CCS52A2/FZR1, a cell cycle regulator, is an essential factor for shoot apical meristem maintenance in *Arabidopsis thaliana*

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**Abstract**

**Background:** Cell division and cell fate decisions regulate organ formation and function in plant growth and development. It is still unclear how specific meristematic regulatory networks operate with the cell cycle machinery to translate stem cell identity and maintenance into cellular behavior. In this study, we address these questions by analysis of a shoot apex defective mutant, namely *xcm9*.

**Results:** Phenotypic analysis of the *xcm9* mutant reveals concomitant premature termination of floral shoots with frequent bifurcation of the shoot apices, stems, and flowers. Microscopic observations show irregular cell organization in shoot apical meristems of *xcm9*. Positional cloning revealed that *xcm9* is a loss of function allele of the *CCS52A2/FZR1* gene, which has previously been implicated in root development. Expression analysis demonstrated that *CCS52A2* maintains a higher transcriptional expression level in actively dividing tissue. Genetic studies indicated that the *CCS52A2* gene functions together with *WUSCHEL (WUS)* and *CLAVATA3 (CLV3)* in regulating the development of the shoot meristem, and also contributes to this regulation together with the chromatin remodeling pathway. In addition, fewer *xcm9* cells express *CYCLIN B1:1*, showing that cell cycle progression is disrupted in the mutant.

**Conclusion:** We propose that the *CCS52A2* gene is a mediator that functions together with meristematic genes to regulate meristem organization, and cross-functions with chromatin regulators in cell cycle progression during shoot apical meristem development.

**Background**

In higher plants, the vast majority of structures and organs, such as leaves, stems, roots, and flowers, are formed postembryonically from groups of undifferentiated cells, called meristems. In many species of plants, certain meristems are maintained throughout life. The activities of the root apical meristem (RAM) and the shoot apical meristem (SAM) determine root and shoot structure and function, respectively. Stem cells are confined to the centers of shoot and root apices, and their proliferation is maintained by signals that cells receive from the local environment [1]. They undergo precisely controlled division, which must be rapid enough to replenish cells lost to differentiation, but restricted enough to prevent overproliferation of undifferentiated cells. Adjacent to the stem cells, several cells in the SAM and RAM form the organizing center (OC) and quiescent center (QC), respectively. These cells coordinate with neighboring cells to establish the balance between proliferation and differentiation in the meristem niche [2].

Several meristematic genes form feedback networks that control this dynamic balance. In *Arabidopsis*, *WUS* encodes a transcription factor expressed in the OC, and its expression promotes the identity of distal meristem cells as stem cells, which themselves are characterized in part by *CLV3* expression [3,4]. *CLV3* encodes a peptide hormone expressed in the central apical surface of shoot and floral meristems and is necessary for controlling the size of the central zone (CZ) in SAMs [5,6]. *WUS* and *CLV3* form a feedback regulation loop: *CLV3* acts as mobile intercellular signal to negatively regulate *WUS*.
transcription in the OC via the receptor proteins CLV1/CLV2/CORYNE (CRN), while WUS positively regulates CLV3 expression [7–9]. Similar to the shoot meristem, the root QC maintains the stem state of the surrounding cells and prevents these cells from differentiating [10]. WOX5 is the functional homolog of WUS expressed in the QC [11], and CLE40 is a CLV3-related peptide expressed in differentiated stele and columella cells [12,13]. Like the CLV3-WUS network in the shoot, CLE40 and WOX5 form a self-regulating network that controls the proliferation and differentiation of stem cells in the root [14].

Besides these vital meristematic regulatory genes, the organization and maintenance of cells in meristems are also modulated by several cell cycle control genes. It is still unclear whether the cell cycle machinery acts largely independently in regulating meristem organization, or acts by receiving the signals from meristemetic pattern genes via unknown mechanisms. The cell division cycle protein CDC5, cyclin D3 (CYCD3), HBT/CDC27B, and the cyclin-dependent kinases A;1 (CDKA;1), CDKB2;1 and CDKB2;2 have been reported to be necessary for SAM and/or RAM development [15–19]. These proteins are among numerous cell cycle regulators, including other cyclins, CDKs, CDK inhibitors and CDCs, that precisely control the mitotic cell cycle during the four cell phases and several checkpoints to accomplish DNA replication and subsequent division. Once the need for the cell cycle regulators ends, they are degraded by ubiquitin-mediated proteolysis. Anaphase-Promoting Complex (APC), which functions as an E3 ubiquitin ligase that marks target cell cycle proteins for degradation by the 26 S proteasome, plays an important role in the phase transition of the cell cycle [20].

Some genes that are not considered to be part of the cell cycle regulator class of proteins also contribute to the development of meristems and are involved with the normal sequence of the cell cycle. Among these are the MGOULN 1 (MGO1) and FASCIATA 1 (FAS1) genes, which play critical roles in the fundamental organization and/or functioning of both the SAM and RAM [21]. fas1 and mgo1 are characterized by fasciated stems and short roots [22,23]. FAS1 is one subunit of Arabidopsis chromatin assembly factor-1 (CAF-1), which shows a conserved activity for chromatin assembly at the DNA replication fork in S phase. Loss of function of FAS1 alters the epigenetic marks at promoters of genes involved in activation of the G2 damage checkpoint, leading to inhibition of mitosis progression [24]. MGO1 is homologous to type IB topoisomerase, which has been reported to stabilize the epigenetic state of developmentally regulated genes and to affect gene expression in conjunction with the chromatin remodeling pathway in Arabidopsis [25]. An unknown mechanism is likely to be present that integrates the meristematic regulating network with the cell cycle machinery to translate SAM identity and maintenance into cellular behavior. The exact nature of this linkage and the mediator or mediators between these two networks is an important research question.

In this article, we report the isolation of a novel SAM defective mutant, xcm9, which displays clear signs of bifurcation and premature termination under our conditions. Positional cloning revealed that the XCM9 gene encodes an activator of APC/C, namely CCS52A2. RT-PCR, promoter analysis and in situ hybridization assays show that the CCS52A2 gene is broadly expressed in all organs tested, but has higher expression specifically in the shoot apices and root tips. Our genetic studies indicate that the CCS52A2 gene functions together with WUS and CLV3 in stem cell regulation, and also contributes to this regulation together with the chromatin remodeling pathway. Monitoring of CYCB1:1 expression revealed that cell cycle progression is disturbed in xcm9. We propose that the CCS52A2 gene is a mediator that regulates meristem organization, functions together with meristematic genes and cross-functions with chromatin regulators in cell cycle progression during SAM development.

Results

Mutations in the XCM9 gene disrupt SAM development and maintenance

While screening for phenotypic mutants from MYB-family T-DNA insertion lines, we identified a shoot apex-defective mutant segregating in SALK_074403 from the SALK T-DNA collection [26]. This mutant was named xcm9. xcm9 mutants demonstrate premature termination (Figure 1A, B) and bifurcations of shoot apices (Figure 1C, D, G). This premature termination is evident in the primary shoot and lateral inflorescences of xcm9, which have an average of 6.97 (range from 1–14, n = 39) flowers on main stems and 9.68 (range from 1–20, n = 39) flowers on lateral inflorescences, compared with an average of 39 (range from 28–46, n = 9) flowers on WT. In most cases, xcm9 plants prematurely terminated with fewer flower buds and a senesced SAM (Figure 1B). But in some groups of shoot apices, the terminus had a flower stalk with a single flower or two fused flowers without an apparent SAM (Figure 1E, F), suggesting that the SAM is completely consumed by flower production at the terminus. Those single or several-fused flowers in the terminus always exhibited fewer flower organs (petals and sepals) and showed curved or fasciated stigmas (Figure 1E). In some extreme cases, the floral SAM did not generate any flowers but only terminated as a cluster of tiny buds, which were arrested during early stages of flower development (Figures 1H, I and 2A–D). Bifurcations were frequently observed in reproductive shoot apices (Figure 1G), flowers (Figure 1C) and stems (Figure 1C).

Thus while only 12.8% of reproductive shoot apices of
ccs52a2-3 (n = 39) was observed to bifurcate, we can infer that more bifurcations occurred in the rosette during early vegetative development, since some xcm9 mutants had more than one leaf initiation center. Subsequent results from local gene expression supported this (see below).

Besides the defective shoots, xcm9 was reduced in height (Figure 1A), stem diameter (Figure 2E), siliques length (Figure 2F) and leaf size (Figure 2G). The number of seeds per siliques in xcm9 was reduced, but the seed size was about 1/5 larger in both length (448.7 ± 23.8 μm for WT, 556.6 ± 42.8 μm for xcm9, p < 0.05) and in width (252.9 ± 26 μm for WT, 321.9 ± 24.1 μm for xcm9, p < 0.05).

Cell organization of SAM is disordered in xcm9

To determine whether the defects of shoot apices in mutants were preceded by the abnormal development of the SAM, we visualized the cellular morphology of mutant meristems by semi-thin section. Cell organization was disrupted in the L1 and L2 layers of the SAM in 8-day-old xcm9 plants compared with the highly organized cells in WT (Figure 3C, D). The width of SAMs in mature embryos and 8-day-old seedlings of xcm9 were broadened to 41.3 ± 0.8 μm and 108.5 ± 5.8 μm, compared with 34.2 ± 0.6 μm and 75.2 ± 4.5 μm in the WT, respectively (Figure 3A-D). In addition, counting of cell numbers in mutant SAM semi-thin sections showed that there was no significant difference (student t test p = 0.15 > 0.05, n = 6) with the WT. Examination of the average cell areas in both the L1 layer and throughout the SAM both indicated that the enlargement of the SAM was mainly due to an increase of the meristem cell areas in the xcm9 mutant in comparison with WT (Figure 3J, K). We also compared the floral SAMs between xcm9 and WT. The flowering-stage SAMs from primary stems of xcm9 were smaller (Figure 3F, H) than that of WT (Figure 3E, G), which is in contrast to SAMs in embryos and seedlings. Scanning electron microscopy (SEM) was used to investigate the overall structure of flowering-stage SAMs. The results indicate that the xcm9 SAM in the primary floral shoot was smaller than WT, which is consistent with results from semi-thin sections; this may be due to premature termination of the floral shoot in xcm9. Bifurcated SAMs were usually observed in lateral floral shoots, and each of the splitting SAMs was surrounded by a ring of floral primordia (Figure 3I). All of the above suggests the size and cell organization of the SAM were both affected in conjunction with the phenotype of defective shoots in xcm9.

A 2.6Kb deletion of At4g11920 caused the shoot apex defect

To test if the mutant phenotype of XCM9 gene cosegregated with the kanamycin resistance harbored in the T-DNA insert, we grew the mutant seeds on MS medium
with kanamycin and found that they are kanamycin-sensitive, indicating that the kanamycin resistance gene may be silenced or lost. The T-DNA website (signal.salk.edu) [26] indicates that the T-DNA was inserted in the 3rd exon of the At1g18960 gene, which encodes a myb-like HTH transcriptional regulator family protein. Primers were designed from the flanking sequences of the T-DNA insertion to confirm its existence. Expected amplification was obtained, indicating that the kanamycin resistance gene was silenced in the mutant. Segregation analysis indicated that the T-DNA insertion did not cosegregate with the mutant phenotype, suggesting that the mutant phenotype is not caused by the T-DNA insertion.

To clone the gene that was disrupted in the xcm9 mutant, the xcm9 mutant was crossed to ecotype Landsberg erecta (Ler) for mapping. All F₁ plants displayed a WT phenotype, whereas the F₂ progeny segregated for xcm9 and WT seedlings, indicating that the xcm9 mutation is recessive in a single nuclear gene ($\chi^2 = 0.06 < \chi^2_{0.05(1)} = 3.84$). Bulked segregant analysis mapped the xcm9 locus to the interval between CIW5 and CIW7 on chromosome 4. Further fine mapping located the XCM9 locus between the two short sequence length polymorphism (SSLP) markers on bacterial artificial chromosomes (BACs) F7L13 and T4C9. Additional markers were used to fine-map the locus between SSLP markers on BAC clones T26M18 and F16J13 (Figure 4A). Sequencing of the genomic DNA amplified from this 47Kb region revealed a 2.6Kb deletion that starts from the 5th exon of At4g11920 and extends to the intergenic region between At4g11920 and At4g11910 in the xcm9 mutant.

To verify whether the deletion in At4g11920 caused the shoot apex defects in xcm9, we next performed a complementation test by inserting a 9.7Kb genomic DNA fragment containing the 1.5Kb upstream sequence, the full-length coding region and 1.2Kb downstream sequences of At4g11920 (which includes the At4g11910 coding region) into heterozygous plants of xcm9 (Figure 4B), because homozygous xcm9 plants exhibit a strong phenotype and do not produce enough flowers for transformation. We found that all transgenic plants obtained in a homozygous xcm9 background were phenotypically indistinguishable from WT plants, confirming that the apex defect phenotypes observed in xcm9 were caused by the deletion of either At4g11910 or At4g11920. In addition, two SALK lines, SALK_001978 and SALK_073708C were identified with T-DNA insertions in exon 7 and 6 of At4g11920, respectively (Figure 4B). These two mutants showed similar phenotypes to xcm9, and were designated as xcm9-2 and xcm9-3, respectively. These results together confirmed that the mutant phenotypes were caused by the mutation of At4g11920.

The At4g11920 gene consists of nine exons and eight introns (Figure 4B) and encodes a putative CDH1/CCS52A2/FZR1 protein (CDH1, also known as HCT1 for Homolog of CDC Twenty; CCS52A2, also known as a 52 kDa protein encoded by a Cell Cycle Switch gene),
Figure 3 The microstructures of SAMs in *xcm9*. (A) and (B), SAM sections of mature embryos in WT (A) and *xcm9* (B). Bar = 10 μm. (C) and (D), SAM sections of 8-day-old seedlings in WT (C) and *xcm9* (D). Bar = 50 μm. (E) and (F), SAM sections of 28-day-old inflorescence shoots in WT (E) and *xcm9* (F). Bar = 10 μm. (G) to (I), Microstructures of inflorescence shoots of WT (G) and *xcm9* (H, I) by scanning electron microscopy (SEM). Bar = 50 μm. The floral SAM in primary shoot of *xcm9* (H) was smaller than that of WT (G). The floral SAM in lateral shoot of *xcm9* (I) shows bifurcation. Arrowhead indicates the two SAMs in one floral shoot. (J) and (K), The ranges of cell areas of 1st layer cells and all cells in SAMs of mature embryo (J) and 8-day-old plants (K).
which contains WD40 repeats and is a component of APC/C, acting as a co-activator and substrate recognizer [27,28]. Therefore, the xcm9 mutant was renamed ccs52a2-3. xcm9-2 corresponds to ccs52a2-1, and xcm9-3 was renamed ccs52a2-2 [29]. In Arabidopsis, CCS52A2 was reported as stabilizing root meristem maintenance by acting in the distal region of the root and regulating the mitotic state of the QC [30]. Additionally, CCS52A2, under the regulation of the transcription factor EF1/DEL, promotes endoreduplication in cells of mature leaves [29].

In Arabidopsis, three CDH1/FZR homologs, AtCCS52A1, AtCCS52A2 and AtCCS52B, have been previously identified [31]. All of the CCS52 (A1, A2 and B) proteins show a similar structure, with conserved C-box, CSM (Cdh1-specific motif), the IR motifs (APC binding domain) and the CBM motif (mitotic RVL cyclin binding motif) [31]. Importantly, ccs52a2-3 lost the conserved potential CDK phosphorylation sites, IR and CBM motifs of CCS52A2, suggesting that this allele is a possible null mutant.

CCS52A2 mRNA is broadly expressed
RT-PCR analysis indicated that CCS52A2 had a broad expression profile including roots, rosette leaves, stem, cauline leaves, inflorescences and siliques (Figure 5P). The expression of CCS52A2 was also examined in 8-day-old plants by a histochemical GUS assay, using transgenic plants expressing the CCS52A2::GUS fusion protein under the control of the CCS52A2 native promoter. Strong expression was observed in shoot apices and root tips (Figure 5A-D). To determine the expression region of CCS52A2 mRNA, in situ hybridization was carried out. The results were consistent with that of the GUS assay, presenting strong signals in the entire SAM (Figure 5H, J), lateral leaf primordia in 8-day-old seedlings (Figure 5J), flower primordia (Figure 5H), and pollen (Figure 5E, F, L-N) of different stages in 28-day-old plants.

The expression patterns of WUS and CLV3 were altered in the ccs52a2-3 mutant
The disordered organization of SAM in ccs52a2-3 and high expression of CCS52A2 in the shoot apex prompted us to investigate whether the expression patterns of two essential meristematic genes, WUS and CLV3, are affected in the mutant. qRT-PCR results using dissected SAM from 10-day-old seedlings indicated that WUS was expressed at a higher level in ccs52a2-3, while CLV3 maintained slightly higher mRNA expression levels in ccs52a2-3 mutants when compared with WT (Figure 5G).
To further examine the expression patterns of these meristematic genes in the SAM, mRNA \textit{in situ} hybridization assay of \textit{WUS} and \textit{CLV3} genes were carried out. The expression of \textit{WUS} in WT is restricted in a small region composed of a few cells in the center of the SAM (Figure 6A, D) [4]. However, in the SAM of 8-day-old \textit{ccs52a2-3} seedlings, \textit{WUS} expressed in one enlarged domain at higher levels (Figure 6B), or in two distinct nearby domains (Figure 6C), suggesting that enlarged or multiple OC zones are formed, which is consistent with the broadening SAMs and bifurcations of the stem. In contrast to seedlings, the reproductive SAMs of the mutant had either no detectable or decreased expression of \textit{WUS} (Figure 6E) in comparison with WT (Figure 6D), which suggests that the size reduction and premature termination of the floral shoot meristems in \textit{ccs52a2-3} mutants may be caused by a loss of \textit{WUS} activity.

In WT, \textit{CLV3} mRNA accumulates in a small zone of cells in the first three layers at the meristem apex (Figure 6G, I) [6]. Compared with WT, the SAMs of 8-day-old \textit{ccs52a2-3} seedlings that were examined exhibited increased \textit{CLV3} expression (Figure 6H). However, most of the 28-day-old \textit{ccs52a2-3} plants (n = 10) had a slightly wider but shallower expression of \textit{CLV3} in the reproductive SAM sections compared to limited expression in the central surface of WT SAM (Figure 6I), while some didn’t show a significant difference with WT.

To verify the results from \textit{in situ} hybridization, we monitored the expression of \textit{WUS} and \textit{CLV3} using GUS reporters (\textit{pWUS::GUS} and \textit{pCLV3::GUS}) in WT and \textit{ccs52a2-3}. The \textit{pWUS::GUS} assay presented a similar expression pattern as \textit{in situ} results: enlarged and sometimes dual expression domains in the \textit{ccs52a2-3} background (Figure 6K). In addition, the \textit{pCLV3::GUS} location in \textit{ccs52a2-3} SAM exhibited two nearby domains of \textit{CLV3} expression in 6 of 22 8-day-old seedlings examined (Figure 6M), which indicated that two separate \textit{WUS-CLV3} supporting systems of meristem organization had been established as early as the 8-day-old stage. It was also discovered that \textit{CLV3} showed broad expression in some first layer cells that flank the major expression zone in 7 of 22 SAMs of \textit{ccs52a2-3} seedlings (Figure 6N), while the remaining 9 of 22 \textit{ccs52a2-3} SAMs exhibited a single \textit{CLV3} expression region. Additionally, the floral SAMs in main stems of \textit{ccs52a2-3} always displayed decreased \textit{CLV3} expression (Figure 6P), but in some lateral branches multiple \textit{CLV3} expression regions were observed (Figure 6Q). These were consistent with the observations of multiple SAMs in lateral branches (Figures 1G, 3I) and smaller SAMs in main stems (Figure 3F, H).
**ccs52a2-3** is synergistic to **wus** and **clv** mutants

To test if genetic interactions exist between **CCS52A2** and the **WUS, CLV1** and **CLV3** genes, we created the double mutants **ccs52a2-3 wus-1**, **ccs52a2-3 clv1-1**, **ccs52a2-3 clv1-4** and **ccs52a2-3 clv3-2** by crossing **ccs52a2-3** with **wus-1**, **clv1-1**, **clv1-4** and **clv3-2**, respectively. **clv1-4** and **clv3-2** produce enlarged shoot and floral meristems relative to WT and are considered the strongest alleles of **clv1** and **clv3** mutants (Figure 7A, F) [5, 32]. Double mutants with **clv3-2** had a novel phenotype: **ccs52a2-3 clv3-2** plants (n = 48) consistently had many more rosette leaves with larger shoot meristems (Figure 7E) and exhibited a delayed transition from vegetative phase to reproductive phase compared to WT and single mutants. Additionally, **ccs52a2-3 clv3-2** double mutants displayed severe fasciation in reproductive SAMs and stems (Figure 7B, C). More severe bifurcations (7 shoot apices on average and sometimes more than 30 in a single stem, depending on nutritional conditions) were present in **ccs52a2-3 clv3-2** double mutants compared to **ccs52a2-3** single mutants, which generally produced 2 or 3 shoot apices from bifurcations. We were unable to count the exact number of shoot apices resulting from such extreme bifurcations because of the twisted stems.

**CLV1** is the direct downstream target of **CLV3** in the **CLV3-WUS** pathway [9], so **ccs52a2-3 clv1-2** was expected to have similar phenotype to **ccs52a2-3 clv3-2**. Observations of **ccs52a2-3 clv1-1** and **ccs52a2-3 clv1-4** double mutants showed similar fasciation in shoot apices as in the **ccs52a2-3 clv3-2** double mutants. The **ccs52a2-3 clv1-4** (n = 39) displayed bifurcation at a very high frequency, over 50% under our conditions, compared to 12.8% for **ccs52a2-3** (n = 39). All **ccs52a2-3 clv1-4** double mutants were classified into three types according to the width of fasciated stems (Figure 7G-1). Notably, type I had the widest major stem, followed by type II and then the type III. The type I and type II double mutants had no lateral branches originating from the rosette leaves, but type III did. Most of the **ccs52a2-3 clv1-4** double mutants prematurely terminated in the formation of several flowers in lateral branches, which signifies that **clv1-4** can’t rescue premature termination in **ccs52a2-3** by enlarging SAM size in the absence of **CLV** function. Furthermore, flowers in both **ccs52a2-3 clv3-2** and **ccs52a2-3 clv1-4/clv1-1** double mutants appeared to be typical of **clv** flowers, with increased floral organ number, including 4–8 carpels and 5 petals. The severe SAM bifurcations and fasciation in **ccs52a2-3 clv3-2** and **ccs52a2-3 clv1-4** double mutants revealed a synergistic interaction between **CCS52A2** and the **CLV** pathway, with **CCS52A2** being involved in independent pathways but in the same general process of SAM development and maintenance as **CLV3** and **CLV1**.

**WUS** is another important gene in the organization and identity of SAM cells, as **WUS** creates a feedback regulation loop with **CLV1** and **CLV3** [7]. The primary
Shoot meristem of \textit{wus-1} seedlings fails to maintain itself, and cells of the meristem differentiate, halting further shoot growth until adventitious meristems form to initiate further leaves and shoots [3,4]. To test the interaction of \textit{WUS} with \textit{CCS52A2}, we constructed the \textit{ccs52a2-3 wus-1} double mutant. 34\% of 12-day-old progeny of \textit{ccs52a2-3/+ wus-1/+} showed a defective SAM that resembled the \textit{wus-1} single mutant, which was higher than the 24\% of \textit{wus-1/+} self progeny (Table 1). Thus \textit{ccs52a2-3 wus-1} double mutant seedlings (Figure 7M) appeared phenotypically identical to \textit{wus-1} single mutants, displaying serious defects in SAM organization, including the inability to establish normal shoot meristems followed by the development of secondary adventitious-like meristems, which resulted in more rosette leaves (Figure 7K). But the SAM defects in most older \textit{ccs52a2-3 wus-1} double mutants were enhanced, producing more rosette leaves than those in \textit{wus-1} single mutants and delaying transition from the vegetative apex into the reproductive apex as is seen in \textit{wus-1}. 100\% of the 42-day-old \textit{ccs52a2-3 wus-1} plants (\(n = 30\)) did not develop determinate inflorescence shoots, compared with 75\% of \textit{wus-1} single mutants (\(n = 42\)) harboring flowering shoots at the same age under our conditions (Table 1).

**Table 1 Shoot Meristem Defects in \textit{wus ccs52a2-3} Plants**

| Genotype of parent plants | Seeding meristem | n | Arrested (%) | Genotype | Inflorescence shoots | 42-day-old | n | Indet. (%) | Det. (%) |
|---------------------------|------------------|---|--------------|----------|----------------------|------------|---|------------|----------|
| \textit{wus-1/+}          |                  | 103| 24           | \textit{wus-1} |                      | 47         | 47| 25         | 75       |
| \textit{ccs52a2-3}        |                  | 37 | 0            | \textit{ccs52a2-3} |                  | 34         | 34| 0          | 100      |
| \textit{ccs52a2-3 wus-1/+} |                  | 105| 34           | \textit{ccs52a2-3 wus-1} |                  | 30         | 30| 100        | 0        |

Primary seeding shoot meristem was analyzed in 12-day-old plants. det., determinate shoot. Indet., indeterminated shoot.
CCS52A2 controls cell size and ploidy in differentiated cells during cell division

In a previous study, CCS52A2 was found to control the endocycle, as indicated by the low Endoreduplication Index (EI) of mature leaves of the knockout lines CCS52A2KO (analysis with ccs52a2-1 mutant) and the increased number of cells with a high DNA ploidy level in CCS52A2OE leaves [29]. ccs52a2-3 mutants appeared dwarfed and had smaller leaves, stems and siliques in comparison with WT (Figures 1A and 2E-G). We hypothesized that these phenotypes might be due to decreased cell quantities and/or smaller cell sizes, so we analyzed the cell size and number of mature 5th leaves. In comparison to WT, the 5th mature leaves of ccs52a2-3 mutants show a decrease of 13.58% in mesophyll cell size (Figure 8B) and a decrease of 70.61% in total mesophyll cell number (13039.6 ± 2964.3 in ccs52a2-3; 44361.8 ± 6767.2 in WT, Student t test p < 0.05), and a significant cell size decrease (2226.4 ± 61.6um² in ccs52a2-3; 2576.2 ± 113.7um² in WT, p < 0.05) was also detected in abaxial epidermal cells (Figure 8D). To test if the cell size decrease corresponds with lower ploidy levels, we measured the nuclear size of the abaxial epidermal cells in leaf peels. Statistical analysis demonstrated that the mutant leaf cells with 16C ploidy made up a significantly lower percentage than in WT, while the mutant leaf cells of 2C ploidy showed a slightly higher percentage in the mutant (Figure 8E). The Cycle Value (CV) of 5th leaves in the ccs52a2-3 plant was lower (p<0.05) than the WT (Figure 8F) [33], consistent with lower EI of CCS52A2KO leaves determined by flow cytometry in the previous report [29]. Therefore, we believed that loss of CCS52A2 function results in the decrease in cell size and ploidy level in mature leaves. However, as measured by flow cytometry, the CV of SAM cells in 8-day-old ccs52a2-3 seedlings was unaffected in comparison with WT (data not shown). Despite the enlarged cell area of meristem cells in mutant SAMs (Figure 3I, K), the cell ploidy was not correspondingly changed, which means, unlike in the leaf, CCS52A2 did not induce the onset of endocycle in the shoot meristems. The earlier published data of the RAM in CCS52A2 knockout lines (ccs52a2-1, ccs52a2-2) also showed that CCS52A2-mediated control over root development did not involve endoreduplication [30].

CYCB1;1 was revealed as one of the possible substrates of APC/C to interact with CCS52s (A1, A2 and B) in Arabidopsis [31]. Since CCS52A2 acts at G2/M to control mitosis exit and endocycle entry, we checked the expression

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**Figure 8 Cell size and ploidy change in the mature leaves of ccs52a2-3 plants.** (A) and (B), Comparison of cell size of mesophyll cells in 5th mature leaves of 3 week old WT (A) and ccs52a2-3 (B) plants. Bar = 50 µm. (C) and (D), Comparison of cell size of abaxial epidermal cells in 5th mature leaves of 3 week old WT (C) and ccs52a2-3 (D) plants. (E), Ploidy distribution was determined by isolating nuclei from abaxial epidermal cells in 5th mature leaves (n = 5). Mean values ± SD are shown. (F), The comparison of Cycle Value of 5th mature leaves in WT and ccs52a2-3 (n = 5). Mean values ± SD are shown.
of the G2/M transition reporter gene CYCB 1;1 in cccs2a2-3 mutants. We monitored GFP reporters in the root tips of marker lines harboring CYCB1;1::GFP in a cccs2a2-3 mutant background by confocal microscopy. The results showed that the number of RAM cells expressing CYCB1;1 was dramatically reduced, and the range of cells expressing CYCB1;1 in root was shorter in 7-day-old cccs2a2-3 compared with WT (Figure 5R). In addition, qRT PCR was carried out to quantify the expression levels of CYCB1;1 in the shoot. The seedling SAM of cccs2a2-3 showed lower expression level in comparison with that of WT (Figure 5S). Reduction of CYCB1;1 expression in both the SAM and RAM indicated that the cell cycle process was disturbed in cccs2a2-3 mutants.

CCS2A2 interacted with MGO1 and FAS1
The mgo-1 and fas1-1 mutants display fasciation and bifurcation in both vegetative and reproductive development [22,23], similar to the phenotype of cccs2a2-3 mutants. The similarity of the phenotypes compelled us to figure out whether these genes act in the same pathway and/or affect the same downstream processes as CCS2A2. To study their genetic relationship we constructed cccs2a2-3 fas1-1 and cccs2a2-3 mgo1-1 double mutants. The cccs2a2-3 mgo1-1 double mutants were indistinguishable from mgo-1 (Figure 9A, C) under our conditions, suggesting that MGO1 may be epistatic to CCS2A2. The cccs2a2-3 fas1-1 double mutants possessed many characteristics of both parents, but can be easily distinguished from either single mutant (Figure 9D-I). They have narrow leaves and disrupted floral phyllotaxy as in fas1-1, with bifurcation and premature termination characteristic of cccs2a2-3. However, the bifurcation ratio of inflorescences is higher than cccs2a2-3 single mutants (71% of cccs2a2-3 fas1-1, n = 32; 12.8% of cccs2a2-3, n = 39). Many cccs2a2-3 fas1-1 double mutants displayed a large number of rosette leaves with a bushy appearance in the mature plant (Figure 9E). The novel phenotype of cccs2a2-3 fas1-1 double mutants revealed that FAS1 and CCS2A2 are involved in the same downstream process.

Discussion
CCS2A2 is necessary for the normal structure and function of the SAM in Arabidopsis
This article demonstrates that loss of CCS2A2 function in cccs2a2-3 mutants led to novel morphological defects in the SAM. The cccs2a2-3 mutant displays concomitant premature termination of floral shoots with frequent bifurcation of the shoot apices, stems, and flowers. These defects in meristems indicate that the CCS2A2 gene is essential for the proper maintenance and function of the SAM in plant development. Interestingly, the vegetative meristem and inflorescence meristem were larger and smaller than that of WT, respectively. One possible explanation for this is that the double WUS expression zones in the 8-day-old mutant are indicative of bifurcation originating at this time. Lateral splitting results two smaller SAMs, each with a reduced WUS expression domain, and which later give rise to smaller inflorescence SAMs that terminate prematurely.

Expression changes of the major SAM regulator genes WUS and CLV3 in cccs2a2-3 were observed. Ectopic CLV3 expression was detected in the first layer of the SAM (Figure 6N), and the presence of more than one WUS and/or CLV3 expression region in a single SAM of cccs2a2-3 seedlings reveals a rare maintenance pattern in higher plant meristems. All of these abnormal expression patterns of SAM regulators indicate that malfunction of CCS2A2 affects cell identity in meristems. Previously published data show that the CCS2A2 gene plays a role in QC identity in root development, as the

Figure 9 Morphology of mature mgo-1, cccs2a2-3, cccs2a2-3 mgo1-1, fas1-1, and cccs2a2-3 fas1-1. (A) mgo-1. The arrowhead indicates the bifurcations in stem. (B) cccs2a2-3. The arrowhead indicates premature termination in the primary shoot. (C) cccs2a2-3 mgo1-1 double mutant. The arrowhead indicates similar bifurcations in the stem as the mgo1-1 single mutant. (D) fas1-1. (E) and (F) cccs2a2-3 fas1-1 double mutant, demonstrating bushy leaves and enhanced bifurcation. Arrowheads indicated disturbed flower phyllotaxy and two stems of bifurcation. (G) to (I) Floral shoot apex of fas1-1 (G), cccs2a2-3 (H) and cccs2a2-3 fas1-1 (I). The cccs2a2-3 fas1-1 showed premature termination in floral shoot in comparison with fas1-1 single mutant at the same age. All bars = 1 cm.
RAM organizing genes show irregular expression domains and locations and a failure to maintain the low mitotic activities of cells in QC zone in ccs52a2-1 root [30]. We here provide evidence that the expression of SAM organizers are also altered in ccs52a2-3, and duplicate individual CLV3-WUS regulatory centers are sometimes generated in the mutant SAM. One possible explanation for this is that the proliferation of cells in QC zone results from a partial loss of cell division control, leading to formation of new and separate CLV3-WUS regulation systems in a single SAM of the mutant. Considering the role of CCS52A2 in the root, we give a hypothesis is that the duplicated OCs may somehow result from the failure to maintain low mitotic levels in certain SAM cells, with more cells available to fulfill certain roles.

**CCS52A2 synergistically interacts with SAM organizers**

We suspect that CCS52A2 regulates the development of meristems by indirectly interacting with the WUS and CLV3 genes. This hypothesis is firstly supported by up-regulated expression of CLV3 and WUS in the SAM of ccs52a2-3 seedlings (Figure 5G). Additionally, the more severe phenotypes seen in ccs52a2-3 clv3-2 and ccs52a2-3 clv1-4 double mutants indicate that CCS52A2 may function in separate genetic pathways but are involved in the same process with CLV1 and CLV3, and disrupting multiple modes of cell division regulation in meristems leads to a greater likelihood of a very large loss of cell division control and massive overproliferation. The finding of more severe bifurcations from the larger SAMs (compared with any single mutants) of ccs52a2-3 clv double mutants reveals that the bigger the size of the SAM, the more bifurcations occurred. clv single mutant exhibits an expanded SAM but has not been reported to undergo bifurcation.

The enhanced wus-1 phenotype in ccs52a2-3 wus-1 double mutant indicated that CCS52A2 contributes to meristem establishment and leaf initiation together with WUS; however, CCS52A2 also acts in an independent pathway as well.

**CCS52A2 functions in the SAM and stimulates the onset of endocycle in mature leaves**

Previous studies suggest that CCS52A2 is an activator of APC/C, which mediates protein proteolysis during mitosis [30,31,34]. Therefore, it could regulate SAM maintenance and development by regulating cell division. Our hypothesis is consistent with cell cycle impairment, as indicated by the lower CYCB1;1 expression in RAM and SAM of ccs52a2-3 (Figure 5R). However, the enlargement of the SAM is mainly due to enlarged cell areas and not increased cell number, which indicates that cell proliferation is still under control in mutant SAMs even if impairment exists in the cell cycle. CCS52A2 has also been reported to promote the exit from the cell cycle into the endocycle, and leads to endoreduplication by association with APC/C in *Medicago truncatula* [28,34]. In *Arabidopsis*, similar effects are seen in CCS52A2 knockout (KO) and overexpression (OE) lines [29]. However, in this paper, the unchanged CV (corresponding to ploidy level) of the SAM indicates that the enlarged stem cells are not due to the entrance into endocycle in ccs52a2-3. This is consistent with previous studies that CCS52A2 is not correlated with the endocycle in the RAM in CCS52A2KO, which exhibits a normal Endoreduplication Index in the mutant [30]. But in mature leaves, the presence of smaller cells with lower CV of cells in ccs52a2 mutants indicates a role for CCS52A2 in these tissues. Taken together, these studies further supports the idea that CCS52A2 only has obvious effects on modulating endocycle onset in differentiating cells and not in undifferentiated cells.

**CCS52A2 was involved in chromatin remodeling**

Plants carrying a mutation in the MGO1 gene exhibit some phenotypes similar to ccs52a2-3 mutants [22], such as bifurcation of stems. *MGO1* encodes an *Arabidopsis* Type IB DNA Topoisomerase, which functions in the relaxation of supercoiled DNA and acts in a number of different DNA metabolic processes, including replication, transcription, repair, and histone decondensation [36,37]. It has been reported that MGO1 cooperates with chromatin regulators and is necessary for the maintenance of several epigenetically regulated genes [25]. The indistinguishable phenotypes of ccs52a2-3 mgo1-1 double mutants compared to the mgo1-1 single mutant revealed that these two genes might be involved in the same pathway. Recently, it was found that mutation of *MGO1* suppresses ectopic *WUS* activity and enhances stem cell defects in *wus* mutants [25]. Likewise, lack of CCS52A2 enhances the SAM defect in *wus-1*, which further supports a functional relationship between MGO1 and CCS52A2, and imply that their functions may converge to a common downstream process with *WUS* in stem cell regulation [25]. MGO1 and CCS52A2 proteins are both factors regulating cell cycle processes. It would be interesting to further investigate the molecular nature of the link between CCS52A2 and MGO1.

fas1 is another fasciated mutant that displays stem bifurcation, as do ccs52a2-3 and mgo1. *FAS1* encodes
In this study, we showed that mutations in the 
ccs52a2-3 fas1-1 epigenetic marks of their promoters [24]. The novel phenotypes of 
css52a2-3 fas1-1 double mutants demonstrate the two genes have a synergistic interaction in SAM development. The shared phenotypes of plants that have defects in either FAS1, MGO1 or 
CCS52A2 reveal a possibility that these three genes might be involved in the same downstream processes. Thus, FAS1 modulates the epigenetic states of genes involved in the G2 damage checkpoint, while MGO1 acts in DNA repair and maintains the expression of several epigenetically regulated genes [24,25]. Additionally, studies on nodule organogenesis indicate that CCS52A proteins mediate the degradation of mitotic cyclins in the proper phases of the cell cycle to inactivate CDKs, eventually blocking the G2 to M transition and thereby triggering endoreduplication [39]. Previous expression profile analysis of different cell samples in the SAM reveals an enrichment of DNA repair and chromatin modification pathways in stem cells, which suggests that the maintenance of flexible chromatin may facilitate the dynamic balance of gene expression during SAM development [40]. These results lend further evidence to the hypothesis that CCS52A might have a function related to triggering the G2/M transition, and that the signal pathways of CCS52A2, FAS1, and MGO1 are all involved in epigenetic maintenance and the activation of the G2 checkpoint.

Conclusions
In this study, we showed that mutations in the CCS52A2 gene disrupted the normal structure and function of the SAM in Arabidopsis. CCS52A2 modulates the expression domains of the meristem regulatory gene WUS and CLV3. Moreover, double mutant analyses illustrate that CCS52A2 synergistically interacts with SAM organizers. The cell cycle was disturbed in the absence of CCS52A2, as indicated by reduced expression of CYCB1; 1. CCS52A2 is involved in control of cell size and ploidy in differentiated cells during cell division. Furthermore, CCS52A2 is involved in chromatin remodeling with FAS1 and MGO1, which were previously reported to be involved in the activation of the G2 checkpoint and epigenetic maintenance. We propose that CCS52A2 regulates meristem organization, functions together with meristematic genes and cross-functions with chromatin regulators in cell cycle progression during SAM development.

Methods
Plant materials and growth conditions
ccs52a2-2 and ccs52a2-3 mutants (Col ecotype) in the CCS52A2 gene were obtained from the SALK_074403, SALK_073708 and SALK_001978, respectively, from the Arabidopsis Biological Resource Center (ABRC). wus-1, clv1-1, clv1-4, clv3-2, CLV::GUS marker line, WUS::GUS marker line (all in Ler ecotypes), mgo1-1 (Wassilewskija, Ws ecotype) and fas1-1 (Ler ecotype) were used. In the case of double mutants with wus-1, clv1-1, clv1-4, clv3-2, CLV::GUS marker line, and WUS::GUS marker line, we analyzed only plants that also harbored the erecta mutation. To ensure there were no background effects with the ccs52a2-3 phenotype, we crossed ccs52a2-3 with wildtype Ler accession, and selected ccs52a2-3 erecta plants for examination. ccs52a2-3 mutation showed no significant difference in phenotype between these two ecotypes. All of the plants for morphological analysis were grown in soil in growth chambers under 14-h-light/10-h-dark photoperiods (120μmolm−2 s−1) at 24/22 degrees C. Seedlings used for microscopic studies were grown on Murashige and Skoog (MS) medium on vertically oriented plates in growth chambers under the same light conditions. The F1 of all double mutants were morphologically similar to WT. Seeds from plants with the ccs52a2-3 phenotype in the F2 population were chosen to harvest, and clv, wus, fas1 or mgo1 phenotypic plants were selected as homozygous double mutants in the F3 population. In F1 and F2 population of the double mutant, the clv, wus and ccs52a2-2-3 mutation was also genotyped by sequencing, which were carried out with the following primers: DWUSL: 5’-CTACACCGGTTGATGTGATC-3; DWUSR: 5’-TCATGCAAGCTCAGGTACTG-3; DCLV1L: 5’-GA TTCTTTGTATGATCCGG-3; DCLV1R: 5’-ACATCAC TCTTCTCGTCCAC-3; ccs52a2-3 mutation was genotyped by checking the PCR product in agarose gel with primers: 35R-2: 5’-GGTGATTAGTTCATCCATCCATCCGTTATAC-3; DWUSL: 5’-CTACACCGGTTGATGTGATC-3; ACTINR: 5’-GACAGTGATGATGCATG-3.

RT-PCR and Real-time PCR
RNA was extracted from various tissues using the Trizol method, and 2 μg of RNA was used with M-MLV reverse transcriptase (Promega) in an RT reaction to cDNA. The expression level of each gene was normalized against the expression level of ACTIN2. Reverse-transcript PCR was carried out with the following primers: CCS52A2R: 5’-TCGTAACACATCCATCATCT-3; CCS52A2F: 5’-TAAGACGGAAACGCGAGGT-3; and ACTINF: 5’-GGGATTAGAAGTTGGGAAT-3; ACTINR: 5’-CA ABGACGTAGTATGATG-3. Primers for qRT PCR reactions: qWUSR/F, qCLV3R/F were synthesized by previously report [41]; qCCS52A2F: 5’-ACACCGGCGACGAGTGA-3’; qCCS52A2R: 5’-AGCAGTGACCACCAGAGTGA-3’.
were screened from 12-day-old T1 generation by geno-
heterozygote plants by the floral dip method
strain GV3101 by electroporation and transformed into
mids were introduced into
excised from BAC T26M18 (provided by ABRC) with re-
plasmids were introduced into Col or ccs52a2-3 heterozygote by
the floral dip method [43,44].

Tissue sectioning and GUS staining
Tissue was fixed in FAA (3.7% formaldehyde: 50% etha-
ol: 5% acetic acid) overnight and embedded with Tech-
notiv 7100 (Heraeus Kulzer) following the protocol from
manufacturer. The sections were cut to 2um and stained
with 0.1% toluidine blue. The slides were washed with
70% and 100% ethanol and embedded with a coverslip
by neutral resins after drying in air. Histochemical GUS
was performed as described previously (Schoof et al.,
2000) with minor modifications (elongated the staining
time to 8–10 hours).

In Situ Hybridization
The shoot apices of 8-day-old seedlings and 28-day-old
plants were fixed for 3 h in FAA (3.7% formaldehyde: 50% ethanol: 5% acetic acid) solution after vacuum filtra-
tion and embedded in paraplast embedding medium (Oxford Labware, St. Louis, MO). Samples were sec-
tioned at 8um thickness and placed onto Probe-On Plus
microscope slides (Fisher Scientific). Tissue preparation,
hybridization and detection procedures were performed
as described [13,42]. All the probes were generated with
the DIG RNA Labeling Kit T7/SP6 (Roche) and T3 RNA
polymerase (Promega), following the manufacturer’s
instructions. The template of the CLV3 probe was the
first 291 bp of the CLV3 coding sequence and was
cloned into the pCRBlunt vector (Invitrogen). The
template of the WUS probe was the first 664 bp of the
WUS coding sequence and was cloned into the pBluescript
KS vector (Fermentas). The CCS2A2 probe was gener-
ated by cloning the 340 bp of 5’ UTR sequence into
pGEMTeasy (Promega), with the primers CCS25UL: 5’-
ACTCGAGTCACGAATCAA-3’ and CCS25UR: 5’-
AG-3’; qACTIN2F: 5’-GGCTCTCTTTAAACCCAAAG
GC-3’; qACTIN2R: 5’-CACACCATCACCAGAATCCA
GC-3’.

Construction of Transgenes
To generate the CCS2A2p::CCS2A2::GUS con-
structs, the 1.6 Kb promoter region of CCS2A2 was
amplified using primers PG1R: 5’-GTCAAGCCTTTGT
TAAACCCTTGAAGGCCTCG-3’ and PG1L: 5’-CCGCTCA
GATTACGTGTTCGTC ATCCAG-3’, digested with
Hind III and Xbal and inserted into pBI121. Then a
1.5Kb CDS region was amplified from cDNA using prim-
ers p1ATGL2: 5’-GGCTCTAGATGGAAGAAGAT
GAATCAAC-3’ and p1ATGR2: 5’TATGGAATCCACAC
CG GATTGTGGTT CT-3’, digested with BamHI and
Xbal and inserted into pBI121 after the promoter. All
the plasmids were introduced into Agrobacterium tume-
faciens strain GV3101 by electroporation and trans-
formed into wild type (Col) or ccs52a2-3 heterozygote
by the floral dip method [43,44].

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
YJL carried out all of the experiments (except as noted below), constructed all of the figures, and wrote the manuscript. WY participated in molecular cloning and promoter-GUS analysis. BBL isolated the xcm9 mutant and carried out preliminary mapping of the gene. XJZ participated in the screening the double mutant. KDL and MPR designed the experiments and supervised the work. All authors read and approved the final manuscript.

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