APC/C^{CCS52A} complexes control meristem maintenance in the *Arabidopsis* root

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Plant organs originate from meristems where stem cells are maintained to produce continuously daughter cells that are the source of different cell types. The cell cycle switch gene *CCS52A*, a substrate specific activator of the anaphase promoting complex/cyclosome (APC/C), controls the mitotic arrest and the transition of mitotic cycles to endoreduplication (ER) cycles as part of cell differentiation. *Arabidopsis*, unlike other organisms, contains 2 *CCS52A* isoforms. Here, we show that both of them are active and regulate meristem maintenance in the root tip, although through different mechanisms. The *CCS52A1* activity in the elongation zone of the root stimulates ER and mitotic exit, and contributes to the border delineation between dividing and expanding cells. In contrast, *CCS52A2* acts directly in the distal region of the root meristem to control identity of the quiescent center (QC) cells and stem cell maintenance. Cell proliferation assays in roots suggest that this control involves *CCS52A2* mediated repression of mitotic activity in the QC cells. The data indicate that the *CCS52A* genes favor a low mitotic state in different cell types of the root tip that is required for meristem maintenance, and reveal a previously undescribed mechanism for APC/C mediated control in plant development.

CDH1 | cell differentiation | endoreduplication | quiescent center | stem cells

Plant growth and development depend on the persistent activity of meristems, allowing continuous postembryonic organogenesis. In the root tip, meristem maintenance is controlled by different mechanisms that involve the maintenance of stem cells in the root meristem (RM) and spatial control over mitotic exit at the RM-elongation zone (EZ) border.

In the distal RM, stem cells are maintained in an undifferentiated state by the quiescent center (QC) cells (1). The QC represents a center of mitotic inactive cells resting in an extended G_1 phase (2). The stem cells around the QC divide according to strict spatial rules, and provide cell progenies that detach from the QC and differentiate into different root cell types (3). The auxin-PLETHORA (PLT) pathway provides positional information to set up the QC and surrounding stem cells whose activities depend on WOX5 and SHORT ROOT (SHR)-SCARECROW (SCR) transcription factors (4–7).

As cells reach the RM-EZ border, they start to expand and terminally differentiate. Recently, it has been demonstrated that the spatial boundary of the RM and EZ is controlled by the rate of meristematic cell differentiation at this border (8). The transition involves exit from the mitotic cycle to the endocycle (9). In eukaryotes, endoreduplication (ER) onset requires inhibition of mitotic cyclin-dependent kinase (cdk) activities (10–12). This inhibition can be achieved by multiple mechanisms, but mostly by the degradation of mitotic cyclins by the anaphase promoting complex/cyclosome (APC/C) (13–15). The APC/C is an ubiquitin ligase that regulates cell cycle progression from metaphase to S phase by targeted degradation of numerous cell cycle proteins (16). In human and animal systems, this complex has received much attention due to its implication in human diseases and broad therapeutic applications (17).

Substrate specificity of the APC/C complex is achieved by WD40 activator proteins such as CDH1. CDH1/FZR/SRW1 controls APC/C activity during G_1 progression, ER, and cell differentiation in yeast and animal cells (13, 14, 18, 19). In plants, information on the APC/C is still limited. The plant CDH1/FZR/SRW1 orthologue, CCS52A has been shown to control ER in *Medicago* leaf petioles and nodules (15, 20), and in *Arabidopsis* rosette leaves (21). *Arabidopsis* has 2 CCS52A isoforms (22). In this work, we studied the function of CCS52A1 and CCS52A2 in root development, and show that they regulate meristem maintenance by different mechanisms.

Results

***CCS52A1* and *CCS52A2* Regulate Meristem Size in Roots.** In the seedling stage, the *Arabidopsis ccs52a1* and *ccs52a2* mutations resulted in strikingly different and adverse root growth phenotypes. The *ccs52a1* roots were longer than WT roots, whereas root growth was strongly impaired in *ccs52a2* seedlings (Fig. 1 *A* and *B*).

Root growth depends on the production of new cells in the RM and their subsequent expansion and differentiation. This balance determines meristem size. Meristems in the *ccs52a1* roots contained more dividing cells, and were in average 50 μm longer than in the WT roots (Fig. 1 *C* and *D*), indicating that mitotic activity was preserved in a longer region from the QC cells than in the WT roots. Also, the ER index (mean number of duplication cycles per nucleus) was reduced in *ccs52a1* roots, indicating a delayed onset of ER (Fig. 1*E*).

In contrast, the RM was significantly smaller, and contained less dividing cells in *ccs52a2* than in WT or *ccs52a1* roots (Fig. 1 *C* and *D*). However, absence of *ccs52a2* had no measurable effect on the endoploidy level of the root (Fig. 1*E*). Thus, *CCS52A2* mediated control over root development did not involve ER.

Reintroduction of the WT *CCS52A1* or *CCS52A2* genomic

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. At4g22910/NM_118420.4 (*CCS52A1*) and At4g11920/NM_117262.2 (*CCS52A2*)].

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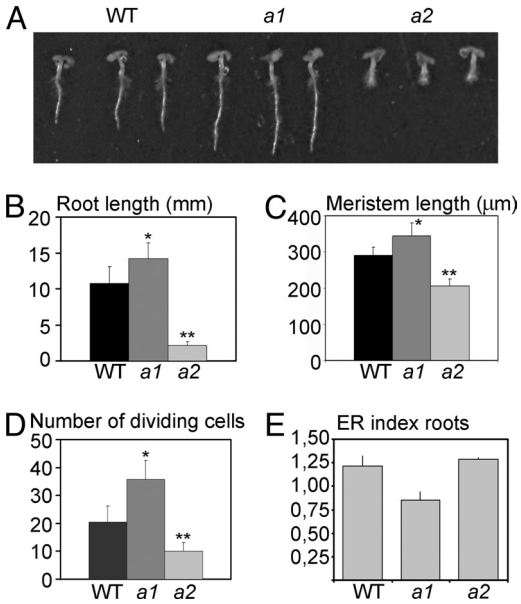

Fig. 1. Root growth in the *ccs52a1* (*a1*) and *ccs52a2* (*a2*) mutants. (*A*) At 4 days, root growth is slightly stimulated in *ccs52a1* mutants and strongly reduced in *ccs52a2* seedlings. (*B–D*) Root length (*B*), meristem size (*C*), and number of dividing cells per meristem (*D*) are inversely affected in *ccs52a1* and *ccs52a2* roots. Student's *t* test: *, $P = 0.0012$ (*B*), $P = 0.0004$ (*C*), $P < 0.0001$ (*D*); **, $P < 0.0001$ (*B–D*). All measurements were done on 25–30 seedlings (mean ± SE). (*E*) The ER index of 4-day-old roots is reduced in the *ccs52a1* mutants compared with WT and *ccs52a2* roots. Samples were prepared from 40 to 50 roots, and measurements were repeated 3 times (mean ± SE).

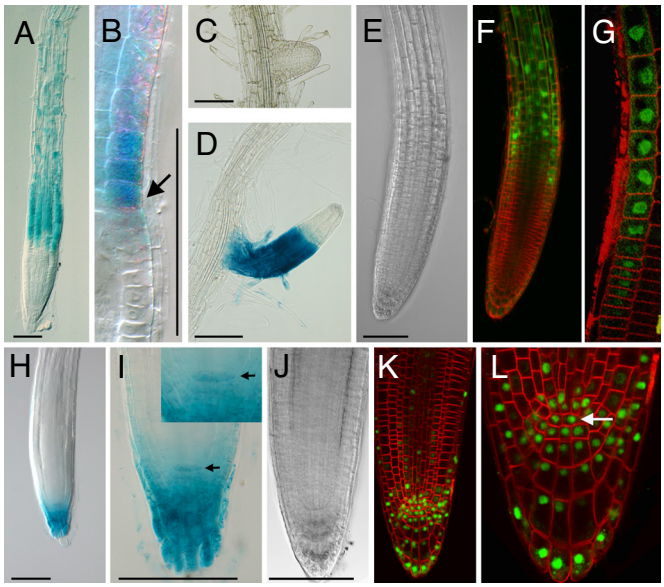

Fig. 2. *CCS52A1* and *CCS52A2* are expressed differently along the root growth axis. (*A–D*) *CCS52A1* promoter driven GUS expression occurs in the EZ (*A*), and appears in the first cells that start to expand above the RM (arrow in *B*). In lateral roots, GUS staining is absent in lateral root primordia (*C*), whereas it appears once a differentiation zone is formed (*D*). (*E–G*) CCS52A1-GFP protein fusion labels nuclei in the EZ of FM4-64 stained roots. (*H* and *I*) GUS expression from the *CCS52A2* promoter occurs mainly in the columella and QC cells, and is also present in stem cells. (*J–L*) CCS52A2-GFP protein fusion is present in the nuclei of distal cells of the root tip. Arrows in *I* and *L* indicate the QC cells. (Scale bars: 100 μm.)

region rescued the *ccs52a1* and *ccs52a2* mutant root growth defects, respectively, as well as the dwarf plant phenotype of the *ccs52a2* mutant (Fig. S1 *A–C*). Also, plants expressing RNAi constructs targeted to the *CCS52A2* transcripts phenocopied the *ccs52a2* T-DNA insertion mutants, confirming that the observed root phenotype was the result of a nonfunctional *CCS52A2* gene (Fig. S1 *D* and *E*).

***CCS52A1* and *CCS52A2* Are Expressed at the Proximal and Distal Borders of the RM, Respectively.** To understand the different phenotypes and functions of the *CCS52A* genes in roots, we studied their expression by promoter β-glucuronidase (GUS) fusions. In 4-day-old seedlings, *CCS52A1* was expressed in the differentiating cell files of the root EZ (Fig. 2*A*). In the first postmitotic cells that showed signs of rapid elongation (Fig. 2*B*), gene expression was activated at the RM-EZ border. In lateral roots, GUS staining was found once the lateral root cells started to elongate (Fig. 2 *C* and *D*). Thus, expression of *CCS52A1* correlated with the transition from the mitotic state to cell differentiation and the occurrence of ER in the root EZ. This finding was in line with the observed delay in mitotic exit and ER in *ccs52a1* mutant roots. In contrast to *CCS52A1*, the *CCS52A2* promoter was active in the root tip, predominantly in the root cap and the QC cells, and a nonnegligible activity was also found in the surrounding stem cells (Fig. 2 *H* and *I*). To confirm the GUS expression analysis, we generated transgenic plants that expressed GFP C-terminal to the *CCS52A1* or *CCS52A2* genomic region, comprising the endogenous promoter, 5′ UTR, introns and exons (Fig. 2 *E–G* and *J–L*). GFP signal was present in the EZ for CCS52A1 and in the QC cells, surrounding stem cells and columella for CCS52A2, in agreement with the GUS expression analysis. Also, these fusion proteins were functional, because they rescued the mutant phenotypes, and could, thus, be used for adequate subcellular localization. Both CCS52A1 and CCS52A2 were nuclear, which corresponded to the reported active form of the yeast and human orthologues (23, 24).

***CCS52A2* Is Required for Meristem Organization and Maintenance of QC Identity and Stem Cells.** In the *ccs52a2* roots, differentiated root hair cells emerged in close vicinity of the meristem, indicating that the RM was consumed (Fig. 3 *B* and *D*). Also, the regular differentiation pattern of root cell types was disturbed, and cells were irregular in size and shape compared with WT (Fig. 3 *E* and *F*). In the stem cell region, cells were disorganized, and the QC and stem cells were morphologically indistinguishable (Fig. 3*F Inset*). Also, distal differentiation markers such as starch granules appeared more proximal in the RM, indicating consumption of columella stem cells (CSC) (Fig. 3*H*). To test whether *CCS52A2* contributes to QC activity, the QC184 marker was used, which in WT roots showed a strong and highly localized expression in the QC (Fig. 3*G*). In the *ccs52a2* roots, the QC184 expression was either absent or strongly diminished and diffuse (Fig. 3*H*), indicating that CCS52A2 is essential for proper function of the QC. This finding is in agreement with the observed atypical cellular organization of QC and stem cells in the mutant root (Fig. 3*F Inset*), and together with the appearance of root hairs just above the RM, indicates that CCS52A2 is essential for QC identity and maintenance of stem cells and meristem size.

Defects in meristem organization and maintenance were also observed in the shoot apical meristem (SAM), where GUS expression from the *CCS52A2* promoter was found (Fig. S2). In contrast to *ccs52a1* and WT, the *ccs52a2* adult plants were severely stunted (Fig. S2 *I* and *J*), indicating that the CCS52A2 function in apical meristems is essential for proper plant development.

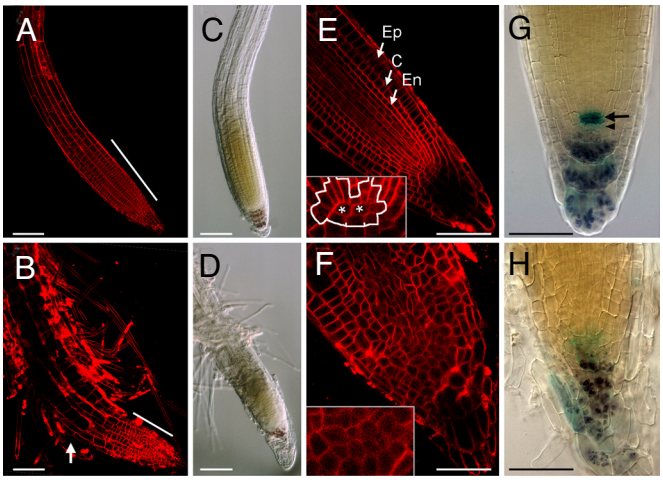

Fig. 3. Defects in meristem organization and maintenance in *ccs52a2* mutant roots. (*A* and *B*) Cellular organization in 4-day-old WT (*A*) and *ccs52a2* (*B*) FM4-64 stained roots. Bars (*A* and *B*) and arrow (*B*) indicate the RM and the position of the first root hairs, respectively. (*C* and *D*) Lugol staining of cleared WT (*C*) and *ccs52a2* (*D*) roots. (*E* and *F*) Cell organization in the RM is disturbed in *ccs52a2* (*F*) compared with WT (*E*). Ep, epidermis; C, cortex; En, endodermis. Insets show the QC (asterisks) and surrounding stem cells (white contours) in WT and the corresponding region in mutant RMs. (*G* and *H*) QC184 GUS expression in the QC of WT (arrow in *G*) is strongly reduced in *ccs52a2* (*H*). Arrowhead indicates the CSC in WT (*G*). Starch granules are visualized by lugol staining. [Scale bars: 100 μm (*A–D*), 50 μm (*E–H*).]

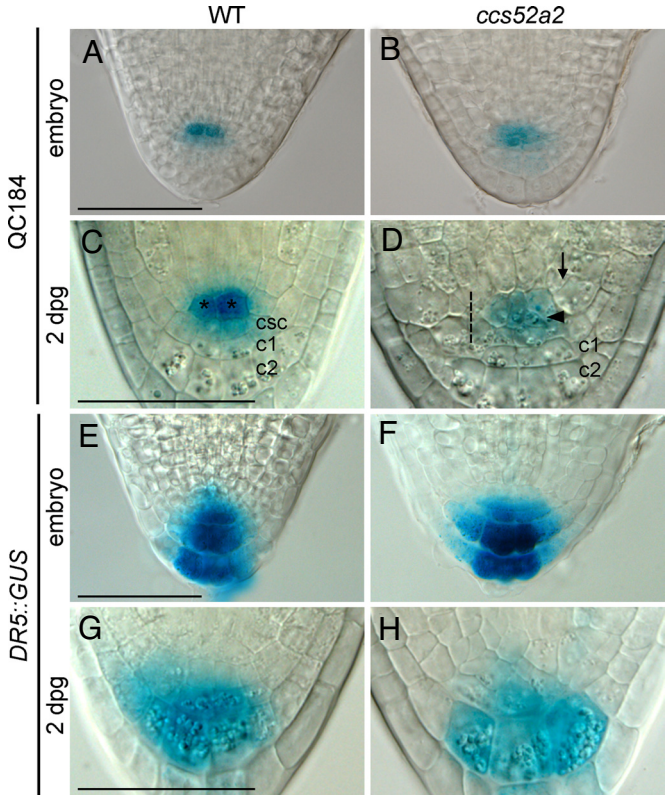

Fig. 4. CCS52A2 specifically affects QC and stem cells after germination. (*A*, *C*, *E*, and *G*) WT and (*B*, *D*, *F*, and *H*) *ccs52a2* RMs. (*A–D*) Expression of the QC184 marker is intact in mature *ccs52a2* embryos (*B*), and becomes diffuse at 2 dpg (*D*). The arrow in *D* denotes a premature periclinal cell division of the cortex/endodermis initial, and the arrowhead an irregular cell division in the QC area. The dotted line indicates cells with starch granules at the position of the CSC and QC. (*E–H*) The auxin maximum as indicated by *DR5*::*GUS* expression remains intact in *ccs52a2* both in the embryo (*F*) and at 2 dpg (*H*). (Scale bars: 50 μm.)

The *ccs52a2* RM Phenotype Originates in the Stem Cell Region After Germination. The expression analysis suggested that CCS52A2 specifically affects cells in the stem cell region. However, defects in meristem organization and stem cell maintenance may result from deregulated cell cycle events throughout the RM. To distinguish between these possibilities, we followed the *ccs52a2* RM phenotype during the first days after germination.

In mature *ccs52a2* embryos, the cellular organization in the RM and the expression of the QC184 marker were similar to WT, indicating that CCS52A2 acts postembryonically (Fig. 4 *A* and *B*). The first visible defects in RM organization were observed at 2 days postgermination (dpg). In all tested roots, cells in the stem cell region became disorganized, whereas the rest of the RM was not affected (Fig. 4*D*; Fig. S3*A*). The most consistent effect was the deformation of QC cells and a diffuse QC184 expression domain. Also, irregular cell divisions were observed in the QC and stem cells (Fig. 4*D*). Starch granules that at this stage normally only occur in the c1 and c2 differentiated columella cell layers, appeared in the *ccs52a2* RM more proximal, in the cells corresponding to the CSC layer and the diffuse QC184 domain (Fig. 4 *C* and *D*, dotted line). These observations indicate that already at this stage QC identity and stem cell maintenance are lost. At 3 dpg, the whole distal part of the RM was disorganized, and at 4 dpg, cellular organization over the whole RM was disrupted, and expression of the QC184 marker was almost abolished (Fig. 3 *F* and *H*; Fig. S3 *B* and *C*).

These data suggest that CCS52A2 acts directly in the distal RM to maintain QC identity and stem cells after germination, and does not primarily affect cell divisions in the proximal meristem.

Auxin Accumulation Affects the *CCS52A2* Expression Pattern in the RM. In the distal part of the root, the auxin maximum is required for QC and stem cell specification (25, 26). Because the *CCS52A2* expression domain largely corresponds with the auxin maximum, we tested whether *CCS52A2* is required for the localization or perception of this auxin maximum. Both in the embryo and at 2 dpg, when the first defects occurred in the stem cell region of *ccs52a2* roots, the auxin maximum was intact and similar between WT and *ccs52a2* (Fig. 4 *E–H*). Only at 3 and 4 dpg, the *DR5*::*GUS* expression expanded more proximal (Fig. S3 *B* and *C*). These findings suggest that the *ccs52a2* mutation does not primarily affect the localization of the auxin maximum.

To test whether the auxin response was normal, we treated seedlings with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), which caused a similar induction of *DR5*::*GUS* in WT and in *ccs52a2* roots (Fig. 5 *A* and *B*). Also, the expression of several early auxin response genes after treatment with 10 μM indoleacetic acid (IAA) was similarly induced in WT and *ccs52a2* mutants, indicating that the auxin response was not affected in *ccs52a2* roots (Fig. 5*E*).

Inversely, we asked whether *CCS52A2* expression depends on auxin accumulation by shifting the auxin maximum more proximal in the RM with naphthylphthalamic acid (NPA) (25). Treatment of seedlings expressing *CCS52A2*::*GUS* with NPA resulted in a proximal expansion of the GUS signal in the RM (Fig. 5*D*), which was not observed in control DMSO treated roots (Fig. 5*C*). Also, auxin treatments resulted in a spreading of the defined distal *CCS52A2* expression pattern into the proximal RM without increasing the *CCS52A2* expression level (Fig. S4). The results indicate that auxin accumulation merely contributes to the pattern of *CCS52A2* expression in the root tip.

Expression of RM Organizers in *ccs52a2*. QC specification and stem cell maintenance depend on the transcription factors *PLT* and

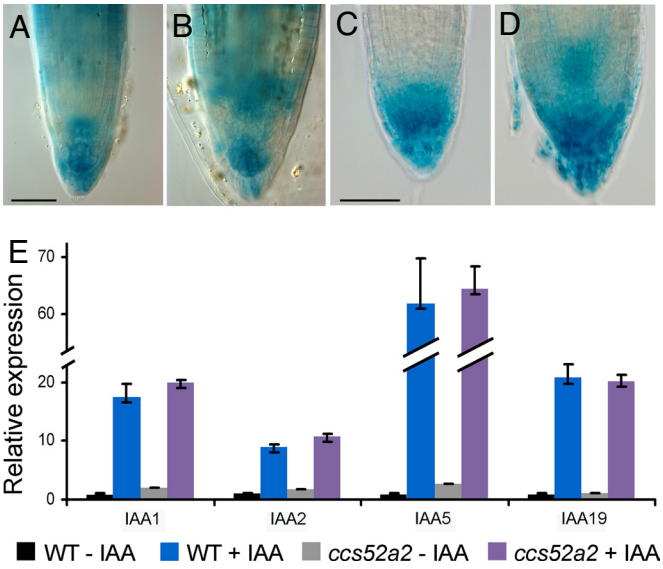

Fig. 5. The *CCS52A2* expression pattern is affected by auxin accumulation. (*A* and *B*) Increased expression of *DR5*::*GUS* in response to a 5-h treatment with 5 μM 2,4-D in WT (*A*) and *ccs52a2* (*B*) roots. (*C* and *D*) Promoter *CCS52A2* driven GUS expression (*C*) expands proximally in response to treatment with 5 μM NPA (*D*). (Scale bars: 50 μm.) (*E*) Induction of auxin response gene expression as revealed by quantitative RT-PCR after 2-h treatment with 10 μM IAA in WT and *ccs52a2* roots. Histogram represents the quantification of specific PCR amplification products normalized to the constitutive *ACT2* expression.

WOX5. We studied the expression pattern of these genes by whole mount in situ hybridization in *ccs52a2* RMs at 3 dpg, when the whole distal part of the RM is disorganized, as shown in Fig. S3*B*.

The *PLT1* gene is expressed in response to auxin accumulation in the distal part of the RM (Fig. 6*A*), where it provides positional information to set up the stem cell niche (7). In *ccs52a2* roots, the *PLT*1 expression domain was smaller and often shifted proximally in the root tip (Fig. 6*B*). Similar shifts occurred in *DR5*::*GUS* expression at this stage (Fig. S3*B*), indicating that *PLT1* expression still responds to auxin accumulation in absence of *CCS52A2*.

Recently, *WOX5* was shown to be required for stem cell maintenance (4). *WOX5* expression was found in the QC cells of WT RMs and in a corresponding region in *ccs52a2* roots that was occasionally irregular in size (Fig. 6 *C* and *D*). Because QC and stem cells are not maintained in *ccs52a2* RMs, these data show that *PLT1* and *WOX5* are insufficient to maintain a stem cell region in absence of *CCS52A2*.

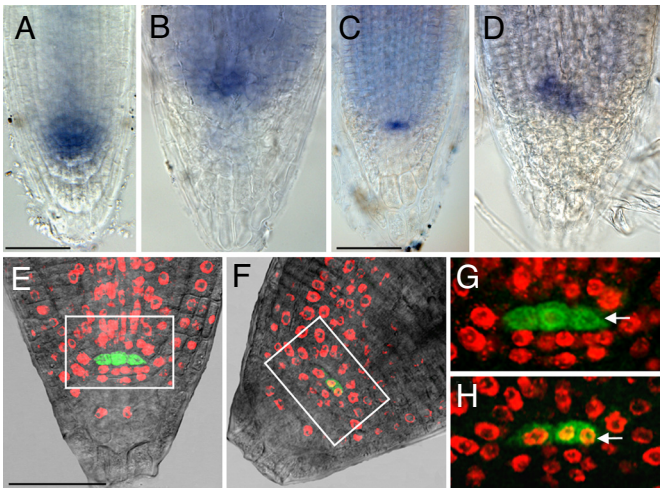

Fig. 6. Altered QC cell properties in *ccs52a2* mutant roots. (*A–D*) Whole mount in situ hybridization of the RM organizers *PLT1* (*A* and *B*) and *WOX5* (*C* and *D*) in WT (*A* and *C*) and *ccs52a2* roots (*B* and *D*). (*E–H*) EdU incorporation assays in WT (*E* and *G*) and *ccs52a2* (*F* and *H*) RMs [*G* represents the outlined area in *E*, and *H* represents the outlined area in *F* (Magnification: 2×)]. Red fluorescent EdU positive nuclei in the QC cells of *ccs52a2* indicate that cell division was activated in these cells in contrast to WT. The *WOX5*::*ER-GFP* marker was used to identify the QC cells (arrows). (Scale bars: 50 μm.)

Mitosis Is Activated in QC Cells of *ccs52a2* RMs. QC cells are considered as mitotic silent cells that divide only occasionally to self-renew (2). Because CDH/FZR type activators are important regulators of cell cycle progression, we checked whether cell cycle activities were altered in the QC cells of *ccs52a2* mutant roots. To this end, we cultured 1-dpg seedlings for 24 h in the presence of EdU, a nucleoside analogue of thymidine. Incorporation of EdU in the nuclei is indicative for S-phase progression and, thus, cell division in the RM. After coupling of the EdU with the Alexa Fluor 647 substrate, we found that in WT, most cells of the RM, including the stem cell region, had incorporated EdU during this 24-h growth period. Only the QC cells marked with green fluorescence of *WOX5*::*ER-GFP* remained free of red fluorescent nuclei, illustrating their mitotic inactivity (Fig. 6 *E* and *G*). In contrast, positive nuclei were frequently observed in the *ccs52a2* QC cells (1/2 in *ccs52a2* vs. 1/7 in WT, $n = 40$) (Fig. 6 *F* and *H*), indicating that mitosis is activated in the QC cells in absence *CCS52A2*.

***CCS52A* Specificity in Root Development Is Mainly Transcriptionally Regulated.** In roots, *CCS52A1* and *CCS52A2* are expressed in different regions along the root growth axis that correspond to their proposed functions in regulating ER at the RM-EZ border and QC identity/stem cell maintenance in the distal RM, respectively. The different expression patterns suggest that functional divergence of the 2 genes could depend on their respective promoter activities. To test this idea, the *CCS52A1* gene was introduced in the *ccs52a2* mutant under control of the *CCS52A2* promoter. This construct restored the root growth and cellular organization of the RM in the *ccs52a2* seedlings (Fig. S5 *C* and *F*). Also the aerial parts of mature plants were recovered and were similar to WT plants (Fig. S5 *G* and *I*), indicating that CCS52A1 and CCS52A2 proteins are functional homologues, and that their functional divergence in root development arises from differences in their promoter activities.

Discussion

Promoter Activity Determines *CCS52A* Functional Specificity. *Arabidopsis* is unique in having 2 *CDH1*/*FRZ*/*CCS52A* genes. Here, we show that these APC/C activators have pivotal roles during root development. Remarkably, the different root phenotypes provoked by the absence of *CCS52A1* and *CCS52A2*, strongly correlated with their complementary expression patterns in roots. Also, expression of the *CCS52A1* gene from the *CCS52A2* promoter complemented the *ccs52a2* phenotype. These data suggest that the functional diversity observed between the 2 *CCS52A* genes mainly depends on their different promoter activities. This idea is also supported by the fact that only the *CCS52A2* promoter, but not *CCS52A1* is controlled by the atypical E2F transcriptional repressor E2Fe/DEL1 (21).

CCS52A1 and CCS52A2 interact with APC/C core subunits *in planta*, indicating that they are functional components of the *Arabidopsis* APC/C (22). Similarly, the APC/C core subunit CDC27/APC3 is also represented by 2 isoforms in *Arabidopsis*, CDC27A and CDC27B/HOBBIT (HBT), which display differences in gene expression and function (27, 28). Together, these data show that multiple APC/$C^{CCS52A\text{-}CDC27}$ complexes exist in

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Arabidopsis with different localizations and functions during plant development. This situation is in contrast to yeast, *Drosophila*, and vertebrates, where all APC/C subunits, including the activators, are represented by single copy genes and functional plasticity of the APC/C arises from differences in subunit composition and stochiometry (29–31).

***CCS52A1* and *CCS52A2* Regulate Meristem Size Through Different Mechanisms.** In roots, both *CCS52A1* and *CCS52A2* regulate meristem size, although through different mechanisms. *CCS52A1* controls meristem size by stimulating ER in the EZ. The gene was activated in the first postmitotic cells of the root EZ, where its activity restricted meristem size and, thus, contributed to the spatial determination of the RM-EZ border.

In contrast, *CCS52A2* regulated meristem maintenance and structure by acting directly in the QC and stem cells of the RM. This finding is supported by the observation that expression of *CCS52A2* and the first visible defects of the *ccs52a2* mutation were restricted to the corresponding region of the RM. The atypical cell morphology and the appearance of starch granules in this region, loss of the highly localized QC184 expression domain, and mitotic activation of QC cells at 2 dpg indicated that *CCS52A2* is crucial for QC identity and stem cell maintenance after germination. Irregularities in the cellular organization of the proximal RM and in the auxin maximum only occurred at later stages, and therefore, were secondary defects of the *ccs52a2* mutation.

Major regulators of QC specification and stem cell maintenance, such as *PLT1* and *WOX5*, were still expressed in *ccs52a2* RMs. Absence of a stem cell region in *ccs52a2* RMs indicates that these regulators were insufficient to maintain QC identity and stem cells in absence of *CCS52A2*, confirming that *CCS52A2* is a crucial factor in the maintenance of QC identity and stem cells.

***CCS52A2* Function Provides a Previously Undescribed Mechanism for APC/C Mediated Control over Plant Development.** Previous reports on APC/C subunits in plants have illustrated their requirement for cell division, cell expansion, ER, meristem formation, and gametogenesis (27, 28, 32–34). Also, the CCS52A type activators have been shown to regulate ER and cell size in plants, which in *Medicago* was an integral part of the symbiotic cell differentiation program (15, 20, 21). The data presented here illustrate the functional specialization of the *Arabidopsis CCS52A1* and *CCS52A2* in roots, and show a mechanism for APC/C mediated control in plant development.

Defects in organization and maintenance of the shoot and RM have also been observed in *hbt* mutants (27, 28). In contrast to CCS52A2, the HBT protein is present throughout the RM. Weak alleles and clonal analysis of the *HBT* gene indicated that the HBT primary function in the root is to mediate cell division and cell expansion (28, 34). In these aspects, CCS52A2 differs significantly from HBT and other APC/C subunits, because it is specifically required in the distal area of the RM for QC identity and stem cell maintenance.

APC/C$^{CDH1/FZR/CCS52A}$ Complex As Cell Cycle Regulator or Director of Cell Fate? In animal systems, both cell cycle progression and regulation of differentiation factors are attributed functions for CDH/FZR type activators. CDH1/FZR controls G_1/S transition, and maintains G_1 arrest by degradation of cell cycle proteins such as CDC6, SKP2, and mitotic cyclins (14, 35, 36). In *Arabidopsis* roots, the QC forms a center of mitotic inactive cells that are kept in a prolonged G_1 phase, which is required for their maintenance (2). Possibly, CCS52A2 maintains QC identity by keeping their mitotic activity low. In support of this hypothesis, we found that cell division was activated in the QC cells in absence of *CCS52A2*. As a result, QC identity was lost, and stem cell and meristem maintenance were disrupted.

In *Caenorhabditis elegans*, FZR has been shown to have an inhibitory effect on the proliferation of seam cells, a stem cell-like lineage (37). In *Arabidopsis*, the stem cells in the RM divide less frequently than the remaining meristematic cells (3). Because the EdU incorporation assays did not allow us to quantify mitotic events in the stem cells, we cannot rule out at this stage whether *CCS52A2* also directly affects mitotic activities in the stem cells.

Interestingly, the retinoblastoma protein related (RBR) protein has been shown to regulate stem cell maintenance in the *Arabidopsis* RM (38). RBR and its related protein in animal cells (pRB) have conserved functions in regulating G_1/S transition and cell cycle exit (38, 39). Recently, interaction of pRB with CDH1 in HeLa cells was shown to enhance APC/C^{Cdh1}-mediated degradation of SKP2 during G_1 exit (19). It would be interesting to find out whether a similar link exists between RBR and APC/C^{CCS52A2} in *Arabidopsis* that is required for stem cell maintenance in the RM.

Studies in *Drosophila* and mammalian cells indicate a more direct involvement of CDH1-type activators in regulating cell differentiation. For example, murine CDH1 coordinates lens differentiation by targeting the transcriptional corepressor SnoN for degradation on TGF-β signaling (40). In *Drosophila*, FZR contributes to glial-neuron cell fate by targeting loco for degradation (41). Similarly, CCS52A2 function could promote the QC and/or stem cell fate. In this scenario, mitotic activation of the QC cells would then be the indirect effect from the loss of QC cell fate.

Identifying target genes through which CCS52A2 maintains QC identity will distinguish between the 2 scenarios. The promoter swap rescue experiment supports the first, because it implies similar cell cycle functions for CCS52A1 and CCS52A2, namely favoring a low mitotic state at the proximal border of the RM and in the QC, respectively, but with a different functional outcome.

Materials and Methods

Plasmids and Constructs. To construct promoter::GUS fusions, the promoter regions of the respective genes were cloned into pGEMTeasy vectors and transferred as SalI/NcoI fragments into pCAMBIA1381z vectors (for primers used, see Table S1). For complementation assays, the complete genomic regions of *CCS52A1* and *CCS52A2* were cloned via pENTR/D-TOPO vectors (Invitrogen) into the Gateway vectors pHWGL7 (*CCS52A1*) and pBWGL7 (*CCS52A2*) (42). To express the *CCS52A1* gene from the *CCS52A2* promoter, the corresponding PCR products were fused in a sewing PCR, cloned into the pBWGL7 vector.

For translational GFP fusions, genomic regions without stop codons of *CCS52A1* and *CCS52A2* were cloned into the pK7FWG2 and the pB7FWG2 vectors, respectively (42), in which the 35S promoter was removed with a SacI/SpeI digest.

Plant Stocks and Manipulations. The *ccs52a1* (*ccs52a1-2*) and *ccs52a2* (*ccs52a2-1*; SALK_001978) mutants of *Arabidopsis thaliana* are Garlic Syngenta and SALK T-DNA lines, respectively (21). The QC184 promoter trap line was obtained from the Nottingham Stock Center (stock number CS9209) and the INRA T-DNA collection, respectively. The *WOX5*::*ER-GFP* line was provided by Ben Scheres. Plants were grown on ½ MS medium, containing 1% sucrose, pH 5.7 and 0.8% agar, and placed under 18- and 6-h day/night cycle at 22 °C.

RNA Expression Analysis. For RT-PCR, RNA preparation, cDNA synthesis, and real-time RT-PCRs were performed according to standard protocols. For detailed information, see *SI Materials and Methods*.

Whole mount in situ hybridization was performed as described (43). Probes for *PLT1* and *WOX5* were prepared as published (4, 7).

Flow Cytometry. Nuclear DNA content (15) was measured on 50 roots per sample with a Partec CyFlow SL3 cytometer and the FlowMax software. The ER index was calculated as described (21).

Histology and Microscopy. For whole mount and starch granule visualization, roots were stained with 1% lugol and cleared in choral hydrate (44). GUS

activity in transgenic marker lines was visualized by staining 2–16 h at 37 °C according to ref. 44, using 0.5 mM $K_4Fe(CN)_6$ and 0.5 mM $K_3Fe(CN)_6$.

Root length was measured from the root tip until the root/hypocotyl border using ImageJ software. Meristem length was measured until the first cells showing signs of rapid elongation, and the number of dividing cells per RM was counted by the number of mitotic figures per RM after staining with 2.5 μg/mL DAPI in PBS for 30 min.

For EdU incorporation assays, seedlings were grown in liquid MS containing 10 μM EdU (Click-iT EdU Alexa Fluor 647 Imaging Kit; Invitrogen). After growth, seedlings were fixed in 3.7% formaldehyde in PBS, pH 7.4 for 15 min. Samples were then washed in 3% BSA in PBS, treated with 0.5% Triton in PBS for 20 min, and washed again. Coupling of EdU to the Alexa fluor substrate occurred in the dark in the Click-iT reaction mixture, prepared according to manufacturer's instructions, and observations were done under the confocal microscope.

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