QUIRKY regulates root epidermal cell patterning through stabilizing SCRAMBLED to control CAPRICE movement in Arabidopsis

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SCM, a leucine-rich repeat receptor-like kinase, is required for root epidermal cells to appropriately interpret their location and generate the proper cell-type pattern during Arabidopsis root development. Here, via a screen for scm-like mutants we describe a new allele of the QKY gene. We find that QKY is required for the appropriate spatial expression of several epidermal cell fate regulators in a similar manner as SCM in roots, and that QKY and SCM are necessary for the efficient movement of CPC between epidermal cells. We also show that turnover of SCM is mediated by a vacuolar degradation pathway triggered by ubiquitination, and that QKY prevents this SCM ubiquitination through their physical interaction. These results suggest that QKY stabilizes SCM through interaction, and this complex facilitates CPC movement between the epidermal cells to help establish the cell-type pattern in the Arabidopsis root epidermis.
The specification of distinct cell fates is a critical process in the development of multicellular organisms. In many cases, cell fate decisions are influenced by the relative position of a cell to its neighbors, indicating that cell–cell communication is crucial. A simple model system for the study of cell fate specification is found in the Arabidopsis root epidermis, which is composed of two cell types, root hair-bearing cells (hair cells) and non-hair cells, that are patterned in a position-dependent manner. The epidermal cells located outside a periclinal cortical cell wall (N position) contacting a single cortical cell differentiate into non-hair cells, while the epidermal cells located over an anticlinal cortical cell wall (H position) contacting two underlying cortical cells differentiate into hair cells.

Many genes are known to influence cell fate specification in the Arabidopsis root epidermis. GLABRA2 (GL2), which encodes a homeodomain-leucine zipper (HD-Zip) protein, is expressed in developing epidermal cells in the N position and specifies the non-hair cell fate. WEREWOLF (WER) and CAPRICE (CPC) encode an R2R3 MYB-type transcription factor and an R3 single homeodomain-leucine zipper (HD-Zip) protein, is expressed in developing epidermal cells in the N position and specifies the hair cell fate. Interestingly, WER is a direct positive regulator of CPC which triggers SCM degradation in vacuoles. To understand how SCM functions in root epidermal cell patterning, we used a genetic approach to search for new regulators acting in the SCM signaling pathway. We identified a mutant with an scm-like root mutant phenotype, and found that it is an allele of the QUIRKY (QKY) gene. QKY acts upstream of SCM which triggers SCM degradation in vacuoles.

### Results

#### Isolation of a new allele of qky

We screened a population of ethylmethanesulfonate (EMS) mutagenized seeds harboring the GL2p:GFP marker and isolated a mutant showing defects in position-dependent root epidermal patterning and expression of the GL2p:GFP marker (Supplementary Fig. 1a, b and Table 1). We confirmed that this phenotype is caused by a single nuclear recessive mutation by analyzing the F1 and F2 offspring from a cross with wild-type plants. Through a bulk segregant analysis, we found that the mutation is linked to a marker (nga111) on chromosome 1, which is near the QKY gene previously reported to affect root epidermal cell patterning. Allelism testing (by crossing this new mutant with qky-822 and with scm-216) showed

### Table 1 Specification of cell types in the root epidermis

| Genotype         | H at H pos (%) | N at H pos (%) | H at N pos (%) | N at N pos (%) |
|------------------|----------------|----------------|----------------|----------------|
| Wild type        | 98.0 ± 1.0     | 2.0 ± 1.0      | 0.0 ± 0.0      | 100.0 ± 0.0    |
| qky-16           | 74.5 ± 4.0     | 25.5 ± 4.0     | 18.2 ± 3.2     | 81.8 ± 3.2     |
| qky-16 QKYp:QKY  | 95.3 ± 0.6     | 4.7 ± 0.6      | 4.3 ± 1.2      | 95.7 ± 1.2     |
| qky-16 QKYp:QKY-GFP | 98.0 ± 1.0     | 2.0 ± 1.0      | 0.3 ± 0.6      | 99.7 ± 0.6     |
| qky-16 CO2p:QKY-GFP | 71.5 ± 5.5     | 28.5 ± 5.5     | 21.4 ± 6.2     | 78.6 ± 6.2     |
| qky-16 SHRp:QKY-GFP | 69.4 ± 4.5     | 30.6 ± 4.5     | 22.5 ± 6.4     | 77.5 ± 6.4     |
| qky-16 SCRp:QKY-GFP | 72.3 ± 4.0     | 27.7 ± 4.0     | 28.2 ± 5.8     | 71.8 ± 5.8     |
| scm-2            | 96.0 ± 1.5     | 4.0 ± 1.5      | 0.0 ± 0.0      | 100.0 ± 0.0    |
| qky-16 scm-2     | 66.6 ± 6.2     | 33.4 ± 6.2     | 24.5 ± 5.3     | 75.5 ± 5.3     |
| RHD3p:QKY        | 97.0 ± 1.5     | 3.0 ± 1.5      | 0.0 ± 0.0      | 100.0 ± 0.0    |
| RHD3p:QKY qky-16 | 95.0 ± 1.2     | 5.0 ± 1.2      | 2.0 ± 1.0      | 98.0 ± 1.0     |
| RHD3p:QKY qky-16 | 71.5 ± 6.2     | 28.5 ± 6.2     | 22.6 ± 3.0     | 77.4 ± 3.0     |
| scm-2            | 30.3 ± 1.5     | 69.7 ± 1.5     | 0.7 ± 0.6      | 99.3 ± 0.6     |
| qky-16 cpc-1     | 20.7 ± 1.5     | 79.3 ± 1.2     | 2.7 ± 1.2      | 97.3 ± 1.2     |
| SCMP:SCMPm:GFP   | 91.7 ± 2.5     | 8.3 ± 2.5      | 3.3 ± 0.6      | 96.7 ± 0.6     |
| SCMP:SCMPm:GFP   | 78.0 ± 2.7     | 24.0 ± 2.7     | 17.3 ± 4.0     | 82.7 ± 4.0     |

Values represent means ± standard deviations (SD) of three independent experiments. In each experiment, ten 4-day-old seedlings were examined for each strain except for the qky-16 mutant (eleven 4-day-old seedlings were examined for this strain).

H at H pos hair cells at H position, N at H pos non-hair cells at H position, H at N pos hair cells at N position, N at N pos non-hair cells at N position.
that the mutant phenotype was complemented by scm-2 but not complemented by qky-8 (Supplementary Fig. 1c). We sequenced the QKY coding region in the genomic DNA from this mutant, which revealed a nonsense mutation at the 870th codon (Supplementary Fig. 1d). In addition, we discovered that a QKY genomic DNA fragment including 1.2 kb 5′- and 1 kb 3′-flanking sequences (QKYp:QKY) completely rescued the abnormal root epidermis phenotype of the new mutant (Supplementary Fig. 1e and Table 1). Together, these results indicate that this newly identified mutant is a new allele of the QKY gene, and we named it qky-16.

Expression of cell fate regulators in the qky mutant root. To determine the regulatory relationship between QKY and previously identified transcriptional regulators of the root epidermis pathway, we examined the promoter activity of WER, CPC and EGL3 using transcriptional reporter genes (WERp:GFP, CPCp:GUS, and EGL3p:GUS) in the qky-16 mutant. In the wild-type root, the WERp:GFP and the CPCp:GUS are preferentially expressed in the N-position cells, while EGL3p:GUS is preferentially expressed in the H-position epidermal cells. In the qky-16 mutant, the position-dependent expression pattern of these three genes was disrupted, causing reporter gene-expressing cells in roots to be specifically expressed cells to be produced at each position (Supplementary Fig. 1a), which is similar to the expression patterns in the scm-2 mutant root. These results suggest that, like SCM, QKY acts early during root epidermis development to enable cells to interpret their relative position and appropriately regulate expression of the downstream transcription factor network.

QKY expression pattern in the root epidermis. To evaluate QKY gene expression, we generated transgenic plants bearing a QKYp:GUS transcriptional reporter construct (GUS gene fused to the same QKY regulatory sequences used for the genomic DNA complementation described above). These plants exhibit strong GUS accumulation in the stele cells and the ground tissue in the root meristematic region, as well as a lower GUS level in developing root epidermal cells and root cap cells (Fig. 1a), consistent with previous reports of widespread expression of QKY in all organs. The QKY protein has been reported to accumulate in the PM in most of the tissues in the root using an N-terminal eGFP fusion to QKY (eGFP-QKY). We generated translational fusions of GFP to the N-terminus or C-terminus of QKY and expressed these under the control of the QKY regulatory sequences (the same 5′ and 3′ regions used in the QKYp:GUS construct), but these constructs failed to complement the qky-16 mutant root phenotype (Supplementary Fig. 1a). Therefore, we tested several versions of reporter constructs containing GFP in-frame with the QKY coding sequence, and found that a translational reporter construct with the GFP sequence inserted between the end of the first transmembrane domain and the beginning of the phosphoribosyltransferase (PRT) domain (QKYp:QKY-GFP) was able to completely rescue the qky-16 mutant phenotype (Supplementary Fig. 1a and Table 1). As expected, the fluorescence signal in this line was detected at the PM of the cells in most of the root tissues including the epidermis, which is similar to the SCM (SCMp:SCM-GFP) localization pattern (Fig. 1b). The QKY-GFP signal was unevenly distributed in the PM, which is consistent with the previously reported PD-localization of QKY. Interestingly, we discovered greater accumulation of QKY-GFP in the H-position cells than the N-position cells of the root epidermis (Fig. 1b), implying a preferential role for QKY function in the differentiating hair cells during root epidermis patterning.

Due to the broad expression of QKY in root tissues and a report suggesting non-cell autonomous action of QKY in aerial tissues, we tested the long-range action of QKY in regulating root epidermal cell patterning. We expressed QKY-GFP only in the stele, endodermis, cortex, or epidermis/lateral root cap tissues of the qky-16 mutant by using the SHORTROOT (SHR)27, SCARECROW (SCR)28, CO229, or WER promoter, respectively. We observed the fluorescence signal only in the tissue(s) where each promoter was expected to be active, and we found that only the WERp:QKY-GFP construct was able to rescue the qky-16 mutant phenotype in the root epidermis (Fig. 1c and Table 1). These results indicate that QKY influences root epidermal cell patterning in a tissue autonomous manner. Notably, SCM has been reported to act in a cell autonomous manner in the root epidermis.20

Genetic interaction between QKY and SCM. Given the similar mutant phenotypes, gene expression patterns, protein accumulation, and epidermis action for QKY and SCM, we sought to examine the relationship between these two genes. First, we tested for possible genetic interaction by generating the qky-16 scm-2
double mutant by a genetic cross. The abnormal epidermal cell pattern in this double mutant was not significantly different from the abnormal pattern in each of the single mutants (Fig. 2a and Table 1). Similarly, the double mutant exhibited disruption in the position-dependent expression of the GL2p:GUS marker that was comparable to the single mutants (Fig. 2b). QKY overexpression using the promoter of the ROOT HAIR DEFECTIVE 3 (RHD3) gene, which shows strong activity ubiquitously in the root (RHD3::QKY)30, was able to rescue the qky-16 mutant root phenotype completely, but it was not able to rescue the scm-2 mutant phenotype and did not affect position-dependent cell-type patterning in the wild-type root epidermis (Fig. 2 and Table 1). Together, these results suggest that QKY and SCM act in a same genetic pathway to regulate root epidermal cell patterning, and that QKY functions upstream of SCM or they function in parallel.

QKY and SCM regulate CPC movement between epidermal cells. The disrupted cell patterning in the qky and scm mutants and the presumed localization of the QKY and SCM proteins at PD23 led us to examine their possible role in the lateral inhibition between epidermal cells mediated by the mobile transcription factor CPC12. First, we examined the root epidermis phenotype of the qky-16 cpc-1 double mutant, and found that it was largely similar to the phenotype of cpc-1 and quite different from the phenotype of the qky-16 mutant (Table 1), implying that the qky-16 mutant phenotype seems to be mediated by CPC at least in part. It was also shown that the scm-2 cpc-1 root phenotype was quite different from the scm-2 mutant phenotype21.

Next, we tested whether QKY or SCM affects CPC movement using the SHRp:CPC-GFP line, in which stele-produced CPC-GFP fusion protein is able to move to the epidermis and preferentially accumulate in the H-position cells21. In both the qky-16 and the scm-2 mutant roots, the CPC-GFP protein produced by the SHRp:CPC-GFP construct was detected in the epidermal cells (Fig. 3a), indicating that QKY and SCM are not essential for radial trafficking of CPC. However, preferential accumulation of CPC-GFP in the H-position cells was disrupted in these mutants (about 40% of CPC-GFP accumulating epidermal cells were located at the N-position while about 10% in the wild-type root) (Fig. 3b), suggesting that QKY and SCM are required for preferential CPC trafficking or accumulation into the H-position cells.

We used a biolistic particle delivery system to produce GFP or the CPC-GFP fusion protein in a single epidermal cell and monitor its movement. When GFP alone was delivered and expressed in an epidermal cell of wild-type leaves, the GFP signal was also detected in a neighboring cell in more than 70% of the cases after 4 h (Fig. 3c and Supplementary Fig. 2a). Similar mobility of GFP was observed in the qky-16 or scm-2 mutants, consistent with a previous report, suggesting no general effect of QKY and SCM on GFP movement between cells23. The CPC-GFP fusion protein (35S::CPC-GFP) was also detected in neighboring cells in about 70% of the cases in wild-type leaves, but only in 46 and 38% of the cases in the qky-16 and scm-2 mutant leaves, respectively (Fig. 3c), implying that QKY and SCM affect CPC-GFP movement between epidermal cells. To determine whether SCM and QKY are involved in export of CPC-GFP from the CPC-GFP expressing cell, we coexpressed SCM-CFP or QKY-CFP together with CPC-GFP in the same leaf epidermal cell (in the scm-2 or qky-16 mutant, respectively) and analyzed CPC-GFP mobility. We found that CPC-GFP protein movement was not enhanced by coexpression of SCM or QKY (Fig. 3c and Supplementary Fig. 2b), implying that SCM and QKY do not affect export of CPC, so they rather may be involved in the import of CPC. This possibility is consistent with the greater abundance of SCM and QKY in the H-position cells of the root epidermis which preferentially accumulate the mobile CPC protein (Fig. 1b)20. It appears unlikely that SCM and QKY affect CPC movement through direct interaction, because we found that neither the cytoplasmic domain of SCM (SCMCD) nor the three domains of QKY divided by the two transmembrane domains (QKYD1, QKYD2, and QKYD3) are able to interact with CPC in yeast cells (Fig. 4a and Supplementary Fig. 3).

Given that CPC mobility is also important for proper trichome spacing in the shoot epidermis31, we examined the leaf surface of the qky, scm, and qky scm mutants and discovered instances of adjacent “twin” trichomes (indicating defective CPC-mediated lateral inhibition) in the qky-16 (0.54%, n = 925), the scm-2 (0.55%, n = 900), and the qky-16 scm-2 mutant (0.70%, n = 849), which was never observed in the wild-type leaves (n = 1376) (Supplementary Fig. 1f). This result further supports the view that QKY and SCM influence CPC movement between epidermal cells.

QKY and SCM interact through their extracellular domains. It has been reported that QKY and SCM physically interact with each other using the FRET-FLIM method and the yeast
two-hybrid assay. We further confirmed this interaction using communoprecipitation of epitope tagged full-length proteins (QKY-FLAG and SCM-MYC) expressed in tobacco leaves (Fig. 4b). In addition, we used the yeast two-hybrid assay to determine that the SCM extracellular domain interacts with the N-terminal domain 1 of QKY (QKYD1) (Fig. 4a–c) rather than the SCM cytoplasmic domain which was previously reported to be a QKY-interacting domain. Further, we found that a mutated version of the SCM extracellular domain (at a proline-repeat region; SCMEm2) was not able to interact with the QKYD1 (Fig. 4a–d). We also found that this mutated version of SCM (SCMp:SCMEm2-GFP) was not able to complement the scm-2 mutant phenotype (Fig. 4e and Table 1). Interestingly, a mutation in a different proline-repeat region in the extracellular domain (SCMem1; SCMp:SCMem1-GFP) did not affect the functionality of SCM nor its interaction with QKY (Fig. 4a–e and Table 1).

These results suggest that the QKYD1 could be an extracellular domain that interacts with the SCM extracellular domain. However, it was reported that the cytoplasmic domain of SCM interacts with the QKYD1, leading to the suggestion that this is a cytoplasmic domain. To examine the topology of QKY at the PM, we isolated protoplasts from QKYp:QKY-GFP qky-16 or SCMp:SCM-GFP scm-2 plants, applied protease K to digest the exposed extracellular domain of the membrane-spanning proteins, and performed western blot analysis with total protein extracts using anti-GFP antibodies (Fig. 4f). The protease K-treated cells yielded a low level of full-length proteins (130 kD for SCM-GFP and 170 kD for QKY-GFP) and a large amount of truncated proteins (70 kD for SCM-GFP and 40 kD for QKY-GFP) in the western blot analysis, implying that the kinase domain of SCM (including the transmembrane domain) and the domain 2 of QKY (including the two transmembrane domains) fused to GFP were protected. We further evaluated the topology of QKY using the bimolecular fluorescence complementation (BiFC) assay (Fig. 4g). We fused the N-terminal half of EYFP (EYFPn) to the C-terminus (cytoplasmic domain) of SCM (SCM-CFP) for all our experiments. We observed fluorescence complementation with the QKY-EYFPc-V1 or SCM-CFP fluorescence. On the other hand, QKY-EYFPc-V2 and SCM-CFP fluorescence complementation was not observed. Therefore, the SCM-CFP fluorescence did not show fluorescence complementation with QKY-EYFPc-V1 or QKY-EYFPc-V3 which has the EYFPc at the end of the N-terminus or the C-terminus of QKY, respectively (Fig. 4g).

Together, these results suggest that the domain 1 and domain 3 of QKY are extracellular domains, and that domain 1 is used by QKY to interact with SCM and regulate epidermal cell patterning.
**Fig. 4** Interaction between QKY and SCM proteins. **a**, Structure of the QKY and the SCM proteins. Schematic representation of QKY (left) shows the N-terminal domain (D1) and the other two domains (D2 and D3) separated by two transmembrane domains (TM). Schematic representation of SCM (right) shows the extracellular domain (ED) containing a proline-rich domain (PRD), and the cytosolic domain (CD), which are separated by one transmembrane domain (TM). **b**, Coimmunoprecipitation of QKY with SCM protein. The experiment was performed with total protein extracts from *Nicotiana benthamiana* leaves transiently transformed with SCM-MYC and QKY-FLAG. **c**, Yeast two-hybrid assay with QKY and SCM. The construct combinations were cointroduced into the yeast strain AH109. Transformants were grown on the -L-T (lacking leucine and tryptophan) control plates and the -A-H-L-T (lacking adenine, histidine, leucine and tryptophan) selective plates for 3 days. The combination of WER/GL3 was used as a positive control, and pACT2 (empty vector)/QKY-D1 as a negative control (BD: pAS2-1; AD: pACT2). **d**, Effect of mutations in the SCM PRD on the interaction between QKY and SCM in a yeast two-hybrid assay. Mutations are shown in the right panel of (a). **e**, Effect of mutations in the SCM PRD on the SCM-GFP protein level (top, bar = 100 kDa) and the root hair phenotype (bottom, bar = 100 μm). **f**, Proteinase K protection test. Protoplasts from *N. benthamiana* leaf epidermal cell using a biolistic particle delivery system. Their expression was confirmed by western blot analysis with total protein extracts using polyclonal anti-GFP antibodies. The red and the black asterisks indicate the QKY-GFP protein and the SCM-GFP protein, respectively. Bar = 25 μm.

**QKY regulates the internalization of SCM protein.** To investigate the biological impact of QKY–SCM interaction, we examined SCM-GFP protein accumulation in the *SCMp:SCM-GFP qky-16 scm-2* plant root. We discovered that the level of SCM-GFP protein was much lower than in the *SCMp:SCM-GFP scm-2* plant root (Fig. 5a, b), while SCM promoter activity and SCM-GFP transcript level were not affected by the *qky* mutation (Supplementary Fig. 5). On the contrary, the *scm* mutation did not significantly affect QKY-GFP protein accumulation and QKY-GFP transcript level (Fig. 5a and Supplementary Fig. 5). In addition, the fluorescence intensity from the SCMl-1-GFP (which fails to interact with QKY) was much weaker than the intensity from SCM-GFP and from SCMl-1-GFP even though their transcript levels are similar (Fig. 4c and Supplementary Fig. 6). These results suggest that interaction between QKY and SCM is important for the stabilization of SCM protein.

Therefore, we examined the effect of MG132 (an inhibitor of proteasome and cysteine protease), lactacystin (a proteasome-specific inhibitor), and E-64d (a cysteine protease inhibitor) on SCM-GFP protein level. Treatment of MG132 and E-64d increased the SCM-GFP protein level in *qky-16 scm-2* and in *scm-2* mutant roots, while lactacystin did not affect SCM-GFP accumulation (Fig. 5c), indicating that SCM is rapidly degraded in a cysteine protease-dependent manner rather than by a proteasome in the *qky-16 scm-2* mutant. Interestingly, the major protein reacting with the anti-GFP antibodies in the *qky-16 scm-2* mutant root had higher molecular weight (MW) (red asterisk) than the SCM-GFP protein in the *scm-2* mutant root (black asterisk) (Fig. 5b). Upon treatment with MG132 and E-64d, the high MW SCM-GFP band (red asterisk) showed greater accumulation in the *qky-16 scm-2* mutant root compared with the same sized SCM-GFP in the *scm-2* mutant root (Fig. 5c).
SCM-GFP preferentially accumulated in the PM, and E-64d treatment increased internal accumulation of SCM-GFP in subcellular membranous compartments much more in the qky-16 scm-2 mutant root than in the scm-2 mutant root (Fig. 6a). Because E-64d is known to inhibit the fusion of late endosomes with vacuoles in Arabidopsis, we examined whether SCM-GFP accumulation in late endosomes increased after E-64d treatment. We introduced the UBQ10p:mCherry-RabF2a, a marker of late endosomes, into the SCMp:SCM-GFP qky-16 scm-2 plant by a genetic cross, and discovered that a small portion of the internal SCM-GFP colocalized with mCherry-RabF2a without E-64d treatment (DMSO) (Pearson’s correlation coefficient = 0.112727; Fig. 6b). E-64d treatment caused strong colocalization with vacuoles in the prevacuolar compartment with the SCM-GFP in the vacuole in the qky-16 scm-2 mutant root, while it caused slightly increased vacuolar accumulation of SCM-GFP in the epidermis of the scm-2 mutant root (Fig. 6c).

Next, we examined the effect of TyrA23, an inhibitor of endocytosis, on the SCM-GFP accumulation and found that TyrA23 treatment greatly increased accumulation of SCM-GFP at the PM in the qky-16 scm-2 mutant root, while it only modestly increased SCM-GFP at the PM in the scm-2 mutant root (Fig. 6d).

These results indicate that SCM is internalized from the PM by endocytosis and degraded in the vacuole, and that QKY prevents this internalization and vacuolar degradation.

**QKY prevents SCM protein monoubiquitination.** Given the observed size difference between SCM-GFPs from the SCMp:SCM-GFP scm-2 and the SCMp:SCM-GFP qky-16 scm-2 roots (Fig. 5b), the accumulation of high-MW SCM-GFP following MG132 treatment (Fig. 5c), and reports showing that ubiquitination is a signal for internalization and degradation of PM proteins, we examined SCM-GFP for possible ubiquitination. Western blot analysis of immunoprecipitated proteins from seedlings treated with MG132 using anti-GFP antibodies and anti-ubiquitin antibodies (P4D1) showed that the high-MW SCM-GFP which accumulated following MG132 treatment was ubiquitinated, while the low-MW SCM-GFP protein was not (Fig. 7a). However, antibodies specific for polyubiquitination (FK1) did not react with this high-MW SCM-GFP (Fig. 7a), while both of the antibodies reacted with the positive control BR11-GFP (which is known to be polyubiquitinated). Considering the homogeneous MW of the ubiquitinated SCM-GFP, this result suggests that the SCM-GFP is monoubiquitinated.

To evaluate the importance of ubiquitination for SCM degradation and QKY’s potential role in preventing SCM ubiquitination, we generated an SCM construct with all 22 lysine codons in the cytoplasmic domain replaced with arginine codons (SCMm22KR). This construct was transiently expressed (35Sp:SCMm22KR-GFP) in the leaf epidermis of wild-type and qky-16 mutant plants, and SCM-GFP protein was immunoprecipitated and subjected to western blot analysis (Fig. 7b). Unlike wild-type SCM-GFP, the SCMm22KR-GFP did not yield a high-MW SCM-GFP band in the qky-16 mutant root; rather, the size is similar to the nonubiquitinated form. Indeed, this SCMm22KR-GFP did not react with anti-ubiquitin antibodies (P4D1). Furthermore, the fluorescence intensity from the SCMp:SCMm22KR-GFP qky-16 scm-2 plant root was not significantly weaker than the intensity from the SCMp:SCMm22KR-GFP scm-2 plant root unlike the wild-type version of SCM-GFP, which was confirmed by western blot (Fig. 7c). In addition, the difference in the fluorescence intensity between the N-position cells and the H-position cells was much smaller in the SCMp:SCMm22KR-GFP qky-16 scm-2 root and SCMp:SCMm22KR-GFP scm-2 root than in the SCMp:SCM-GFP scm-2 root (Fig. 7d). Although the fluorescence intensities in the root of those two plant lines were comparable to the SCMp:SCM-GFP scm-2 line, SCMp:SCMm22KR-GFP was not able to completely rescue the abnormal root epidermis phenotype of the scm-2 mutant (Supplementary Fig. 7). This result is reminiscent of the effect of overexpressed SCM in the scm-2 mutant (35Sp:SCM scm-2). Altogether, these results suggest that monoubiquitination, which is prevented by QKY in the H-position cells, is necessary for differential accumulation of SCM between cells at each position in the root epidermis.
In each seedling, ten cells were examined epidermal cells (left), and the quantified fluorescence intensities in the PM and in the inner area (right) are shown. The seedlings were incubated with or without E-64d (20 μM) for 2 h. P values for a, b, and c are 0.033501792889072, 0.000000033178503, and 0.000005122822657, respectively. Bar = 10 μm. b Colocalization of internalized SCM-GFP and mCherry-RabF2a. Four-day-old seedlings were treated with or without E-64d (20 μM) for 2 h. The scatterplots show the Pearson’s correlation coefficient (r) between these two fluorescent signals. Typical examples of images and scatterplots are shown (left). Average values of the Pearson’s correlation coefficients from five seedlings are shown in a graph and the error bars indicate standard deviations (right). Statistical significance was determined by unpaired t test, and P values for a is 0.000006164460956. Bar = 10 μm. c Effect of ConA on the SCM-GFP level. Four-day-old seedlings of the two plant lines were incubated with or without ConA (200 nM) for 9 h. Confocal images show internal accumulation of SCM-GFP (left) and the fluorescence intensities in the PM and in the inner area were quantified (middle). The level was also analyzed by western blot (right). P values for a, b, and c are 0.00251027108002, 0.0000000000221023, and 0.000001097753466, respectively. Bar = 10 μm. d Effect of TyrA23 on the SCM-GFP protein level. Four-day-old seedlings of the two plant lines incubated with or without TyrA23 (100 μM) for 10 h. Confocal images show SCM-GFP accumulation (left) and the fluorescence intensity in the PM was quantified (middle). The level was also analyzed by western blot (right). P values for a, b, and c are 0.0000000000079707, below 0.000000000000001, and 0.000651871734356, respectively. Bar = 10 μm. The asterisks in (c) and (d) indicate two different bands reacting with anti-GFP antibodies. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test (a, c, d). Values represent means ± SD of five individual 4-day-old seedlings for (a, c) and of ten seedlings for (d). In each seedling, ten cells were examined.

To identify the ubiquitination sites in the SCM protein, we analyzed the gel mobility of mutant versions of SCM-GFP with single or multiple lysine residues replaced by arginine residues and expressed transiently in qky-16 mutant leaves (Supplementary Fig. 8). The SCM-GFPs with a substitution of Lys-520, Lys-525 or Lys-526, and two substitutions at Lys-525 and Lys-526 showed intermediate gel mobility (between the mobility of wild-type SCM-GFP in the qky-16 mutant and the wild type), and SCM-GFPs with two substitutions at Lys-520 and Lys-526 showed similar mobility to that of nonubiquitinated SCM-GFP. Therefore, we changed all three of these lysine residues to alanine (SCMp:SCM-GFP), expressed this construct transiently in qky-16 mutant leaves, immunoprecipitated with anti-GFP antibodies, and analyzed its gel mobility and ubiquitination (Fig. 7e). This SCMqky-16 SCM–GFP showed similar mobility to that of nonubiquitinated SCM-GFP and did not react with anti-ubiquitin antibodies (P4D1) (Fig. 7e). These results suggest that SCM is modified by multi-monoubiquitination and that these three residues (Lys-520, and at least one from Lys-525 and Lys-526) represent the ubiquitination site.

Discussion
SCM, an atypical LRR RLK, is a critical regulator of cell-type patterning in the Arabidopsis root epidermis16. However, the mechanism and regulation of SCM action had been unclear prior

Fig. 6 The internalization of SCM-GFP is regulated by QKY. a Internal accumulation of SCM-GFP after E-64d treatment. Confocal images of the root epidermal cells (left), and the quantified fluorescence intensities in the PM and in the inner area (right) are shown. The seedlings were incubated with or without E-64d (20 μM) for 2 h. P values for a, b, and c are 0.033501792889072, 0.000000033178503, and 0.000005122822657, respectively. Bar = 10 μm. b Colocalization of internalized SCM-GFP and mCherry-RabF2a. Four-day-old seedlings were treated with or without E-64d (20 μM) for 2 h. The scatterplots show the Pearson’s correlation coefficient (r) between these two fluorescent signals. Typical examples of images and scatterplots are shown (left). Average values of the Pearson’s correlation coefficients from five seedlings are shown in a graph and the error bars indicate standard deviations (right). Statistical significance was determined by unpaired t test, and P values for a is 0.000006164460956. Bar = 10 μm. c Effect of ConA on the SCM-GFP level. Four-day-old seedlings of the two plant lines were incubated with or without ConA (200 nM) for 9 h. Confocal images show internal accumulation of SCM-GFP (left) and the fluorescence intensities in the PM and in the inner area were quantified (middle). The level was also analyzed by western blot (right). P values for a, b, and c are 0.00251027108002, 0.0000000000221023, and 0.000001097753466, respectively. Bar = 10 μm. d Effect of TyrA23 on the SCM-GFP protein level. Four-day-old seedlings of the two plant lines incubated with or without TyrA23 (100 μM) for 10 h. Confocal images show SCM-GFP accumulation (left) and the fluorescence intensity in the PM was quantified (middle). The level was also analyzed by western blot (right). P values for a, b, and c are 0.0000000000079707, below 0.000000000000001, and 0.000651871734356, respectively. Bar = 10 μm. The asterisks in (c) and (d) indicate two different bands reacting with anti-GFP antibodies. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test (a, c, d). Values represent means ± SD of five individual 4-day-old seedlings for (a, c) and of ten seedlings for (d). In each seedling, ten cells were examined.
Fig. 7 QKY regulates the ubiquitination of SCM. a In vivo ubiquitination analysis of SCM-GFP. Immunoprecipitation was performed on protein extracted from MG132-treated (20 μM) SCMp:SCM-GFP scm-2 and SCMp:SCMm22KR-GFP qky-16 scm-2 seedlings and the resulting immunoprecipitates were subjected to immunoblotting with monoclonal anti-GFP (left), anti-Ub P4D1 (middle) and anti-polyUb FK1 (right). b In vivo ubiquitination analysis of SCM-GFP and SCMp:SCM-GFP. Ubiquitination assay was performed as in (a) with the immunoprecipitates from Arabidopsis leaves transiently expressing SCM-GFP or SCMp:SCM-GFP for 12 h with or without MG132 (20 μM) after introducing 35Sp:SCM-GFP or 35Sp:SCMm22KR-GFP by the Biolistic PDS-1000/He system. c GFP accumulation in the root epidermal cells of SCMp:SCM-GFP scm-2, SCMp:SCM-GFP qky-16 scm-2, SCMp:SCMm22KR-GFP scm-2 and SCMp:SCMm22KR-GFP qky-16 scm-2 (left). SCmM22KR indicates that all the lysine residues in the cytoplasmic domain of SCM protein were mutated to arginine residues. Same confocal detection setting was used to compare the fluorescence intensities from different plants. Bar = 25 μm. The protein level was also analyzed by western blot (right). d Aberrant differential accumulation pattern of SCMp:SCMm22KR-GFP between the N-position cells and the H-position cells. Fluorescence intensities were measured in two adjacent cell files from the starting point of elongation zones (left) and the intensities of the four peaks in the N-position cells and the nearest four peaks in the H-position cells were compared (right). Error bars indicate standard deviation from three independent transgenic lines. Five plants were analyzed in each transgenic line. Statistical significance was determined by one-way ANOVA with Bonferroni’s post hoc test, and the P values for a and b are 0.003199883401511 and 0.002495323713490, respectively. AU arbitrary unit, Bar = 25 μm. e Identification of ubiquitination sites in SCM protein. Ubiquitination assays were performed as in (a) with the immunoprecipitates from Arabidopsis leaves transiently expressing SCM-GFP or SCMp:SCM-GFP in which lysine residues at 520, 525, and 526 were substituted by arginine for 12 h with or without MG132 (20 μM) after introducing 35Sp:SCM-GFP or 35Sp:SCMm22KR-GFP by the Biolistic PDS-1000/He system. In (a), (b), and (e), the red asterisk indicates ubiquitinated SCM-GFP and black asterisk indicates nonubiquitinated SCM-GFP.

to this study. Here, we showed that SCM function in root epidermal patterning is dependent on QKY. Like scm mutants, the qky mutant lacked position-dependent cell-type specification (Supplementary Fig. 1). The QKY gene displayed a similar root expression pattern as the SCM gene, and the QKY and SCM proteins both accumulated in the PM, preferentially in the H-position cells (Fig. 1). Furthermore, we found that SCM and QKY are likely to act in the same genetic pathway (Fig. 2).

We have also shown that QKY and SCM are required for movement and preferential accumulation of CPC in the H-position epidermal cells (Fig. 3). PD are intercellular channels conferring cytoplasmic continuity between plant cells and controlling movement of various molecules by targeted and non-targeted mechanisms. Some plant RLKs including SCM appear to associate with PD38–41 and some of these are suggested to regulate movement through PD39,40. Interestingly, a QKY-related protein (one of the 16 members of the multiple C2 domain and transmembrane region proteins (MCTP) family in Arabidopsis) called FT-INTERACTING PROTEIN1 (FTIP1) shows preferential localization to PD and regulation of specific protein
(FLOWERING LOCUS T (FT)) movement between cells42. FTIP was shown to directly interact with FT in companion cells through its third C2 domain and to control FT export from companion cells to the neighboring sieve elements42. In contrast, we discovered that QKY directly interacts with SCM through its N-terminal region containing C2 domains (Fig. 4) rather than CPC (Supplementary Fig. 3) and seems to control CPC import rather than export between epidermal cells (Fig. 3c). Furthermore, QKY seems to regulate the movement of CPC indirectly, as QKY is a regulator of SCM protein accumulation (Figs. 5 and 6).

Our discovery of QKY-dependent stabilization of the SCM protein provides an explanation for the close relationship between QKY and SCM. We show that QKY directly interacts with SCM and generates high levels of nonubiquitinated SCM, rather than ubiquitinated SCM which is internalized and degraded in the vacuole (Figs. 4–6). The precise mechanism by which QKY generates nonubiquitinated SCM is unclear. QKY may promote SCM deubiquitination or it may prevent SCM ubiquitination. The former possibility appears unlikely, because although QKY directly interacts with SCM through their extracellular domain (Fig. 4), QKY has no discernible protease domain nor shows any similarity to known deubiquitinating enzymes. The effect of QKY on SCM stability may also explain the previously reported preferential accumulation of SCM in the H-position cells of the root epidermis20. Notably, a nonubiquitinated mutant version of SCM (SCMmK520/525/526R) expressed under the control of the SCM promoter accumulated highly in both epidermal cell positions (H cell and N cell), even in the absence of QKY (Fig. 7c, d). This implies that the observed preferential H-cell accumulation of wild-type SCM is due to preferential SCM stabilization in the H-cell position as a result of the greater QKY accumulation in the H-position cells. Accordingly, an important future goal is to explore the mechanism responsible for preferential H-position cell accumulation of QKY.

In mammals and yeast, monoubiquitination and K63-polyubiquitination generally govern trafficking of membrane proteins, whereas K48-polyubiquitination generally regulates proteasome-dependent protein degradation43,44. In plants, diverse forms of ubiquitination appear to be used for the degradation of different membrane proteins. Polyubiquitination has been reported to be important for internalization and degradation of LRR RLKs, such as BRI1 and FLAGELLIN SENSING2 (FLS2)37,45. Other membrane proteins have also been reported to be ubiquitinated. The multi-monoubiquitination of ION-REGULATED TRANSPORTER 1 (IRT1) drives its internalization to early endosomes (EE)46 and subsequent K63-polyubiquitination of the multi-ubiquitin moieties is required for its vacuolar degradation47. BOR1, a boron transporter protein, is mono- or diubiquitinated and degraded in the vacuole48. We found that SCM is multi-ubiquitinated, and this promotes its internalization and vacuolar degradation (Figs. 6c, d and 7). Taken together, mono-ubiquitination seems to be sufficient for the internalization of membrane proteins in plants, as observed in nonplant organisms43,44, but multi-monoubiquitination and K63-polyubiquitination may increase the efficiency of internalization, as also shown in yeast49,50, and may be responsible for the vacuolar targeting. It is possible that SCM is also polyubiquitinated, which may have gone undetected due to its low level. Here, however, we showed that blocking SCM degradation in vacuoles resulted in greater accumulation of multi-monoubiquitinated SCM, implying that multi-monoubiquitination plays a major role in the vacuolar degradation of SCM even though we cannot rule out the possibility of polyubiquitination.

Together, these results provide an updated model for cell fate specification in the Arabidopsis root epidermis. In this model, QKY protein preferentially accumulates in the H-position cells of the root epidermis and stabilizes SCM protein in these cells by preventing its ubiquitination. The preferential accumulation of SCM at PD in the H-position cells facilitates import of CPC protein from the neighboring N-position cells. The accumulation of CPC in the H-position cells inhibits WER function5. In turn, feedback regulation (negative regulation by WER and positive regulation by CPC on SCM expression20) further establishes preferential accumulation of SCM protein in H-position cells, which ultimately results in the cell-type pattern. This model is consistent with the inability of QKY overexpression to rescue the scm-2 mutant phenotype (Table 1) and the report that increasing SCM gene copy number shows a mild reduction of the qky mutant phenotype23. It is also consistent with the finding that the scm mutation and the qky mutation did not cause a significant increase in hair cell specification in the N-position cells in the absence of CPC (Table 1)21. However, we also found that ubiquitously expressed QKY in the wild type did not cause ectopic hair cell fate specification in the N-position cells (Table 1), which indicates that an additional H-position factor might be required for effective stabilization of SCM by QKY.

**Methods**

**Plant materials and growth conditions.** The ver-1, cpc-1, scm-2, and qky-8 alleles were described previously4,8,12,22. The GL2-GUS, WER:GFP, CPC-GUS, EFL3-GUS, SCM:mGFP, SCMp:SCM-GFP and SHR:GFP reporter gene lines were also described previously6,7,10,13,16,25,31. For plant growth, seeds were sterilized, germinated and grown vertically on agarose-solidified medium containing mineral nutrients53.

**Analysis of epidermal cell patterning.** For each line, we analyzed at least ten 4-day-old seedlings and performed three independent experimental repeats. We scored the ten H-position cells and the ten N-position cells for each seedling using differential interference contrast microscopy.

**Gene constructs and plant transformation.** For qky-16 complementation experiments, the genomic DNA fragment containing a 1210-bp 3′ flanking region DNA fragment and a 576-bp 3′ flanking region DNA fragment from QKY were used. To analyze the promoter activity of QKY, the β-glucuronidase gene or the GUS gene was inserted between the same 5′ and 3′ flanking DNA fragments that were used for the complementation test (QKYp:GUS and QKYp:GFP). To visualize the QKY protein, the GFP coding region was inserted at the end of the first transmembrane domain of QKY (QKY-GFP). For the QKYp:QKY-GFP construct, the chimeric QKY-GFP was inserted between the same flanking sequences of QKY. To express QKY-GFP in various tissues, the chimeric gene was fused to each tissue-specific promoter. For the RHD3p:QKY construct, QKY was fused to the 3′ end of the RHD3 promoter30. SCMmK520/525/526R and SCMmK520/525/526R-fusions were fused to the 3′ end of the SCM promoter28. For the BiFC assay, QKY-EYFPc-V1, QKY-EYFPc-V2, QKY-EYFPc-V3, SCM-EYFPc-V1 and SCM-EYFPc-V2 constructs were made by PCR cloning using Gibson assembly technology and expressed under the control of the 3SS promoter. To identify ubiquitination sites, SCM, SCMmK520/525/526R and SCMmK520/525/526R were fused to 5′ end of the GFP gene, and transiently expressed under the control of the 3SS promoter in Arabidopsis leaves. Plant transformation was achieved by electroporating constructs into the Agrobacterium strain GV3101 followed by introduction into Arabidopsis using the floral dip method39. The primers used for the constructions are described in Supplementary Table 1.

**Chemical treatments.** Inhibitors (Sigma-Aldrich) were used at the following concentrations: 200 nM ConcA, 100 μM TyrA23, 20 μM E-64d, 50 μM MG132, 50 μM lactacystin and 10 ng/μl proteinase K.

**Histochemical GUS staining.** GUS activity was examined by observing 4-day-old seedlings stained for 1–12 h in a solution containing 5-bromo-4-chloro-3-indolyl glucuronide12.

**Confocal microscopy.** To analyze GFP expression, seedlings were counterstained with 5 μg/ml propidium iodide (PI) or 5 μM FM4-64 (Invitrogen) and examined using a Zeiss LSM880 confocal microscope5. The PI signal was detected with a 543 nm excitation mirror and a 560 nm long-path filter. The mCherry-RabF2a signal was detected with a 561 nm excitation mirror and a 661 nm long-path filter. Laser intensity settings were kept constant across samples in each experiment.

**Transient gene expression with the particle gun.** Rosette leaves were bombarded with DNA-coated tungsten particles using the Biolistic PDS-1000/He system.
(Bio-Rad) according to the manufacturer’s instructions. Tungsten (M10) particles were coated with 1 μg of the DNA, or with 1 μg of each DNA for co-bombardment. The analysis was carried out after 4–12 h of incubation.

**Immunoprecipitation and western blot analysis.** Total proteins were extracted from 4-day-old seedlings or transiently expressed rosette leaves. For western blot analyses, total proteins were extracted from approximately 100 mg of tissue. The following antibodies were used in these experiments: monoclonal anti-GFP (Clontech 632380, 1/300), polyclonal anti-GFP (abcam ab290, 1/2000), anti-FLAG (Abm G191, 1/3000), anti-CMYC (Calbiochem 0106, 1/3000), anti-ubiquitin (P4D1 (Millipore 05-944, 1/2500)) and anti-polyubiquitin FK1 (Enzo Life Sciences BML-PW8805-0500, 1/1000). Unsupervised blots are shown in Source Data file. One gram of seedlings or transiently expressed rosette leaves was used for the immunoprecipitation experiments. The tissue was ground in liquid nitrogen, re-suspended in 1 ml of immunoprecipitation buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail) and left on a rotating wheel for 30 min at 4 °C. Samples were then centrifuged for 10 min at 20,000 × g at 4 °C. Immunoprecipitations were carried out on 1 mg of total proteins using the E.Zview® Red ANTI-FLAG® M2 Affinity Gel (Sigma) and GFP-Trap-A (Chromotek) according to the manufacturer’s protocol.

**Quantitative real-time RT-PCR.** To measure the steady-state level of transcripts, total RNA was extracted from the root tips of 4-day-old seedlings and treated with RNase-free DNase I. One microgram of total RNA was used for the reverse transcription using the AccuScript High Fidelity 1st Strand cDNA synthesis kit (Agilent Technologies, 200820), and 1 μl of resulting first-strand cDNA (20 μl) was used as a PCR template for the quantitative real-time RT-PCR. Quantitative PCR analysis was performed using SYBR Premix EX Taq (Takara, #RR82LR) with an internal reference to normalize the relative level of each transcript. Each experiment was repeated three times, and each time the experiment included triplicate samples. The raw data underlying the averages are shown in Source Data file.

**Quantification of SCM-GFP accumulation.** Confocal images were obtained from 4-day-old seedlings of the corresponding plant line. Mean fluorescence intensities of SCM-GFP at the PM and in the inner area were measured in the ten H-position epidermal cells from each seedling image by creating a region of interest covering the PM area and entire cytoplasm area, respectively using ImageJ program (Fiji version). The raw data underlying the averages are shown in Source Data file.

**Analysis of colocalization of internalized SCM and RabB2a.** Confocal images of 4-day-old seedling roots were analyzed using the Image-pro 10 software to produce scatterplots and to calculate colocalization of SCM-GFP and mCherry-RabB2a. The scatterplots that were obtained by analyzing all pixels inside five different cells in one seedling produced the Pearson’s correlation coefficient based on the automated Otsu threshold44. The raw data underlying the averages of the Pearson’s correlation coefficient are shown in Source Data file.

**Protease K protection test.** Proteoplasts were isolated from SCM:pSCM-GFP and QKYp:QKY-GFP stable transgenic plants. The protoplasts were incubated in the presence or absence of protease K (1 μg/μl). Then, PMSF and SDS sample buffer were added and denatured at 95 °C to inactivate protease activity.

**Bimolecular fluorescence complementation.** Fifty microliters of Agrobacterium overnight culture was centrifuged, washed, and suspended in a solution containing 10 mM MES, 10 mM MgCl₂, and 200 μM acetosyringone. Coinfiltration of Agrobacterium strains containing the BiFC constructs and the p19 silencing plasmid was carried out. The leaves of 4-week-old Nicotiana benthamiana were infiltrated and subsequently analyzed at day 3 after infiltration using a Zeiss LSM 880 confocal microscope with ZEN software. EYFP signal was detected with a 514 nm excitation mirror and a 520–600 nm band-pass filter.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The source data for Figs. 3–7, Supplementary Figs. 4–6, 8, and Table 1 are provided as a Source Data file. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
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