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ORIGINAL RESEARCH

F-box Protein Arabidillo-1 Promotes Lateral Root Development by Depressing the Functioning of GA_3 in *Arabidopsis*

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Abstract In *Arabidopsis*, Arabidillo-1 and Arabidillo-2 have great sequence homology to *Dictyostelium* and metazoan β–*catenin*/*Armadillo*, which are important to animal and *Dictyostelium* development. Arabidillo-1 and Arabidillo-2 promote lateral root formation redundantly in *Arabidopsis*. Here, we showed that gibberellins (GA_3) has a greater inhibitory effect on lateral root growth from the null mutant *arabidillo-1* than from the wild type, suggesting that the mechanism for Arabidillo-1-regulated modulation of lateral root proliferation is associated with GA_3-metabolic or signaling pathways. Our yeast two-hybrid analysis demonstrated that Arabidillo-1 interacts with ASK2 and ASK11, and that ASK2 can bind with the F-box domain of Arabidillo-1. Therefore, Arabidillo-1 is involved in the ubiquitin/26S proteasome-mediated proteolytic pathway. Based on these results, we conclude that Arabidillo-1 can degrade some positive regulator of the GA_3 signaling pathway through selective protein degradation of ubiquitin/26S. Moreover, that process is believed to be the mechanism for Arabidillo-1 promotion of lateral root development in *Arabidopsis*.

Keywords Arabidillo-1 · Armadillo · F-box · GA · Lateral roots

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Introduction

The Armadillo (Arm-) repeat protein is a type of protein containing tandem repeats of a sequence motif of about 42 amino acids (Peifer et al. 1994; Huber et al. 1997). Such proteins are conserved across eukaryotic kingdoms, and non-metazoa has homologues for members of the animal Arm-repeat proteins family (Coates 2003). In animals, these proteins function in various processes, e.g., cytoskeletal regulation and intracellular signaling (Coates 2003). In plants, their roles include light/gibberellin signaling and trichome development (Amador et al. 2001; Downes et al. 2003; El Refy et al. 2003). Two *Arabidopsis* proteins, Arabidillo-1 and -2, show the greatest sequence homology to metazoan and *Dictyostelium* β-catenin (Coates 2003), acting redundantly to promote lateral root development (Coates et al. 2006).

Selective protein degradation is an important post-transcriptional regulatory process. Through this mechanism, cells can respond rapidly to intracellular signals and changing environmental conditions. In plants, the ubiquitin/26S proteasome-mediated proteolytic pathway is an important system for that process (Sullivan et al. 2003; Smalle and Vierstra 2004). F-box proteins, which contain an F-box motif (approximately 40 amino acids) near their N-terminus, anchor that subunit to the rest of the SCF (a complex of Skp1/Cullin or CDC53/F-box protein) by interacting with the F-box and Skp1. The C-terminus of such F-box proteins usually contains one or more protein–protein interaction motifs that facilitate target identification. Because Arabidillo-1 and Arabidillo-2 have F-box motifs near their N-termini, we can speculate that they are involved in ubiquitin/26S proteasome-mediated proteolytic degradation. Furthermore, the Arm-repeat domains of

Arabidillo-1/2 participate as the surface for protein–protein interactions for substrate identification.

Gibberellins (GAs) regulate a wide range of developmental processes, including fruit and seed development, stem elongation, flowering, leaf expansion, and seed germination (Sun and Gubler 2004; Swain and Singh 2005). Their role in lateral root formation is still unclear. Whereas inhibitors of GA biosynthesis can promote the growth of lateral roots (Berova and Zlatev 2000; Chaney 2003; Watson 2004; Grossi et al. 2005), exogenous application of GA_3 suppresses adventitious root formation (Lo et al. 2008). Modulation of GA metabolism and responses provides an important signaling mechanism that helps plants react to stress through coordinated suppression of their aerial growth and the stimulation of lateral root development (Gou et al. 2010).

Both Arabidillo-1 and gibberellins promote lateral root growth in *Arabidopsis*. Therefore, our objective was to identify possible interactions between the two in regulating lateral root formation, and to investigate ubiquitin/26S proteasome-mediated proteolytic degradation of some positive regulator for the GA_3 signaling pathway in that species.

Materials and Methods

Plant Growing Conditions

Arabidopsis seedlings were grown on a full-strength Murashige and Skoog (1962) medium (MS; pH 5.8) that was supplemented with 1% (*w*/*v*) Suc and 1× Gamborg's vitamins. Seeds were first surface sterilized with a 20% (*v*/*v*) bleach solution, then washed thoroughly with sterile water and placed on MS plates solidified with 1.0% agar. After incubation at 4°C for 2 days in darkness, the seedlings were oriented vertically for growth under continuous cool-white light at room temperature.

RNA Preparation and Reverse Transcription-PCR

Total RNA was obtained from the leaves, flower buds, stems, and roots of 4-week-old plants, using an RNA isolation kit (TaKaRa, Dalian, China). Samples of each tissue type (1 μg) were digested with RNase-free DNase I (TaKaRa) for reverse transcription (RT) with M-MLV reverse transcriptase (Invitrogen, CA, USA). After a 1:10 dilution was made, 1 μl of the synthesized cDNA was used for RT-polymerase chain reaction (PCR). Conditions included 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min; followed by a final 72°C for 5 min. Products were then cloned and sequenced to check identity.

Generation of Constructs and Transformation of *Arabidopsis*

A 2,123-bp genomic fragment (+6 to –2,117 upstream of the predicted start codon for *Arabidillo-1*) was amplified as the *Arabidillo-1* promoter. Afterward, this promoter plus cDNA from *Arabidillo-1* and a GFP sequence were inserted into the pBI101.2 binary vector to make *pArabidillo*:: Arabidillo-1-GFP fusions. The construct was introduced into *Agrobacterium* strain GV3101 and subsequently transformed into wild-type *Arabidopsis* ('Col-0') plants by the floral-dip method (Clough and Bent 1998), and the empty vector was also transformed into wild-type *Arabidopsis* as control.

PCR-Screening of T-DNA Insertion Lines

To identify plants with the T-DNA insertion, we performed PCR analyses with genomic DNA, using one gene-specific

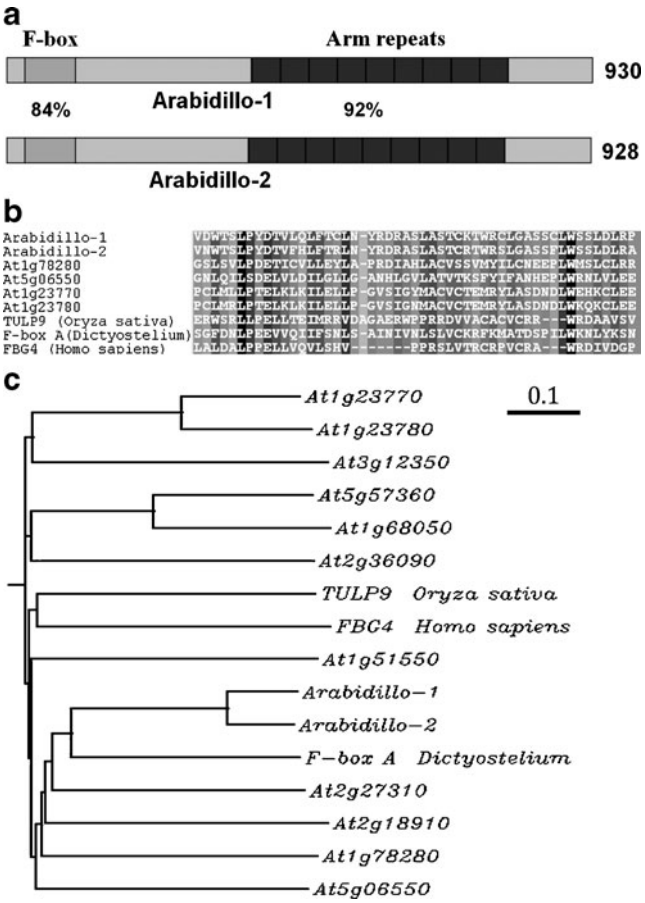

Fig. 1 Structure of Arabidillo-1/2. **a** Both have an F-box domain (sharing 84% amino acid identity) and 9 Arm repeats (sharing 92% amino acid identity). **b** F-box sequences comparison of Arabidillo-1, Arabidillo-2, 4 F-box proteins of *Arabidopsis* (At1g78280, At5g06550, At1g23770, At1g23780), TULP9 (*Oryza sative*), F-box A (*Dictyostelium*) and FBG4 (*Homo Sapiens*). **c** Phylogenetic tree of various F-box proteins. The *bar* represents the branch length equivalent to 0.1 amino acid changes per residue

primer and one T-DNA-specific primer in the left border. Gene-specific primer pairs flanking the insertion site were utilized to determine whether plants were homozygous, while RT-PCR was conducted to test for expression of *Arabidillo-1* in those homozygotes.

Drug Treatments

GA_3 (2 mgml^{-1}; Sigma, USA) was initially dissolved in dimethyl sulphoxide, then added to the MS media to give specific final concentrations. Transformed 3-day-old seedlings that had originally been grown on either a standard MS medium or one supplemented with 2 mgL^{-1} GA_3 were grown for another 1–9 days before their number of lateral roots and length of their main root were recorded. Each experiment was independently performed three times and results were expressed as means±SE.

Microscopy and Image Analysis

Fluorescent specimens were observed with a Zeiss confocal microscope equipped with an epifluorescence UV light filter set. To detect GFP, a 488-nm excitation level and a BP 505–530 filter were used.

Yeast Two-Hybrid Assays

We introduced coding full-length or different domains of *Arabidillo-1* cDNA sequences as well as full-length cDNA sequences of *Arabidillo-2* into the pGBKT7 bait plasmid (Clontech, CA, USA) to produce fusion proteins with the GAL4 DNA binding domain. In addition, 17 ASK cDNAs (ASK1-4, 6–14, and 16–19) were inserted into a pGADT7 prey plasmid containing the GAL4 activation domain (provided by the lab of Yongbiao Xue, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing; Wang et al. 2004). Conditions for yeast transformation, growth, and assays for β-galactosidase activity were as specified in the manufacturer's instructions (Clontech).

Results

Arabidillo-1 is Expressed Throughout the Plant and is Localized to the Nucleus

Arabidillo-1/2 contain nine Arm repeats and share high amino acid identity (Fig. 1a). Both have an F-box motif (residues 45–93 in Arabidillo-1, 38–85 in Arabidillo-2), which also share high amino acid identity (Fig. 1a, b). In addition, Arabidillo-1/2 and F-box A (*Dictyostelium*) belong to the same clade in the phylogenetic tree (Fig. 1c), which suggests they may have similar or related functions in *Arabidopsis* and *Dictyostelium* respectively. To examine the expression patterns of *Arabidillo-1*, we performed RT-PCR with samples from *Arabidopsis* flower buds, stems, leaves, and roots. Products from cDNA templates were sequenced to ensure that the amplified sequences matched the known sequence of *Arabidillo-1*. The *Arabidopsis actin* gene served as a control for RNA extraction and PCR. *Arabidillo-1* mRNA was detected in all tested organs, with roots having the strongest expression (Fig. 2a). Animal Arm β-catenin has both nuclear and

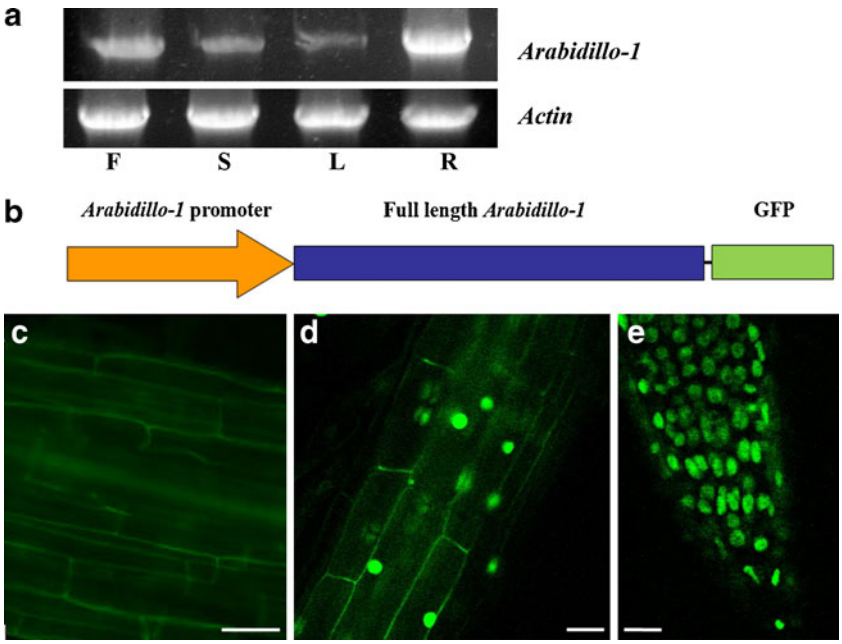

Fig. 2 *Arabidillo-1* is expressed throughout the plant, especially in roots. **a** RT-PCR of *Arabidillo-1* cDNA amplified from flower buds (*F*), stems (*S*), leaves (*L*), and roots (*R*). *Actin* was loaded as control. **b** Structure of Arabidillo-1-GFP fusion proteins. **d**, **e** Arabidillo-1-GFP fusion protein is nuclear protein, **c** the empty vector control of **d** and **e.** *Bar* 50 μm

cytoskeletal functions. To determine where Arabidillo-1 might function in our plant cells, we expressed the full-length protein as GFP fusions driven by an *Arabidillo-1* promoter (Fig. 2b). Arabidillo-1-GFP was detected exclusively in the nuclei (Fig. 2d, e), while the fusion protein was found only in the root cells.

GA_3 Inhibits Lateral Root Development More Strongly in the Null Mutant Arabidillo-1 than in the Wild Type

To examine the relationship among Arabidillo-1, lateral root development, and gibberellin, we obtained an *Arabidopsis* T-DNA insertion line of *Arabidillo-1* (Fig. 3a). No expression of that gene was detected in any of our independent lines (Fig. 3b). When grown on standard MS media, plants of the WT and *arabidillo-1* did not differ in their numbers of lateral roots (Fig. 3c, e, g). However, when treated with 2 mgL^{-1} of GA_3, both the WT and *arabidillo-1* had fewer lateral roots compared with the controls, with gibberellin having a greater inhibitory effect on the latter genotype (Fig. 3d, f, g). By contrast, the lengths of primary roots did not differ among either control or treated plants (Fig. 3h). These data suggest that the mechanism for Arabidillo-1-related modulation of lateral root proliferation is associated with GA_3. Similar to the results reported by Coates et al. (2006), we found no changes during the later stages of lateral root development, implying that Arabidillo-1 promotes such growth in *Arabidopsis* by controlling the *initiation* of lateral roots rather than acting during subsequent stages.

Physical Interaction of Arabidillo-1/-2 with ASKs

Both Arabidillo-1 and Arabidillo-2 have F-box domains near the N-terminal; in general, F-box proteins can directly bind to Skp1 through that domain. We cloned full-length cDNAs of *Arabidillo-1* and *Arabidillo-2* into Gal-4 DNA-binding domain vector pGBKT7 and transferred it into yeast stain HF7C. cDNA clones of 17 ASK genes were amplified by the Xue lab, and all 17 were then cloned into Gal-4 activation domain vector pGADT7 for transformation into the same yeast strain. Our yeast two-hybrid assays showed that Arabidillo-1 could interact with ASK2 and

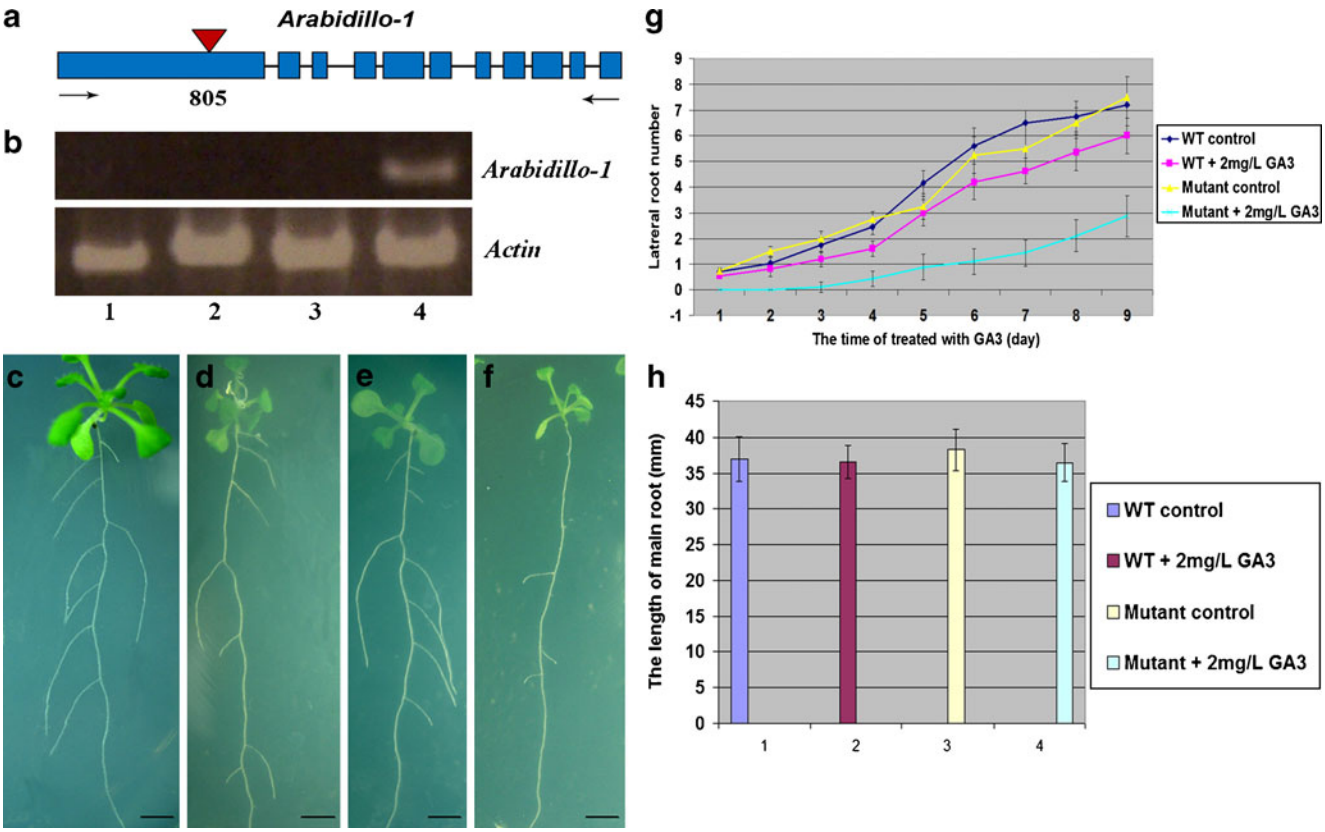

Fig. 3 Influence of GA_3 on insertional mutants. **a** T-DNA insertions (*red*) in *Arabidillo-1*. *Blue boxes* show exons. *Arrowheads* showed location and direction of forward and reverse primers used in RT-PCR. **b** RT-PCR of *arabidillo-1*. cDNA amplified from three independent lines of null mutant (*1*, *2*, *3*) and WT (*4*). *Actin* was loaded as control. **c–f** Differing effects of gibberellin on WT control **c** WT treated with 2 mgL^{-1} GA_3 **d** *arabidillo-1* control **e** and *arabidillo-1* treated with 2 mgL^{-1} GA_3 **f** *Bar* 30 mm. **g** Numbers of lateral roots from WT control, *arabidillo-1* control, WT treated with 2 mgL^{-1} GA_3, or *arabidillo-1* treated with 2 mgL^{-1} GA_3; observations made after 1–9 days of treatment. *H*, lengths of main root from WT control, *arabidillo-1* control, WT treated with 2 mgL^{-1} GA_3, or *arabidillo-1* treated with 2 mgL^{-1} GA_3; observations made after 7 days of treatment

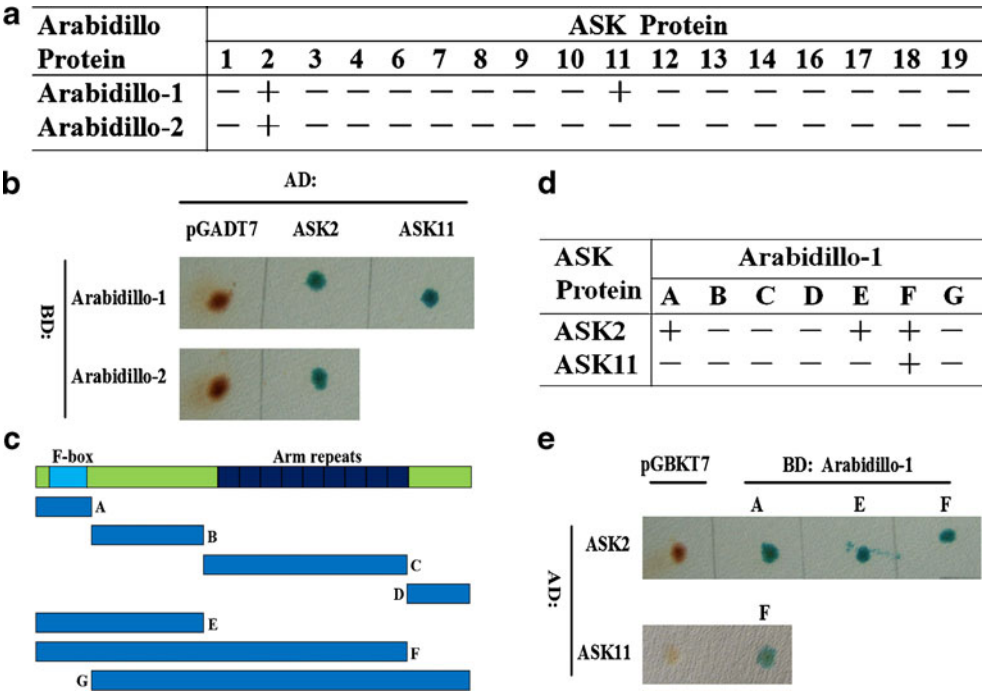

Fig. 4 Physical interactions among Arabidillo-1, Arabidillo-2, and ASK proteins detected by yeast two-hybrid screening. **a** Summary of interactions for ASK preys and Arabidillo-1/-2 baits. Plus (+) and minus (–) indicate positive and negative interactions, respectively. **b** β-galactosidase activities in yeast for interactions of ASK preys and Arabidillo-1/-2 baits. **c** Map of truncated Arabidillo-1. **d** Summary of interactions for ASK2 and ASK11 preys and different truncated Arabidillo-1 baits. Plus (+) and minus (–) indicate positive and negative interactions, respectively. **e** β-galactosidase activities in yeast for interactions of ASK2 and ASK11 preys and different truncated Arabidillo-1 baits

ASK11, while Arabidillo-2 could interact only with ASK2 (Fig. 4a, b). These data showed that, although Arabidillo-1 and Arabidillo-2 share high amino acid identity, they interact with different ASK proteins, possibly because they have different functions in *Arabidopsis*. Taken together, these results suggest that the physical interactions of Arabidillo-1 and Arabidillo-2 with ASKs lead to the formation of diversified functional SCF complexes that target various substrates for ubiquitin/26S proteasome-mediated proteolysis.

ASK Proteins Bind with the F-box Domain of Arabidillo-1

To verify the existence of an ASK2- and Ask11-binding F-box domain for Arabidillo-1, we created seven truncated Arabidillo-1 as bait. These included A (1–100 aa), containing the F-box domain; B (101–347 aa), containing the sequence between the F-box domain and the Armadillo-repeat domains; C (348–750 aa), containing Armadillo-repeat domains; D (751–930 aa), with the C-terminal of Arabidillo-1; E (1–347 aa) and F (1–750 aa), both with F-box domains; and G (101–930 aa), which lacked the F-box domain (Fig. 4c). cDNAs from all seven were cloned into Gal-4 DNA-binding domain vector pGBKT7 and transformed into yeast stain HF7C. Yeast two-hybrid assays showed that truncated Arabidillo-1 A, E, and F can interact with ASK2 but Arabidillo-1 F interacts only with ASK11 (Fig. 4d, e). These data also indicated that ASK2 can bind with the F-box domain of Arabidillo-1. However, it remains unclear why ASK11 F interacts only with truncated Arabidillo-1 F. Several explanations are possible, e.g., another domain of Arabidillo-1 may be involved in the binding of SCF complexes with Arabidillo-1/ASK11, or the assay method was not sensitive enough to detect a weaker interaction between truncated Arabidillo-1 A/E and ASK11.

Arabidillo-1 Cannot form a Homodimer or Heterodimer with Arabidillo-2

Many multi-cellular organisms contain a super protein family of β-catenin/Arm, in which each member has more than one Arm repeat. β-catenin/Arm proteins participate in protein–protein interactions and function in many biological processes. To study whether Arabidillo-1 could form a homodimer or heterodimer with Arabidillo-2, we cloned full-length cDNAs of *Arabidillo-1* and *Arabidillo-2* into Gal-4 DNA-binding domain vector pGADT7 for transformation into yeast stain HF7C. Our yeast two-hybrid assays showed that the Arabidillo-1 failed to interact with either Arabidillo-1 or Arabidillo-2 (Fig. 5). Thus, it was unable to form a homodimer or a heterodimer with the latter. This finding strongly supports our conclusion that the F-box of

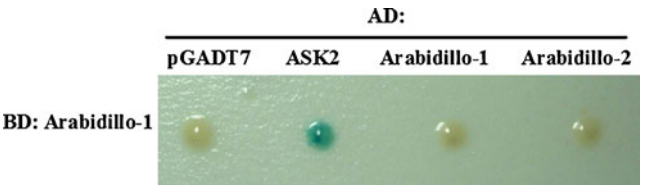

Fig. 5 Arabidillo-1 cannot form homodimer or heterodimer with Arabidillo-2. β-galactosidase activities in yeast for interactions of Arabidillo-1 and Arabidillo-2 preys (ASK2 as positive control) and Arabidillo-1 baits

Arabidillo-1 anchors the subunit to the rest of the SCF complex by interacting with Skp1.

Discussion

The β-Catenin-Related Protein Arabidillo-1 Works with GA_3 to Control Lateral Root Development in *Arabidopsis*

Low levels of GAs are needed for maintaining undetermined meristem cell fates (Sakamoto et al. 2001), whereas high concentrations can promote cell differentiation and expansion (Shani et al. 2006). Gibberellins are required for the de-differentiation and initiation of lateral root meristems; hence, their deficiency and insensitivity in roots can promote the formation of new lateral root primordia. Mutants defective in GA biosynthesis or signaling have enhanced lateral root production (Berova and Zlatev 2000; Busov et al. 2006). Their role in this process has already been reported in GA-deficient and GA-insensitive transgenic *Populus* lines (Gou et al. 2010).

Although single null mutants of *Arabidillo-1* or *Arabidillo-2* do not exhibit any obvious phenotype, double mutants have fewer lateral roots than do wild-type plants (Coates et al. 2006). Our results showed that GA_3 was more detrimental to lateral root development from *arabidillo-1* than from the WT. This suggests that its metabolic or signaling pathways are involved in the mechanism by which Arabidillo-1 regulates the proliferation of lateral roots. Because such pathways have numerous positive and negative regulators, it cannot yet be confirmed which one(s) Arabidillo-1 works with to promote lateral root growth.

Arabidillo-1/2 may be Involved in the Ubiquitin/26S Proteolysis Pathway as a Subunit of the SCF Complex

As components of various SCF complexes, most F-box proteins are associated with ubiquitin/26S proteasome-mediated protein degradation (Hershko and Ciechanover 1998). Using yeast two-hybrid analysis, we found here that Arabidillo-1/2 are able to interact physically with ASK proteins, thereby demonstrating that these proteins are part of those complexes. Most known *Arabidopsis* F-box proteins interact mainly with ASK1, ASK2, and, possibly ASK11 (Risseeuw et al. 2003). Our data also indicated Arabidillo-1 interacts with ASK2 and ASK11, but that Arabidillo-2 interacts only with ASK2. No physical interactions were detected between Arabidillo-2 and ASK11. However, it is possible that the assay method was not sensitive enough to detect any weak interactions. In *Arabidopsis*, the ASK family contains 19 members (Arabidopsis Genome Initiative 2000; Farras et al. 2001), so Arabidillo-1/2 might interact with different members or may have functions other than the targeted degradation of poly-ubiquitinated protein.

Armadillo-1 Regulates Lateral Root Formation in *Arabidopsis* by Degrading Some Positive Regulator of GA_3 Signal Transduction

F-box proteins contain two regions. The F-box motif in the N-terminus interacts with Skp1 proteins while the C-terminal region determines substrate specificity. Both Arabidillo-1/2 contain nine Arm repeats near the C-terminal region. A single Arm repeat has three α-helices (Huber et al. 1997), while multi-Arm repeats can fold together and interact with each other to form a right-handed superhelix, creating a protein–protein interaction surface (Huber et al. 1997; Conti et al. 1998; Daniels et al. 2001). Thus, we propose that Arabidillo-1 and Arabidillo-2 interacts with ASK proteins through the F-box motif in the N-terminus, while the C-terminal region determines substrate specificity via Arm-induced protein–protein interactions.

Our data indicated that Arabidillo-1 in conjunction with GA_3 controls lateral root development, and that the former is possibly involved in the ubiquitin/26S proteolysis pathway as a subunit of the SCF complex. Therefore, we might hypothesize that Arabidillo-1 degrades some positive regulator of the GA_3 signal through ubiquitin/26S proteasome-mediated proteolytic pathway, and that this is the mechanism by which Arabidillo-1 promotes lateral root development in *Arabidopsis*. Our future work will focus on searching for the substrate in the SCF complex for ubiquitin/26S proteolysis, and we will continue to investigate the relationship between that substrate and various GA_3-metabolic or signaling pathways.

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