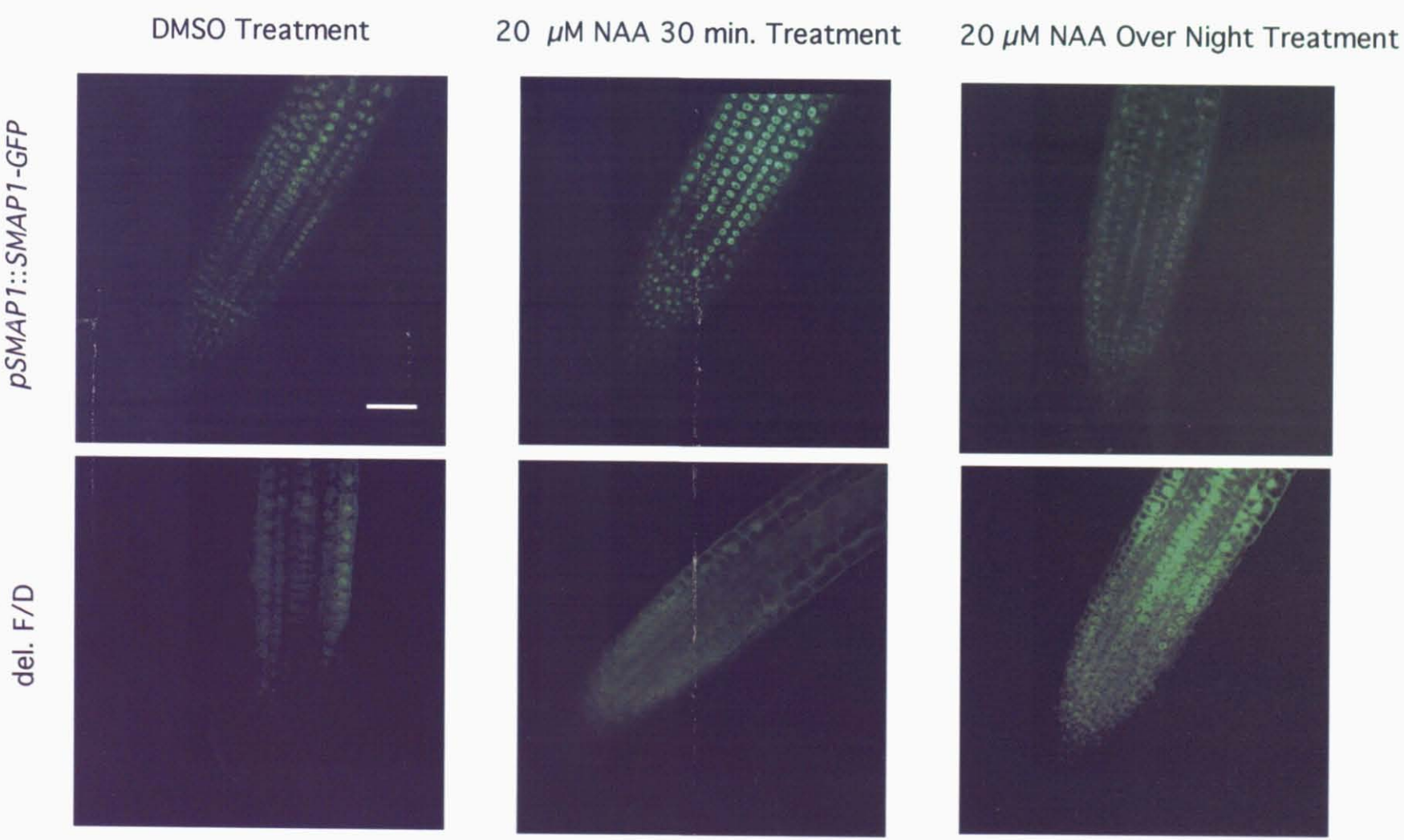
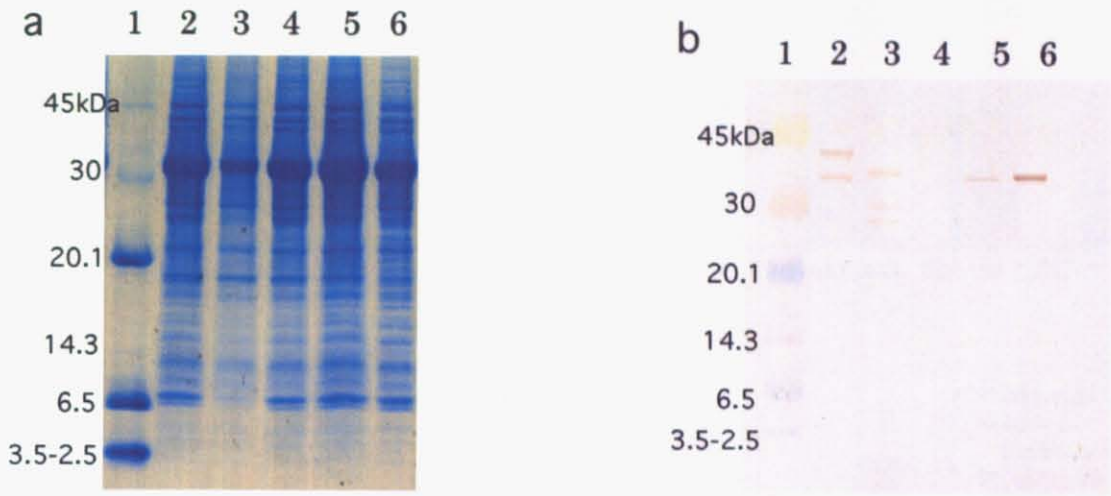


Figure 15. Twenty μm NAA treatment of *pSMAP1::SMAP1-GFP* and del. F/D. Plants on day 7 were dipped in MS + MES media containing DMSO or 20 μM NAA. All pictures are in same magnification, and the bar indicates 50 μm.



Lane:
1. Rainbow Marker
2. PSMAP1::SMAP1-GFP1
3. del. F/D
4. D1
5. D2
6. D3

Figure 16. Immuno blot analysis of SMAP1 GFP-fusion and its deleted derivatives.
To each lane, 15 μg total plant protein was subjected to SDS-PAGE. a. Gel-code-stained gel.
b. Western blot with anti-GFP antibody.

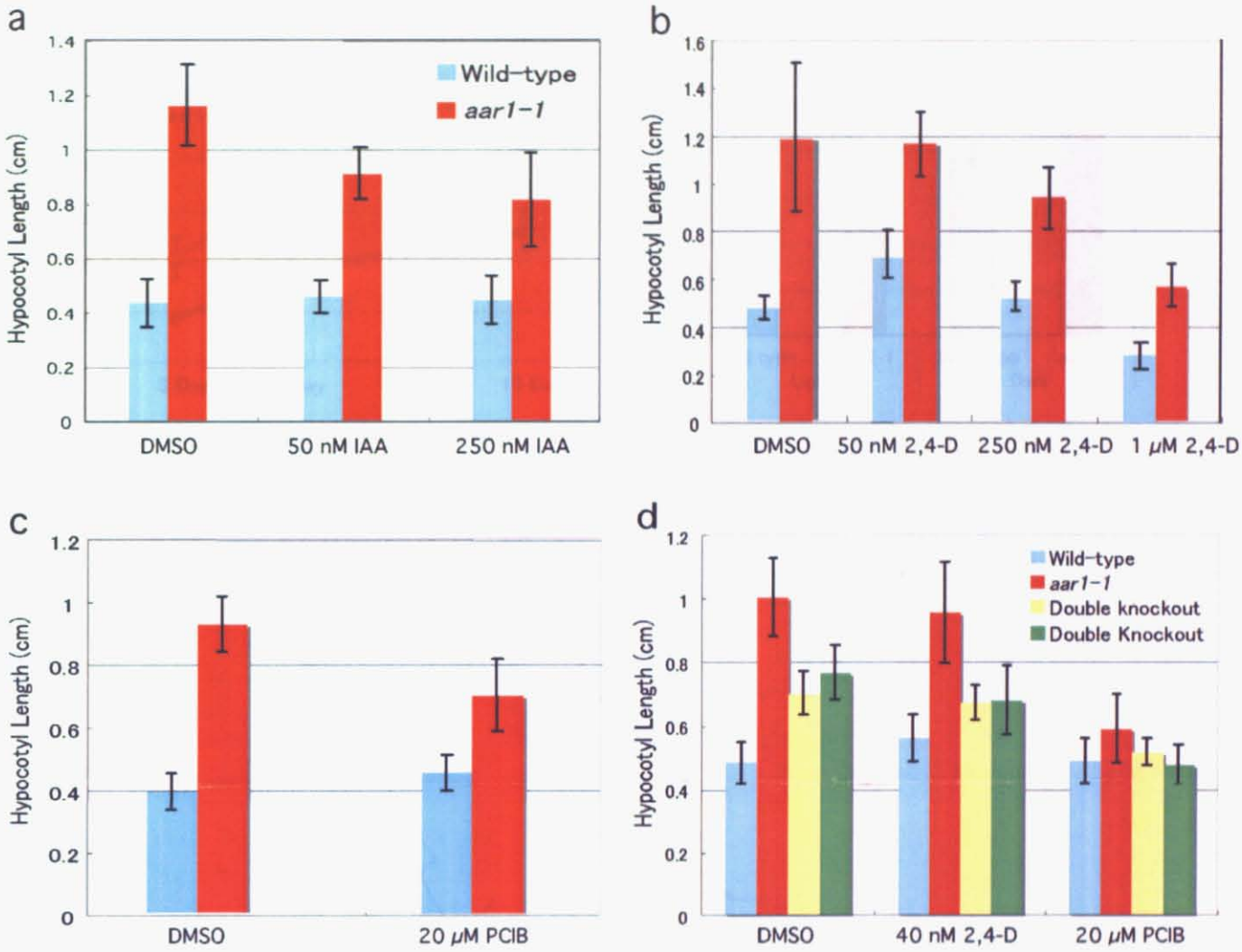


Figure17. Effect of auxin and PCIB on hypocotyl elongation.

Hypocotyl length of 10-day-old plants was measured.

a. Wild-type, *aar1-1*, and two lines of double knockout transgenic plants grown on 40 nM 2,4-D or 20 μM PCIB. b. Wild-type, *aar1-1*, and 35S::SMAP1 lines plated on GM containing DMSO as control, 50 nM, 250 nM IAA , c. 50 nM, 250 nM, 1 μM 2,4-D, and d. 20 μM PCIB.

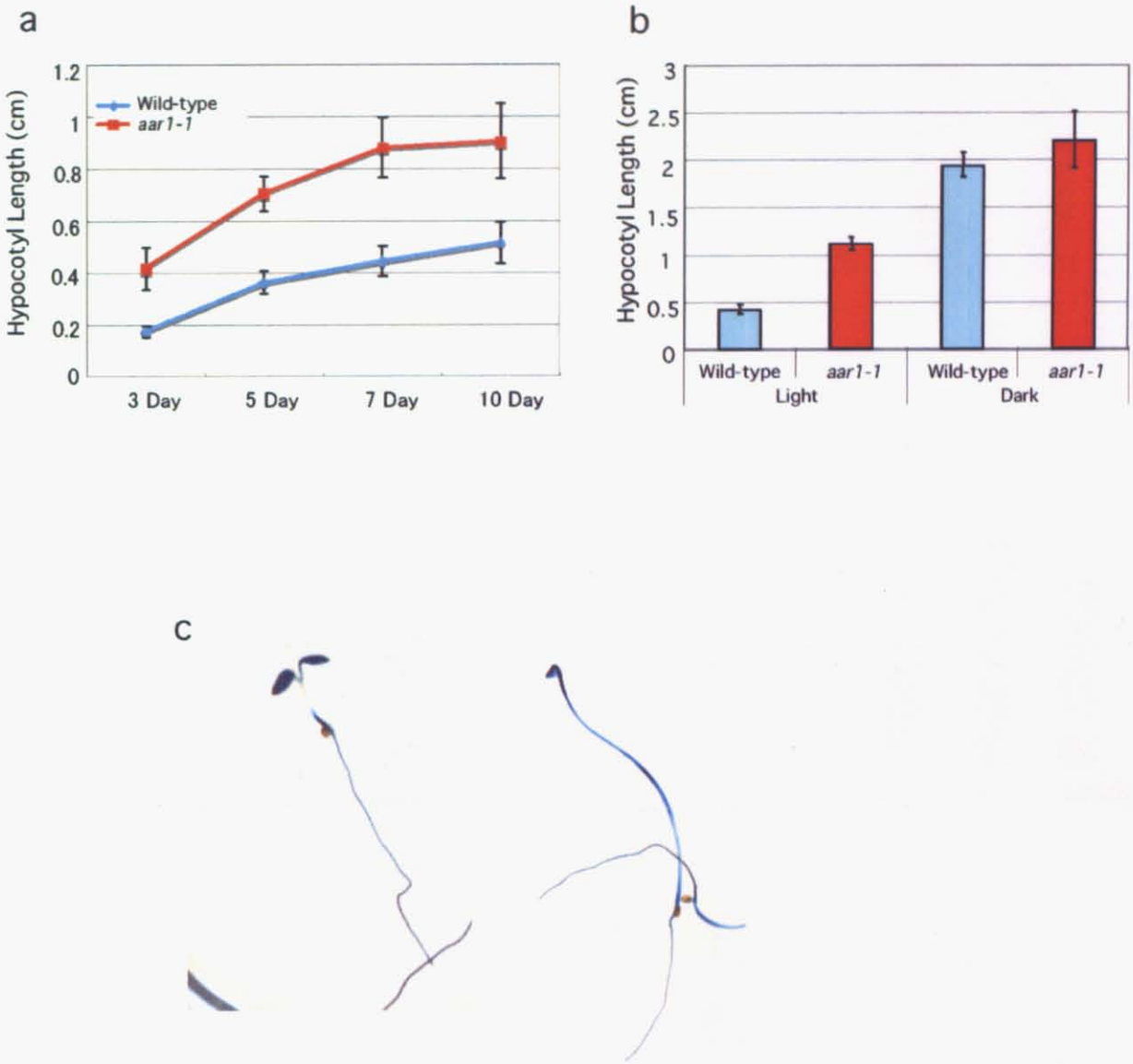


Figure 18. Phenotype in hypocotyl of wild-type and *aar1-1* plants.

a. Hypocotyl length of wild-type and *aar1-1* was compared from Day 3 to 10. b. Wild-type and *aar1-1* was grown in light or dark for 7 day, and their hypocotyl length was compared. c. Expression of P*SMAP1*::*GUS* in 3-day-old light (left) and dark (right) grown plants incubated in GUS buffer for over night in a dark chamber at 37 °C.

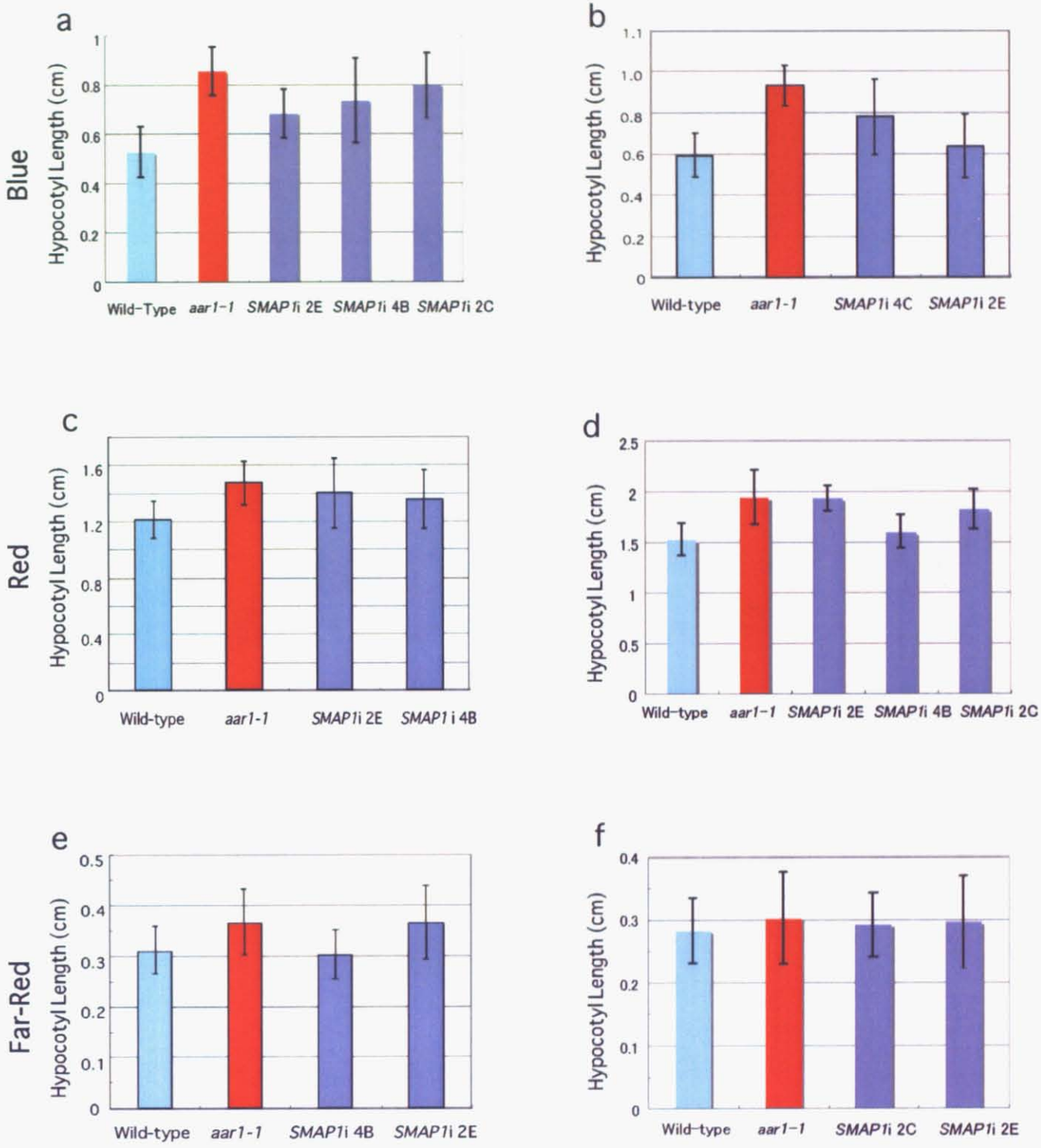


Figure 19. Hypocotyl length in various light conditions.

Hypocotyl elongation under blue, red, and far-red light was measured on day 7 after germination. The light conditions were: a. Blue light 25.8 μ mole $m^{-2}s^{-1}$. b. Blue light 56.0 μ mole $m^{-2}s^{-1}$. c. Red light 20 μ mole $m^{-2}s^{-1}$. d. Red light 15.0 μ mole $m^{-2}s^{-1}$. e. Far-red light 0.76 μ mole $m^{-2}s^{-1}$. f. Far-red light 0.41 μ mole $m^{-2}s^{-1}$.