The ATP-Binding Cassette Transporter ABCB19 Regulates Postembryonic Organ Separation in Arabidopsis

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Abstract

The phytohormone auxin plays a critical role in plant development, including embryogenesis, organogenesis, tropism, apical dominance and in cell growth, division, and expansion. In these processes, the concentration gradient of auxin, which is established by polar auxin transport mediated by PIN-FORMED (PIN) proteins and several ATP-binding cassette/multi-drug resistance/P-glycoprotein (ABCB/MDR/PGP) transporters, is a crucial signal. Here, we characterized the function of ABCB19 in the control of Arabidopsis organ boundary development. We identified a new abc19 allele, abc19-5, which showed stem-cauline leaf and stem-pedicel fusion defects. By virtue of the DIL-VENUS marker, the auxin level was found to be increased at the organ boundary region in the inflorescence apex. The expression of CUP-SHAPED COTYLEDON2 (CUC2) was decreased, while no obvious change in the expression of CUC3 was observed, in abc19-5. In addition, the fusion defects were greatly enhanced in cuc1 cuc3-5, which was reminiscent of cuc2 cuc3. We also found that some other organ boundary genes, such as LOF1/2 were down-regulated in abc19-5. Together, these results reveal a new aspect of auxin transporter ABCB19 function, which is largely dependent on the positive regulation of organ boundary genes CUC2 and LOFs at the postembryonic organ boundary.

Introduction

Throughout the lifespan of most higher plants, new organs are initiated continuously from pluripotent cells in the shoot apical meristem. This essential process is associated with the establishment of boundaries separating the newly formed organs from adjacent tissues [1]. Such boundaries are composed of a specialized group of saddle-shaped cells that are morphologically different from the adjacent cells [2]. The unique shape of these cells is attributed to elongation along the organ boundary, contraction along the axis perpendicular to the boundary, and cell division leading to a new cell wall parallel to the boundary [2–4]. These boundaries emerge at the early stage of primordia initiation, and their positions are determined by signals from the central region of the meristem [1,2,5]. The boundaries act as a barrier to separate and maintain different cell types [1], and, when localized at the base of leaves, they have the potential to produce axillary meristems, which contribute greatly to the overall architecture of plants [6].

In Arabidopsis, a number of genes with a boundary-specific expression pattern have been identified. Among them, CUP-SHAPED COTYLEDON (CUC) genes are well-known NAC domain-containing transcription factors [7–9]. CUC1, CUC2, and CUC3 participate redundantly in embryonic meristem formation and cotyledon separation [7,8]. CUC2 and CUC3 play a significant role in the separation of postembryonic organs, including rosette leaves, stems, and pedicels [8]. It has also been reported that two MYB domain-containing transcription factors, LATERAL ORGAN FUSION1 and LATERAL ORGAN FUSION2 (LOF1 and LOF2), which are specifically expressed at organ boundaries, play critical roles in lateral organ separation [6]. A number of other boundary-specific genes, including JAGGED LATERAL ORGANS (JLO) [10], LATERAL SUPPRESSOR (LAS) [11,12], BLADE ON PETIOLE (BOP) [13,14], REGULATORS OF AXILLARY MERISTEMS (RAX) [15], and LATERAL ORGAN BOUNDARIES (LOB) family genes [16,17], have been shown to be involved in embryonic and/or postembryonic boundary specification.

Several lines of evidence show that auxin plays a significant role in organ patterning and boundary establishment by controlling CUC gene expression [18–21]. Mutations in the putative auxin efflux carrier PIN1 produce naked inflorescence stems resulting from the ectopic expression of CUC2 at a ring-like domain characterized by primordia-specific gene expression [18]. PINOID (PID) and ENHANCER OF PINOID (EXP) regulate PIN1 localization and function to promote cotyledon initiation bilaterally by preventing CUC1, CUC2, and STM from expanding to the primordia during embryonic development [20,21]. MONO-
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**Results**

**ABCB19 is necessary for organ separation at stem-cauline leaf and -pedicel junctions in Arabidopsis**

To characterize novel components in flowering time control, we screened a T-DNA insertion mutant library and identified a mutant with a delay in the transition to flowering (Figure 1A). In addition, the mutant exhibited epinastic cotyledons and wavy roots and hypocotyls at the seedling stage (Figure 1B–E). Furthermore, organ fusion defects occurred at both stem-cauline leaf junctions (the abnormal growth of the proximal part of the cauline leaf fused with the stem) (Figure 1F and G) and stem-pedicel junctions (Figure 1H and I). This fusion, which was seen on the primary and secondary branches, was most obvious on rosette branches. The stem-cauline leaf fusions caused bending of the stem (Figure 1G). Stem-pedicel fusions were more obvious for the first several siliques, and, as a result, the angle between the stem and pedicel was significantly reduced for the first eight siliques (Figure 1J).

**Auxin distribution is altered by ABCB19 mutation**

It was reported that *ABCB19* is required for the basipetal auxin transport out of the shoot apex of seedling and inflorescence [24], and that loss of *ABCB19* function increased auxin retention in the apical tissues of seedling by quantification of endogenous IAA levels and radiotracer studies [42]. Due to the organ separation defects of *abcb19*, we are curious about the endogenous auxin level at the organ boundary region in *abcb19*. However, as a result of the auxin distribution gradient, with levels being highest in the primordia and lowest in the organ boundaries [1], it is difficult to analyze the alteration of auxin levels at the site of organ fusion using the auxin-responsive marker DR5::GUS/GFP. Fortunately, the DII-VENUS (termed domain II fusion with fast maturing variant of YFP, VENUS) marker is more sensitive than DR5::GUS/GFP; images of which are like a photographic negative of auxin levels [43–45]. And there are strong signals at the organ boundary at the inflorescence apical region [43].

Consequently, the DII-VENUS marker was introduced into *abcb19*. We found that the overall fluorescence signal was dropped in *abcb19* compared with wild type plants at the inflorescence apex including the inflorescence meristem (IM) and organ boundary region (Figure 5). As a negative indicator of auxin, the reduction of DII-VENUS indicates that the auxin level is increased at the inflorescence apex in *abcb19*, consistent with the abnormal basipetal auxin transport activity in *abcb19*. Thus, by means of DII-VENUS, we show that the endogenous auxin level is increased both in the organ boundary region and in inflorescence meristem (Figure 5).
ABCB19 Regulates Postembryonic Organ Separation

CUC2 and CUC3 expression is differentially regulated in abcb19

Among the genes that function in postembryonic organ boundary separation, CUC2 and CUC3 of the NAC family are two well-known, important regulators [8]. And it was indicated that the expression of CUC2 are inhibited by auxin [18,19]. To examine the expression of these genes in wild-type and abcb19 plants, CUC2::GUS and CUC3::GUS [46] were crossed into abcb19-5, respectively.

The expression patterns of CUC2::GUS and CUC3::GUS were analyzed at different developmental stages in wild-type and abcb19 plants. In wild-type, CUC2::GUS and CUC3::GUS activity was detected at the organ boundary in cotyledons, stem-cauline leaf junctions, and at the boundary of stem-pedical junctions, consistent with previous in situ results [8]. In abcb19-5, the CUC2::GUS was down-regulated after the plants undergoing the floral transition and after bolting (Figure 4A-B). Furthermore, CUC2::GUS activity was reduced by about 37% in both the stem-cauline leaf junctions and inflorescences of abcb19 plants according to our β-glucuronidase assay results (Figure 4C-D). The CUC2::GUS activity showed similar down-regulation pattern in another allele, abcb19-3 [Figure 4E-F]. However, under the same conditions, the expression of CUC3 as shown by histological staining and a β-glucuronidase assay was not obviously changed in our experiment (Figure 4H, I, and J). The decrease in the CUC2 expression in abcb19 was further confirmed in both abcb19 alleles by determining the level of CUC2 mRNA using the real-time quantitative-PCR (q-PCR) (Figure 4G). Thus, mutations in ABCB19 may specifically affect CUC2 expression by increased auxin level in the organ boundary region, with no or little effect on CUC3 expression, during postembryonic growth.

The genetic relationship between ABCB19 and CUC2 or CUC3 in Arabidopsis

CUC2, but not CUC3, expression was obviously reduced in abcb19-5. Given this, we hypothesized that the organ separation defects in abcb19 cuc3 would be enhanced compared to those in abcb19, while the elimination of cuc2 would not be as efficient as the elimination of cuc3 in terms of phenotype enhancement.

To test this hypothesis, we generated abcb19-5 cuc2-3 and abcb19 cuc3-105 plants. Consistent with our expectations, cuc3-105 enhanced the fusion defects seen in abcb19 dramatically, while cuc2-3 contributed to the observed defects to a lesser extent (Figure 5A-C). The extent of fusion was greatly enhanced at stem-cauline leaf junctions and inflorescence stem-pedical junctions in abcb19-5 cuc3 compared with abcb19-5 (Figure 5A and B) and fusion of the axillary shoot to the main stem was observed in abcb19 cuc3, showing the phenotype equivalence between abcb19 cuc3 and cuc2 cuc3 (Figure 5A, shown by an white arrow) [8]; however, the degree of fusion was still less than that seen in cuc2 cuc3, due to the residual expression of CUC2 in abcb19-5. cuc2 enhanced the fusion defects in abcb19-5 slightly and less effectively than cuc3 (Figure 5A and B). We next determined the frequency (%) of fusion defects at stem-cauline leaf junctions. In primary stem-cauline leaf junctions, the number of fusion events in abcb19-5 cuc3-105 was significantly increased compared with abcb19-5 (Figure 5C); in addition, the number of fusion events in abcb19-5 cuc2 was not significantly different from abcb19-5 (Figure 5C). The rate of fusion in abcb19 cuc3 was even higher than that in cuc2
cuc3, however, the difference was not significant shown by the t-test (Figure 5C).

In general, the lesion of cuc3 significantly reinforced the fusion defects in abcb19 in terms of the degree and frequency of fusions, while cuc2 contributed less. This is largely consistent with the reduced expression of CUC2 (but not of CUC3) in abcb19 (Figure 4).

Other organ boundary-specific genes besides CUC2 may be involved in ABCK19-mediated organ separation

As CUC2 and CUC3 participate redundantly in postembryonic organ separation, each single mutant shows no obvious fusion defect [8]; thus, only reduction in CUC2 in abcb19 does not account for the organ fusion phenotype observed. Since ABCK19 acts as an auxin transporter, the auxin distribution pattern in abcb19 is altered obviously (Figure 3). Auxin is such an important regulator of plant development that a number of factors may be changed to varying degrees at the organ boundaries in abcb19. Variations in these factors together with the down-regulation of CUC2 may contribute to the fusion defect observed in abcb19.

We tested a number of organ boundary-specific factors in abcb19 by semi-quantitative RT-PCR, and observed that BOP was elevated in abcb19 (the elevated BOP expression is similar to the situation in lof1 [6]; LOF1 was reduced slightly and LOF2 was down-regulated obviously; LAS and RAX1 were not distinguishable from the wild type plants (Figure 6). Since it has been shown that the lof1 knock-out considerably enhances the cuc2 phenotype [6], the down-regulation of the two LOFs in abcb19 might at least to some extent explain why the cuc2 phenotype does not match the abcb19 phenotype.

Therefore, these results demonstrate that ABCK19, as an auxin transporter, control a variety of organ boundary genes to guarantee the establishment of the organ boundary.

ETT may function in postembryonic organ separation

Auxin functions mainly through AUXIN RESPONSE FACTORS (ARFs). ETTIN (ETT)/ARF3 are reportedly involved in flower development [47], adaxial-abaxial patterning during leaf development [48], and in the vegetative phase change as the target of trans-acting (ta) siRNA-ARFs (asir-ARF) [49]. We observed that ett-3 showed moderate cauline stem-cauline leaf fusion defects (Figure 7A). When we combined ett-3 with abcb19-5, the extent of fusion was dramatically enhanced (Figure 7A). The rate of
fusion in *abcb19* was also significantly enhanced by *ett-3* (Figure 7B). This suggests that *ABCB19* participates in a pathway parallel with *ETT* to control postembryonic organ separation.

**Discussion**

*ABCB19* participates in postembryonic organ separation in *Arabidopsis*

*ABCB19*, as an auxin transporter [24,29,31,32,39], has been implicated in a multitude of biological processes, including normal growth and development in multiple tissues [24,39], photomorphogenesis [32,40], and gravitropic responses [29,41]. In this study, we generated several lines of evidence showing the novel function of *ABCB19* in postembryonic organ separation based on a mutant identified from our genetic screen. The similar organ separation defects in two alleles of *abcb19* and the appearance of the same defect in *F1* plants from a cross between *abcb19-3/mdr1-3* and *abcb19-5*, as well as transgenic complementation (Figure 1 and Figure 2), all demonstrate the role of *ABCB19* in organ separation control.

When *ABCB19* is knocked out, the auxin concentration is increased in the boundary region, as is shown by the newly developed DII-VENUS marker (Figure 3). This may result in abnormal cell growth and then the organ fusion defects. We also found that *AUXIN RESPONSE FACTOR-ARF3/ETT* is involved in postembryonic organ separation (Figure 7), and that *ABCB19* may participate in a pathway parallel with *ETT* to control postembryonic organ boundary formation.

*ABCB19* plays a role in organ separation by partially regulating *CUC2* and some other organ boundary genes

Previous studies have indicated that auxin plays a critical role in organ boundary establishment by controlling *CUC* gene expression [18–21]. *CUC2* and *CUC3* play redundant roles in postembryonic organ separation [8]. *CUC2* has been frequently reported to be repressed by high auxin concentrations [18,19]. Notably, we found that the expression of *CUC2* was obviously reduced at the postembryonic boundary in *abcb19* compared with wild-type (Figure 4A–G). In contrast, *CUC3* expression was not obviously changed (Figure 4H–J), indicating the differential regulation of these homologs at the transcriptional level by *ABCB19* through the control of auxin distribution. Consistently, it was *cuc3* rather than *cuc2* that enhanced the fusion defects in *abcb19* significantly (Figure 5). Besides *CUC2*, we also found that the some other organ boundary genes, such as *LOF1*, *LOF2*, and *BOP*, were also shown altered expression in *abcb19* (Figure 6). Together, our gene expression and genetic results indicate that *ABCB19* may promote postembryonic organ separation via the regulation of *CUC* and other organ boundary genes, probably through the depletion of auxin at the boundary.
Figure 4. CUC2/3 expression level in WT and abcb19-5. **A**: A longitudinal paraffin section after histological GUS staining of the inflorescence meristem region after the floral transition. Compared with WT, the level of CUC2::GUS activity in abcb19-5 was obviously reduced. **B**: Histological CUC2::GUS staining of the inflorescence, cauline leaves, and axillary branches done after bolting. The CUC2::GUS level was low in abcb19-5. **C** and **D**: β-glucuronidase assay of CUC2::GUS in wild type and abcb19-5 stem-cauline leaf junctions and inflorescences. For **C** and **D**, the values are the mean and standard deviation from three biological replicates (N = 3). The decrease in CUC2::GUS activity in abcb19-5 was significant (Student’s t-test, p = 0.046 in **C** and p = 0.026 in **D**). * Significantly different, P < 0.05. **E** and **F** were similar results as **A** and **B**, respectively, except that these are in abcb19-3. **G**: Relative expression level of CUC2 revealed by real-time quantitative-PCR using about 3 mm region including the stem-cauline leaf junction from the secondary branch. ACTIN2 was used as an endogenous control. Error bars indicate the standard deviation from the three biological replicates. *** Significantly different from the wild type, P < 0.001. **H**: Histological CUC3::GUS staining of the inflorescence, cauline leaves, and axillary branches. The CUC3::GUS level in abcb19-5 was not obviously different from that in WT. **I** and **J**: β-glucuronidase assay of CUC3::GUS in wild-type and abcb19-5 stem-cauline leaf junctions and inflorescences. For **I** and **J**, the values are the mean and standard deviation from three independent biological replicates (N = 3). CUC3::GUS activity in abcb19-5 was not significantly different from that in WT (Student’s t-test, p = 0.152 in **I** and p = 0.756 in **J**). m, meristem; p, pedicel; ab, axillary bud; cl, cauline leaf. Bar = 200 μm in **A** and **E**, 1 mm in **B** and **F** and 5 mm in **H**.

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In summary, we demonstrated that the auxin efflux carrier ABCB19 participates in postembryonic organ boundary specification by partially regulating the NAC family transcription factor CUC2 and some other organ boundary genes.

Materials and Methods

Plant Materials and Growth Conditions

The Arabidopsis thaliana plants used in this work were all in the Columbia-0 (Col-0) background. abcb19-3 (mdr1-3) was kindly provided by Dr. Edgar P. Spalding; abcb19-5, which carries a T-DNA insertion [50], was cloned by TAIL-PCR. abcb19-5 was crossed with CUC::GUSs to produce abcb19-5 CUC::GUSs. In the F2 generation, plants homologous for abcb19-5 that carried CUC::GUS were identified by PCR. In the next generation, thirty seedlings of several different lines were analyzed by GUS staining to identify lines homologous for CUC::GUS. abcb19-5 cuc2-3, which exhibited an abcb19-specific leaf shape and smooth leaf margin (cuc2-3 phenotype), was first identified by leaf appearance and then by PCR analysis. abcb19-5 cuc3-105 was characterized by PCR analysis. abcb19-5 ett-3 was identified by abnormal carpel development (ett-3) and the PCR analysis of abcb19-5.

Seeds were sterilized in 75% ethanol for 1 min, washed three times with sterile water, kept at 4°C for 2 days to promote germination, and then grown on Murashige and Skoog medium. After 8–10 days of growth chamber (Percival CU36L5) under a cool white fluorescent light (160 μmol m⁻² s⁻¹) (16 h of light/8 h of dark, 22°C), the seedlings were transferred to soil and grown in a growth chamber under long-day conditions (16 h of light/8 h of dark) at 22°C and 65% relative humidity.

Plasmid Construction and Plant Transformation

The full-length CDS of ABCB19 was amplified from Arabidopsis cDNA reverse-transcribed from total seedling RNA using the following primers: ABCB19-c-F (5’-CGGGATCCATGTCGGAAACTAACACAACC-3’) and ABCB19-c-R (5’-GGGTACCTCAAATCCTATGTGTTTGAAGC-3’). After sequencing, the ABCB19 CDS was cleaved with BamHI and KpnI and ligated to the pCAMBIA1300 binary vector under the control of the CaMV 35S promoter. The construct was then transformed into GV3101 cells and introduced to abcb19-3 by Agrobacterium tumefaciens-mediated floral infiltration as described previously [51].

Figure 5. Genetic interaction between abcb19 and cuc2, cuc3. A: Fusion defects between the primary stem and cauline leaf in abcb19-5, abcb19-5 cuc2, abcb19-5 cuc3, and cuc2 cuc3. White arrowheads indicate stem-cauline leaf fusion; white arrow shows the fusion of axillary shoot to the main stem. B: Fusion defects between the primary inflorescence stem and pedicel in abcb19-5, abcb19-5 cuc2, abcb19-5 cuc3, and cuc2 cuc3. White arrowheads indicate stem-pedicel fusion. C: The rate of fusion at primary stem-cauline leaf junctions in different genotypes. At least 30 samples were analyzed for each genotype in every biological replicate. The values represent the mean and standard deviation from two independent biological replicates (N = 2). * Significantly different, P < 0.05. Scale bar = 5 mm in A and B.

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Figure 6. Expression of some organ boundary genes analyzed by semi-quantitative RT-PCR. The numbers labeled on the right are the cycle numbers of the corresponding genes in the RT-PCR. The primer sequences were from the reference [6].
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RNA Extraction and Real-Time PCR

Total RNA was isolated using TRI Reagent Solution (Ambion) according to the manufacturer's handbook. Following digestion with RNase-free DNase (Promega) to eliminate DNA contamination, 3 mg of total RNA were used for reverse transcription (Fermentas). Real-time PCR was carried out using Takara SYBR Premix Ex Taq in a 7500 real-time PCR instrument (Applied Biosystems). Primer information:

ACT2-Q-F, 5'-TCCCTCAGCAGATTCGAGGAT-3'
ACT2-Q-R, 5'-AACGATTCTGGAACCTGCCTC-3'
CUC2- Q-F 5'-GAACAACAGCACCCGTCACAG-3'
CUC2- Q-R 5'-GAATGAGTTAAGCCTGCAAGC-3'

Primers used in the transcript analysis:
P1 5'-GAAGCTTGTGGTTCCGTGTTTCC-3'
P2 5'-TCAAATCCTATGTGTTTGAAGC-3'
P3 5'-ATGTCGAAACTAACACACC-3'
P4 5'-GTAAACAGATCTTTGGGTCTTTC-3'

GUS Staining

GUS staining and subsequent Paraplast Plus sectioning were performed as described previously [52]. A β-glucuronidase assay was performed according to the protocol of Jefferson [53].

Confocal Microscopy

Immediately after the plants were bolting, the inflorescences were cut and placed on a slide. Almost all visible buds were cut off and left only the tiny region including the inflorescence meristem. The fluorescent pictures were taken at 40x lens at the excitation of 514 nm on an inverted Zeiss 510 microscope.

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Author Contributions

Conceived and designed the experiments: LM HZ SC XL LL. Performed the experiments: HZ LI HM. Analyzed the data: HZ LL LQ LM YC. Contributed reagents/materials/analysis tools: HZ SC XL LL. Wrote the paper: HZ LM.

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