TITLE

ANGUSTIFOLIA mediates one of the multiple SCRAMBLED signaling pathways regulating cell growth pattern in *Arabidopsis thaliana*

AUTHORS

Su-Hwan Kwak ^{a,*}, Sang-Kee Song ^b, Myeong Min Lee ^c and John Schiefelbein ^d

^a Department of Biology, Long Island University, 1 University Plaza, Brooklyn, New York, USA

^b Department of Biology, Chosun University, Gwangju 501-759, Korea

^c Department of Systems Biology, Yonsei University, 50 Yonsei-ro, Seoul 120-749, Korea

^d Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University Avenue, Ann Arbor, Michigan, USA

* Corresponding author.

E-mail address: su-hwan.kwak@liu.edu (S.H. Kwak).

ABSTRACT

In *Arabidopsis thaliana*, an atypical leucine-rich repeat receptor-like kinase, SCRAMBLED (SCM), is required for multiple developmental processes including root epidermal cell fate determination, silique dehiscence, inflorescence growth, ovule morphogenesis, and tissue morphology. Previous work suggested that SCM regulates these multiple pathways using distinct mechanisms via interactions with specific downstream factors. ANGUSTIFOLIA (AN) is known to regulate cell and tissue morphogenesis by influencing cortical microtubule arrangement, and recently, the AN protein was reported to interact with the SCM protein. Therefore, we examined whether AN might be responsible for mediating some of the SCM-dependent phenotypes. We discovered that both *scm* and *an* mutant lines cause an abnormal spiral or twisting growth of roots, but only the *scm* mutant affected root epidermal patterning. The siliques of the *an* and *scm* mutants also exhibited spiral growth, as previously reported, but only the *scm* mutant altered silique dehiscence. Interestingly, we discovered that the spiral growth of roots and siliques of the *scm* mutant is rescued by a truncated SCM protein that lacks its kinase domain, and that a juxtamembrane domain of SCM was sufficient for AN binding in the yeast two-hybrid analysis. These results suggest that the AN protein is one of the critical downstream factors of SCM pathways specifically responsible for mediating its effects on cell/tissue morphogenesis through cortical microtubule arrangement.

Keywords:

ANGUSTIFOLIA

Arabidopsis thaliana

Leucine-rich repeat receptor-like kinase

Root development

SCRAMBLED

Silique development

1. Introduction

Arabidopsis leucine-rich repeat receptor-like kinases (LRR RLKs) are involved in responses to a wide range of signals such as hormones and environmental stimuli [1-3], and they also are used to regulate many developmental processes [4,5]. The Arabidopsis genome has more than two hundred members of LRR RLK family [5,6]. The variety of pathways that Arabidopsis LRR RLKs regulate is likely much greater than their number because a single LRR RLK has the potential to mediate multiple signaling pathways by responding to multiple ligands and/or act in combination with multiple downstream factors [7]. A current challenge is to define the mechanisms by which LRR RLKs can regulate and coordinate many different downstream processes.

In Arabidopsis, an atypical LRR RLK, SCRAMBLED (SCM), also known as STRUBBELIG, has been shown to influence multiple developmental processes, including root epidermal cell patterning, ovule and flower formation, silique dehiscence, leaf and silique morphogenesis, and inflorescence growth [8-11]. SCM regulates root epidermal cell fate by repressing the expression of *WEREWOLF* (*WER*), a MYB transcription factor, in a cell-position-dependent manner [9,12], which leads to differential expression of a suite of transcription factor genes, including the homeodomain transcription factor *GLABRA2* (*GL2*) in the developing root

epidermal cells [13-17]. Interestingly, the kinase domain of SCM protein lacks autophosphorylation activity, and the kinase activity of SCM is not required for flower organ development and root epidermal patterning [8,10,18]. However, the kinase domain of SCM per se is required for root epidermal patterning, because a truncated SCM protein lacking its kinase domain was unable to rescue the abnormal root epidermal pattern of *scm* mutants [10]. The kinase domain of SCM is also necessary for its role in regulating inflorescence growth [10]. On the other hand, the truncated SCM protein lacking its kinase domain was able to rescue the silique dehiscence defect of the *scm* mutants [10]. These findings imply that SCM participates in multiple development processes using distinct regulatory mechanisms and/or interacting partners for different downstream pathways. SCM is also thought to regulate tissue morphology by cortical microtubules organization because the mutation in the *SCM* gene caused spiral growth of stems and siliques [8,19]. In this work, we observed spiral growth of the *scm-2* mutants in roots as well as in stems and siliques. We also discovered that a truncated SCM protein lacking its kinase domain rescued this spiral growth.

Recently, ANGUSTIFOLIA (AN) was reported to physically interact with the kinase domain of SCM [20]. The *AN* gene influences the arrangement of cortical microtubules that are important for leaf shape and trichome branching, processes that are defective in *an* mutants [21,22]. The *an* mutants also developed twisted siliques and petals [23]. AN is a plant homolog of C-terminal binding protein/brefeldin A ADP-ribosylated substrate (CtBP/BARS) proteins. Although animal CtBP/BARS proteins are known to be transcriptional repressors that bind to the C-terminus of transcription factors, AN is supposed to function in a different way outside of the nucleus [24,25]. Interestingly, the AN protein not only regulates plant morphology via cortical

microtubule arrangement, but also affects transcriptome profiles [19], and it is also involved in abiotic and biotic stress responses [26].

The similarity in certain phenotypes of *scm* and *an* mutants (including leaf morphology and twisted siliques) and the physical interaction between the SCM and AN protein prompted us to examine their relationship in other developmental processes. Specifically, we sought to define the potential role of AN in root morphogenesis, root epidermal patterning, and silique dehiscence. In addition, we tested the importance of particular SCM domains for mediating functional interactions with AN in these developmental processes. Our results suggest that AN is a downstream factor that mediates one of the multiple pathways that SCM regulates. We propose that SCM recruits the AN proteins to the vicinity of the plasma membrane to enable them to control the cortical microtubule arrangement.

2. Materials and methods

2.1 Plant materials and growth conditions

Arabidopsis thaliana seeds were sterilized and germinated vertically on solid agarose medium for four days under continuous light as previously described [27]. The *scm-2* allele contains a transfer DNA insertion (SALK_086357) in the third intron of the *SCM* gene (TAIR: AT1G11130). The *an-t1* allele has a transfer DNA insertion (SALK_026489) in the first intron of the *AN* gene (TAIR: AT1G01510). The *scm-2* allele and *an-t1* allele with Columbia background was crossed with the wild type *GL2::GUS* reporter line that has been generated using Wassilewskija (WS) ecotype [28]. The *scm-2 dKD2GFP* line has been generated by

transformation of the *scm-2 GL2::GUS* line with *dKD2GFP* gene construct [10]. The *dKD2GFP* construct was built in pPZP222 vector with the native *SCM* promoter and coding sequence of *SCM* extracellular domain, transmembrane domain and juxtamembrane domain fused with mGFP5 open reading frame. For silique analysis, plants were grown in growth chamber at 22°C with an 18-h-light/6-h-dark cycle.

2.2 GUS histochemical analysis, cell type analysis and twisting phenotype analysis

Analyzing *GL2::GUS* expression was done as previously described [28]. Four-day-old roots were incubated in GUS staining solution at 37°C for 30 min. We identified the H position and N position by examining the alignment of cortical cells and epidermal cells under a microscope. GUS-expressing and GUS-nonexpressing cells on each position were counted from the meristem region of root tips as in previous work [10]. GUS-expressing cells and GUS-nonexpressing cells were considered to have nonhair cell fate and root-hair cell fate, respectively. For each line, more than 1,000 cells from fifteen to thirty roots were analyzed per each counting. Four-day-old roots that display sporadic spiral growth along the root axis were counted as twisting roots. For each line, twenty three to eighty five roots were analyzed for twisted growth per each counting. Mature siliques that develop tilted or spiral valve margin relative to the axis of silique were counted as twisting siliques. For each line at least two hundred siliques were analyzed collected from five to seven plants.

2.3 Yeast two-hybrid analysis

For the yeast two-hybrid analysis, DNA fragment that encodes SCM intracellular domain including juxtamembrane domain and kinase domain (SCM-INT) was amplified by polymerase chain reaction (PCR) using primers: 5'-GGATCCGTTGGAGATGTTGCAGAAATAAA-3' and 5'-CTCGAGTTAGATCATATGTTGAAGATC-3'. DNA fragment that encodes SCM juxtamembrane domain (SCM-JD) was amplified using primers: 5'-GGATCCGTTGGAGATGTTGCAGAAATAAA-3' and 5'-CTGCAGTTAATTATTTGTGTATTGCTGAAGTGA-3'. The PCR products were verified for their sequences and subcloned into pAS2-1 for the fusion with GAL4 binding domain. A cDNA clone, U15161 from Arabidopsis Biological Resource Center (ABRC) was used as template for the amplification of the *AN* gene. We confirmed that the sequence of U15161 encodes ⁴²⁸Leu and ⁵⁴³Leu instead of ⁴²⁸Ser and ⁵⁴³Val, respectively. The full open reading frame of the *AN* gene was amplified from U15161 and subcloned into pACT2 for the fusion with GAL4 activation domain. The recombinant DNA plasmids were introduced into Y190 yeast cells. The binding of proteins in yeasts were analyzed on solid synthetic defined medium that lacks leucine, tryptophan and histidine supplied with 25 mM 3-aminotriazole at 30°C.

3. Results

3.1 The *scm* and *an* mutants exhibit spiral growth of *Arabidopsis* roots

Given the documented effect of *scm* and *an* mutations on shoot organ twisting, we analyzed whether the orientation of root growth was affected in the *scm-2* mutant and *an-t1* mutant. The *scm-2* is a null mutant that has a transfer DNA insertion in the third intron

(SALK_086357) [9], and the *an-t1* allele has a transfer DNA insertion in the first intron (SALK_026489) [26]. The *an-t1* represents a null allele, lacking detectable AN RNA, and it exhibits the same trichome branching and leaf morphology defects as other described *an* alleles [26]. We discovered that the roots of both *scm-2* and *an-t1* mutants exhibit spiral growth (twisting), similar to the previously reported twisting of their stems and siliques (Fig. 1A-E). Most of the *scm-2* and *an-t1* roots were twisting right-handedly, however; a small fraction of both mutants also exhibited left-handed twisting (Fig. 1B, D). We quantified this root twisting phenotype and found that most of the *an-t1* roots (91.6%) and *scm-2* roots (73.5%) exhibited spiral growth (Fig. 1G). Given that spiral growth of roots is caused by abnormal cortical microtubule arrangement [29,30], it is likely that SCM and AN are important for regulating cortical microtubules throughout the plant body including roots, stems, and flowers.

Next, we analyzed whether the kinase domain of SCM is necessary for its ability to influence root morphogenesis. We examined the root morphology of a *scm-2* mutant line that expresses a truncated SCM protein containing the extracellular, transmembrane and juxtamembrane domains but lacking the kinase domain (dKD2GFP). This line exhibits appropriate expression and accumulation of the dKD2GFP protein in roots, siliques, and inflorescence meristem [10]. Interestingly, the dKD2GFP protein was able to rescue the twisting phenotype of the *scm-2* roots to a significant extent (Fig. 1F, G). This suggests that the extracellular domain, transmembrane and juxtamembrane domains of the SCM protein are sufficient for its ability to regulate cortical microtubules for coordinated root cell expansion.

3.2 The siliques of the an-t1 mutants show spiral growth but dehisce normally

We analyzed siliques of the *scm-2* mutants and *an-t1* mutants and observed a twisted silique phenotype, consistent with previous reports (Fig. 2A-C) [23]. Quantitative analysis showed that 73.5% of *scm-2* siliques were twisted and 83.6% of *an-t1* siliques were twisted (Fig. 2E). The *an-t1* mutants showed a more severe twisting phenotype than the *scm-2* mutants in both roots and siliques. Introduction of the dDK2GFP construct into the *scm-2* mutant significantly reduced the percent of twisted siliques (15.1%; Fig. 2E). Thus, the SCM kinase domain is dispensable for SCM's regulation of cellular growth orientation during silique development, similar to what we had observed for root development.

We also analyzed silique dehiscence in the *scm-2* and *an-t1* mutants. Unlike *scm-2* mutant siliques, the *an-t1* siliques did not show any dehiscence defect (Fig. 2C, F). This suggests that AN does not participate in the SCM pathway that regulates silique dehiscence. Consistent with our prior report, we found that dKD2GFP is able to rescue the silique dehiscence defect of the *scm-2* mutant (Fig. 2D, F) [10].

3.3 AN is not required for root epidermal patterning

To analyze the possible role of AN on root epidermal patterning, we examined the expression pattern of the non-hair cell marker gene *GL2* (using the *GL2::GUS* transgene) in the developing roots of *an-t1*, *scm-2*, and wild-type plants (Fig. 3). Normally, *GL2* is preferentially expressed in the files of epidermal cells that are adopting the non-hair fate (the “N” position), which leads to a characteristic “striping” pattern for the GUS accumulation. We found that *an-t1* roots display the normal cell-file-dependent distribution of *GL2*-expressing and non-*GL2*-expressing cells, whereas the *scm-2* mutant lacks this pattern (Fig. 3A-C). Further, we quantified

the mis-specified cells in each H position and N position and again found that the *an-t1* mutant is similar to the wild-type roots (Fig. 3D). These results suggest that, although AN is involved in root morphogenesis events, it is not required for proper root epidermal patterning.

3.4 The juxtamembrane domain of SCM is sufficient for AN binding

We were intrigued by the ability of the dKD2GFP, which lacks the SCM kinase domain, to rescue the spiral growth of roots and siliques in the *scm-2* mutants. This result implied that AN, which is also involved in spiral growth in these organs, might interact with SCM without using the kinase domain. To examine this, we analyzed the interaction of the AN protein with different domains of the SCM protein using the yeast two-hybrid assay. We discovered that the AN protein is able to physically interact with a SCM fragment including the entire intracellular domain (SCM-INT) as described previously (Fig. 4) [20], and more specifically, it could interact with the small juxtamembrane domain of SCM (SCM-JD) which does not include the kinase domain (Fig. 4).

4. Discussion

The SCM LRR RKL has been proposed to participate in multiple pathways by interacting with distinct factors that mediate specific downstream responses [10]. In this work, we provide evidence to suggest that AN is one of these distinct downstream interacting factors. Both SCM and AN exhibit some common mutant phenotypes related to abnormal cortical microtubule arrangement and spiral growth. This implies that they share functions in regulating these

developmental processes. However, the *an-t1* mutant did not show defects in other *scm* mutant characters, such as root epidermal patterning and silique dehiscence, which indicates that AN does not mediate all SCM-dependent processes. It may be that SCM recruits the AN proteins to the vicinity of the plasma membrane, enabling them to interact with cortical microtubules. This possibility is consistent with the results that the dKD2GFP protein rescued spiral growth of roots and siliques, and that the juxtamembrane domain of SCM binds to AN in the yeast two-hybrid analysis. SCM was shown to require its kinase domain for proper function for root epidermal patterning [10] although the kinase domain of SCM was not needed for AN binding and possibly cortical microtubule arrangement. This represents an example of combinational action by two factors that have multiple functions, and shows how one receptor mediates multiple signaling pathways. The other pathways regulated by SCM might be mediated by different downstream factors that are not identified yet.

The abnormality in cortical microtubules arrangement that was detected in the leaf epidermis seems to cause abnormal leaf morphology in *scm* and *an* mutants [11, 22]. It is also known that the stabilization of microtubules is involved in trichome branching [31]. The trichomes of *an* mutants have a reduced number of branching [21,22], which may be due to failure of microtubule accumulation at the trichome tip [21]. Interestingly, *scm* mutants show normal trichome branching, perhaps due to the lack of *SCM* expression in trichomes [32,33]. It may be that another unknown membrane factor is responsible for interacting/recruiting AN proteins and regulating microtubule distribution in developing trichomes.

The *an-t1* mutants showed more severe twisting phenotypes in roots and siliques than the *scm-2* mutants. This may be explained by the idea that SCM facilitates the function of the AN

proteins by recruiting them to the plasma membrane. In the absence of SCM, a small fraction of the AN proteins might still be able to interact with microtubules in the cortex of cells.

Previously, we suggested that the silique dehiscence defect of the *scm-2* mutant was not likely due to twisted growth because a few non-twisting siliques of the *scm-2* mutant also failed to dehisce [10]. Related to this, the roots and siliques of the *an-t1* mutants exhibit twisting but do not alter root epidermal patterning or silique dehiscence. This supports the argument that twisted growth does not cause the abnormal epidermal patterning and silique dehiscence.

In animals, it is well known that neighboring cells communicate through their networks of extracellular matrix, integrins and cortical cytoskeletons including microtubules [34]. In Arabidopsis, it is possible that SCM might interconnect unknown components of cell wall and cortical microtubules through AN. In particular, cells might coordinate directions of expansion through this communication. In future research, the unknown extracellular components that work as ligands of SCM and the mechanism by which AN regulates the arrangement of cortical microtubules will be important topics for study.

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Figure legends

Fig. 1. Spiral root growth in the *scm-2* and *an-t1* mutants. (A-F) Four-day-old roots from wild type (A), *scm-2* (B, C), *an-t1* (D, E) and *scm-2 dKD2GFP* (F) were examined for their spiral growth pattern. (B) Left and right panel show right-handed and left-handed spiral *scm-2* roots, respectively. (D) Left and right panel show right-handed and left-handed spiral *an-t1* roots, respectively. (G) Quantification of root twisting phenotype. Four-day-old roots of four genetic lines that exhibit spiral growth along the root axis were counted as twisting roots. The values mean the average percent of twisting roots of three independent counting sessions. Error bars represent the standard deviation. Single asterisk indicates a significant difference from the wild-type (WS) control ($P < 0.05$, Student's t test). The double asterisks indicate a significant difference from both the wild-type control and the *scm-2* mutant ($P < 0.05$, Student's t test). Bar = 200 μm in A, B, D and F, and 25 μm in C and E.

Fig. 2. Silique dehiscence and twisting phenotype in the *scm-2* and *an-t1* mutants. (A-D) Representative mature siliques of WS wild-type control (A), *scm-2* (B), *an-t1* (C) and *scm-2 dKD2GFP* (D) plants are shown. (E) Quantification of silique twisting phenotype. Twisting siliques and non-twisting siliques were counted from five or seven plants of each genetic line. The values show the mean average twisting siliques per plant. Error bars represent the standard deviation. Single asterisk indicates a significant difference from the wild-type (WS) control ($P < 0.05$, Student's t test). The double asterisks indicate a significant difference from both the wild-type control and the *scm-2* mutant ($P < 0.05$, Student's t test). (F) Quantification of silique dehiscence phenotype. Dehiscing siliques and non-dehiscing siliques were counted from five or seven plants of each genetic line. The values show the mean average dehiscing siliques per plant. Error bars represent the standard deviation. Single asterisk indicates a significant difference from the wild-type (WS) control ($P < 0.05$, Student's t test). The crosses indicate a significant difference from the *scm-2* mutant ($P < 0.05$, Student's t test). Bar = 2 mm.

Fig. 3. Root epidermal patterning in the *scm-2* and *an-t1* mutants. (A-C) Four-day-old roots of the WS wild-type control (A), *scm-2* (B) and *an-t1* (C) were analyzed for *GL2::GUS* expression. The blue-stained cells adopt the nonhair cell fate. Unlike the *scm-2* mutant root (B), the *an-t1* root (C) is indistinguishable from the wild type (A). (D) Quantification of root epidermal patterning. GUS-expressing cells and non-expressing cells on each H position (black bar) and N position (white bar) were counted. The averages percentage of mis-specified cells on each position from three independent counting sessions are shown. Error bars represent the standard deviation. Single asterisk indicates a significant difference from the wild-type (WS) control ($P <$

0.05, Student's t test). The crosses indicate a significant difference from the *scm-2* mutant ($P < 0.05$, Student's t test). Bar = 50 μm .

Fig. 4. Interaction of the AN protein with SCM intracellular domain (INT) and juxtamembrane domain (JD) in the yeast two-hybrid system. Y190 cells expressing various combinations of proteins were grown on synthetic defined medium that lacks tryptophan and leucine (-T/-L; lower panel) or SD medium that lacks tryptophan, leucine and histidine supplied with 25 mM 3-aminotriazole (-T/-L/-H/3AT; upper panel). SCM-INT represents the intracellular domain of SCM that consists of juxtamembrane domain and kinase domain fused with GAL4 binding domain. SCM-JD represents the juxtamembrane domain of SCM fused with GAL4 binding domain. The full-length AN protein was fused with GAL4 activation domain (AN). AD and BD means GAL4 activation domain and binding domain, respectively.

Figure 1

Figure 2

Figure 3

Figure 4