Rice STOMATAL CYTOKINESIS DEFECTIVE2 regulates cell expansion by affecting vesicular trafficking in rice

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Abstract

Vesicular trafficking plays critical roles in cell expansion in yeast and mammals, but information linking vesicular trafficking and cell expansion in plants is limited. Here, we isolated and characterized a rice (Oryza sativa) mutant, decreased plant height 1-1 (dph1-1), which exhibited a wide spectrum of developmental phenotypes, including reduced plant height and smaller panicles and grains. Cytological analysis revealed that limited cell expansion was responsible for the dph1-1 mutant phenotype compared to the wild-type. Map-based cloning revealed that DPH1 encodes a plant-specific protein, OsSCD2, which is homologous to Arabidopsis (Arabidopsis thaliana) STOMATAL CYTOKINESIS DEFECTIVE2 (SCD2). Subcellular localization revealed that OsSCD2 is associated with clathrin. Confocal microscopy showed that the dph1-1 mutant has defective endocytosis and post-Golgi trafficking. Biochemical and confocal data indicated that OsSCD2 physically interacts with OsSCD1 and that they are associated with intracellular structures that colocalize with microtubules. Furthermore, we found that cellulose synthesis was affected in the dph1-1 mutant, evidenced by reduced cellulose synthase gene accumulation at the transcript and protein levels, most likely resulting from an impaired localization pattern. Our results suggest that OsSCD2 is involved in clathrin-related vesicular trafficking with an important role in maintaining plant growth in rice.
Introduction

Rice (Oryza sativa) is a staple food for over half of the global population and has a pivotal position in ensuring food security. Plant architecture is the foundation of yield and represents a collection of genetically controlled agronomic traits, including plant height, panicle size, tiller number, leaf characteristics, and root morphology (Wang and Li, 2008). Plant architecture is plastic and undergoes numerous changes during the growth cycle (Wang et al., 2018). The cellular basis of plant architecture is coordinately regulated by cell division and cell expansion. Cell division generates new cells by the generation of a new cell wall between daughter nuclei following mitosis (Smith, 2001), while cell growth and expansion is presumably driven by turgor pressure to reach the ultimate size and shape that in turn determines plant morphology (Smith and Oppenheimer, 2005). However, the molecular machineries required for these cellular processes remain unclear, especially in monocotyledonous crop species.

Vesicle-mediated membrane trafficking in plant cells plays a critical role in cell expansion and cytokinesis by maintaining a unique composition of proteins and lipids in the endomembrane system (Bassham et al., 2008; Van Damme et al., 2008). Endocytosis and exocytosis are the main vesicular trafficking pathways and play pivotal roles in cell wall biosynthesis, nutrient uptake, hormone signaling, and pathogen defense during plant growth and development (Kim and Brandizzi, 2014; Zwiewka et al., 2015; Kanazawa and Ueda, 2017; Zhang et al., 2019a). Exocytosis is responsible for the transport of newly synthesized or recycled cargos to the plasma membrane (PM) in expansion cells or to the cell plate in dividing cells (Elliott et al., 2020). Endocytosis in contrast internalizes or retrieves excess materials from the PM and cell plate, and passes these through endosome compartments to vacuoles for degradation or to the trans-Golgi network (TGN)/early endosomes (EEs) for recycling to the PM (Paez Valencia et al., 2016; Rodriguez-Furlan et al., 2019). Clathrin-coated vesicles (CCVs) are vesicle carriers surrounded by a clathrin lattice formed at the PM and TGN/EEs in plant cells (Ekanayake et al., 2019; Narasimhan et al., 2020). Clathrin-mediated endocytosis (CME) is a well-studied endocytosis route in eukaryotes and is recognized as the major endocytic process for the internalization of PM proteins in plant cells (Chen et al., 2011; McMahon and Boucrot, 2011; Reynolds et al., 2018). In addition to involvement in endocytosis, CCVs have been implicated in function to post-Golgi trafficking pathways (Robinson and Pimpl, 2014). How CCV-related endocytosis and exocytosis coordinately regulate plant growth remains largely unknown in rice. Stomatal cytokinesis-defective (scd) mutants including scd1 and scd2 were first characterized based on their developmental defects in stomatal cytokinesis and cell expansion in Arabidopsis (Arabidopsis thaliana) (Falbel et al., 2003; McMichael et al., 2013). AtSCD1 harbors a differentially expressed in normal and neoplastic cell domain, which has been reported to function as a guanine nucleotide exchange factor for specific RAB GTPases to mediate membrane protein targeting in metazoans (Stenmark, 2009; Marat et al., 2011). AtSCD2 encodes a protein with a central coiled-coil domain and a carboxy-terminal domain of unknown function (McMichael et al., 2013). AtSCD1 and AtSCD2 proteins cofractionated with CCVs and colocalized with CCVs/clathrin-coated pits at the PM, suggesting a role in clathrin-mediated membrane trafficking including endocytosis (McMichael et al., 2013). A recent study revealed that AtSCD1 and AtSCD2 together constitute the SCD complex that cooperates with members of the exocyst complex and RabE1 GTPases to mediate post-Golgi trafficking to the PM and cell plate in Arabidopsis (Mayers et al., 2017). However, whether the SCD complex is directly involved in endocytosis remains elusive. There are one SCD1 homolog and three putative SCD2 homologs in the rice genome, but whether and how they SCD proteins function in rice remained unknown.

In this study, we report the isolation and characterization of two rice plant growth retardation allelic mutants. The decreased plant height1-1 (dph1-1) mutant is smaller in almost all respects compared to its wild-type (WT). Microscopic analysis revealed that the dph1-1 mutant phenotype is caused by a reduction in cell size. Molecular cloning showed that DPH1 encodes a homolog of AtSCD2. OsSCD2 is a membrane-associated protein that mainly associates with clathrin. Loss of OsSCD2 function caused defects in endocytosis and post-Golgi trafficking. Biochemical data demonstrated that OsSCD2 forms a functional protein complex with OsSCD1. Furthermore, our studies found that the OsSCD2 mutation led to reduced CESAs accumulation at both transcript and protein levels. Collectively, our data suggest that OsSCD2 is involved in clathrin-mediated vesicular trafficking that has an important role in regulation of plant growth.

Results

Phenotypic characterization of the dph1-1 mutant

To dissect the molecular mechanisms regulating plant architecture in rice, we isolated a mutant with altered plant height. The mutant named dph1-1 was one of two phenotypically similar mutants found in tissue culture-derived japonica rice populations. Compared with its WT Kitaake, the dph1-1 mutant exhibited reduced size in almost every tissue or organ examined throughout the entire growth cycle. For example, a detailed time-course analysis showed that the dph1-1 mutant displayed markedly shorter shoots and roots after germination (Figure 1, A and F). At the mature stage, the dph1-1 mutant had a 31.4% reduction in plant height (Figure 1, B and G), due to smaller panicle length and internodes (Figure 1, C, H, and I). Finally, the dph1-1 mutant produced smaller grain (Figure 1, D and E) with grain length and width decreased by 11.7% and 9.4%, respectively (Figure 1J), which led to a 25.3% reduction in 1,000-grain weight (Figure 1K). Furthermore, statistical analysis showed that grain number per panicle was reduced by 71.1% relative to the WT, resulting from a combination of reduced primary...
and secondary branch numbers (Figure 1, L and M). Overall, the dph1-1 mutation causes a general reduction in all plant characteristics leading to a smaller plant.

**The dph1-1 mutant has restricted cell expansion**

To uncover the cellular basis of retarded growth in the dph1-1 mutant, we conducted cytological analyses of several tissues, including roots, internodes, and spikelet hulls. Analyses of seedling roots revealed that the smaller roots of the dph1-1 mutant were caused by significant reductions in the size of all cell types examined, including endodermal, cortex, sclerenchyma, exodermal, and epidermal cells (Figure 2A and B). Cell length and width of first internode in the dph1-1 mutant were reduced by 21.8% and 26.8%, respectively (Figure 2, C and E). Similarly, cell length and width in the lemma surface were significantly decreased by 27.8% and 33.1%, respectively (Figure 2, D and F). These results indicate that cell expansion is constrained in the dph1-1 mutant.

Flow cytometry analysis of 1-week-old seedlings was performed to investigate the effect of the dph1-1 mutation on cell proliferation. There was no significant difference in cell number in the G1, S, and G2/M phases relative to the WT (Figure 2, G and H), suggesting that the cell number was not significantly affected. Transmission electron microscopy of 1-week-old root cells also indicated that cell differentiation and cytokinesis between WT and dph1-1 mutant were largely comparable (Supplemental Figure S1). Taken together, these results indicate that the dph1-1 mutant cells are competent to proliferate, but exhibited remarkably reduced cell size.

**DPH1 encodes a plant-specific protein homologous to AtSCD2**

We constructed a cross between WT and dph1-1 mutant for genetic analysis. The F2 population segregated 196 normal: 64 mutant plants, suggesting that the dph1-1 involved a recessive nuclear mutation ($\chi^2 = 3.1$; $P_{adj} > 0.05$). Using 1,186 F2 mutant individuals from a cross between dph1-1 and japonica var. IRAT129, the DPH1 locus was narrowed to a physical region of 73 kb on chromosome 1 harboring 9 putative candidate genes (Figure 3A). DNA sequencing revealed a single base substitution of guanine (G) to thymidine (T) at the splicing site of the sixth intron of LOC_Os01g70320 (Figure 3B). This mutation was expected to impair splicing of the sixth intron and lead to frame shifts (Figure 3B). In addition, we identified another allelic mutant designated dph1-2 (Supplemental Figure S2). The dph1-2 mutant contains a 2-bp insertion in the ninth exon causing a premature translation stop (Figure 3B). LOC_Os01g70320 was predicted to encode a protein of 571 amino acid residues that harbors two coiled-coil domains and an unknown function domain SCD2, mutations in dph1-1 and dph1-2 were expected to cause the lack of the SCD2 domain (Figure 3C).

To confirm the identity of LOC_Os01g70320 as the DPH1 gene, we introduced a 10-kb WT genomic fragment spanning the entire coding region of LOC_Os01g70320 and flanking regulatory sequences (i.e. promoter and terminator) into the dph1-1 mutant. The reduced growth attribute was rescued in positive transgenic lines at all developmental stages examined and confirmed by sequencing and statistical analyses (Figure 3, D–K). Furthermore, we raised polyclonal antibodies against DPH1 using the N-terminal 2–20 amino acids as the antigen. The DPH1 antibodies specifically recognized two ~70 kDa bands in total protein extracts from WT but not in dph1-1 and dph1-2 mutants (Figure 3L), indicating the specificity of the DPH1 antibodies and the loss-of-function nature of dph1-1 and dph1-2 mutants. Moreover, knockdown and loss-of-function mutations of LOC_Os01g70320 in the WT background generated by RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology, respectively, exhibited phenotypic defects similar to the dph1 mutants (Supplemental Figure S3). These results provided strong genetic evidence supporting the notion that mutations in LOC_Os01g70320 were responsible for the dph1 mutant phenotypes.

A BLAST search showed that the rice genome encodes two DPH1 homologs, LOC_Os11g31950 and LOC_Os05g05580, which share 50.4% and 38.8% protein identity with DPH1, respectively. Phylogenetic analysis showed that DPH1 homologs were widely present in monocots and eudicots, but not in animals, fungi, or protists (Supplemental Figure S4A), indicating that DPH1 represents a plant-specific protein. We also found that DPH1 is a homolog of AtSCD2, and thus for simplicity we designated the rice DPH1 as OsSCD2. OsSCD2 and AtSCD2 shared two conserved CC domains in the mid-region and a SCD2 domain of unknown function at the N terminus (Supplemental Figure S4B).

Consistent with the pleiotropic phenotypes of the dph1 mutants, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis revealed that OsSCD2 was widely expressed in all tissues examined including roots, leaves, leaf sheaths, stems, and young as well as mature panicles, with relative higher expression in leaves and mature panicles (Supplemental Figure S5A).

**OsSCD2 is associated with clathrin and colocalized with microtubules**

To investigate the subcellular localization of OsSCD2, we introduced into dph1-1 mutant calli a construct, in which OsSCD2 was C-terminally fused with GFP driven by a CaMV35S promoter. The reduced growth phenotype was rescued by the construct (Supplemental Figure S5, B–D), demonstrating that the OsSCD2-GFP fusion protein was biologically functional. Consistent with anti-OsSCD2 antibodies, the anti-GFP monoclonal antibody also recognized two bands at ~90 kDa in transgenic positive but not in WT plants (Supplemental Figure S6A). It is possible that the upper band detected by anti-OsSCD2 and anti-GFP antibodies...
represents a modified OsSCD2/OsSCD2-GFP, similar phenomena is also observed in AtSCD2 immunoblot analysis (McMichael et al., 2013). The results of subcellular fraction assays using the GFP complementation transgenic lines showed that GFP-fused OsSCD2 was present in both the soluble and membrane fractions (Supplemental Figure S6B). The OsSCD2-GFPs were extractable from the membranes by alkali, but not by high salt solution (Supplemental Figure S6C). The solubility efficiency resembled that of peripheral membrane protein GLUTELIN PRECURSOR ACCUMULATIONS (GPA5) (Ren et al., 2020), unlike the integral tonoplast protein γ-Tip (Supplemental Figure S6C). Thus, due to the lack of predictable transmembrane domains, OsSCD2 is more likely to be a peripheral membrane protein localized to the membrane by interaction with other membrane-anchored proteins or lipids.

Visualization of the subcellular localization of OsSCD2 in rice by confocal microscopy using protoplasts from complemented OsSCD2-GFP seedlings in which PM marker PIP2-mCherry was transiently co-expressed showed agreement with the subcellular fraction experiment (Supplemental Figure S6C); OsSCD2-GFP located in the cytosol and punctate structures. The OsSCD2-GFP labeled punctate structures also partially colocalized with the PM marker PIP2-mCherry.
OsSCD2 in rice plant growth

Figure 2 Cell expansion defect in the dph1-1 mutant. A, Median longitudinal sections of the WT and dph1-1 mutant root tips. Different cell types are marked with different colors. vb, vascular bundle; en, endodermis; co, cortex; sc, sclerenchyma; ex, exodermis; ep, epidermis. Bars, 15 μm. B, Average cell size as indicated in (A) (n = 10). C, Longitudinal sections of first internodes of the WT and dph1-1 mutant. Bars, 100 μm. D, Scanning electron microscopy (SEM) images of lemma surfaces of the WT and dph1-1 mutant grains. Bars, 50 μm. E and F, Analysis of cell size as indicated in (C) and (D), respectively (n ≥ 20). G, DNA content of 1-week-old WT and dph1-1 mutant seedlings were analyzed by flow cytometry analysis. H, Quantification of cell numbers in G1, S, and G2/M phase as shown in (G) (n = 3). Values are given as percentages of the total cell population. All data are means ± SE. **P < 0.01; ns, not significant (t test, compared with the WT value).

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(Figure 4A). In agreement with its localization in rice protoplasts, OsSCD2-GFP was detected in the cytosol and punctate organelles in root cells (Figure 4B). Staining by the dye FM4–64, an endocytic tracker for PM internalization and endosomal trafficking, showed that OsSCD2-GFP-labeled punctate compartments partially colocalized with the PM (Figure 4B), thus confirming its PM association. In addition, colocalization with FM4–64-labeled endosomes was detected in a few punctate compartments (Figure 4B).

Furthermore, we conducted immunoelectron microscopy (immuno-EM) analysis on OsSCD2-GFP transgenic roots using polyclonal antibodies against GFP to determine the nature of OsSCD2-GFP-labeled punctate compartments. As shown in Figure 4C, clusters of gold particles were found at or near the PM, TGN, prevacuolar compartment (PVC), and even inside PVCs, but the morphology was difficult to discern due to low electron density. Quantitative immuno-EM analysis showed that gold particles mainly distributed at or near the PM and TGN (Figure 4D). As a control, no or few gold particles were observed in dph1-1 mutant samples (Supplemental Figure S7A).

Interestingly, OsSCD2-GFP-labeled punctate structures were arranged in order and colocalized with microtubule marker mCherry-TUB6 in Nicotiana benthamiana leaf epidermal cells (Supplemental Figure S8A). To confirm these colocalization, we performed immunofluorescence labeling using anti-tubulin antibody in leaf epidermal cells and protoplasts of N. benthamiana as well as expanding leaf sheath cells of OsSCD2-GFP complemented rice plants. As expected, confocal microscopy images showed that OsSCD2-GFP labeled...
Figure 3  Map-based cloning of DPH1. A, Fine mapping of the DPH1 locus. Molecular markers and numbers of recombinant plants are indicated above and below the filled bars, respectively. The candidate ORF is highlighted in red. B, Genomic structure of LOC_Os01g70320 (ORF7). White and black boxes indicate untranslated regions and exons, respectively, and the black lines indicate introns. Mutation sites are marked by arrows. C, Schematic domain structure of the OsSCD2 protein. Putative translation termination sites are indicated by arrowheads. CC, coiled-coil domain; SCD2, the SCD2 domain. D–H, A 10-kb WT genomic fragment of OsSCD2 rescues the phenotypic defect of dph1-1 mutant plants at the seedling (D) and mature (E) stages as well as panicles (F), grain morphology (G and H). Bars, 1 cm in (D, G, and H); 10 cm in (E); 3 cm in (F). Plant samples were photographed together and were shown as a single image. I–K, Statistical analyses of plant height (I) (n ≥ 20), panicle length (J) (n ≥ 30), and seed size (K) (n ≥ 15) of WT, dph1-1 mutant, and complemented transgenic lines. All data shown are means ± se. ** P < 0.01 (t test, compared with the WT value). L, Immunoblot analysis of OsSCD2 protein level in total protein extracts from the WT, dph1-1, and dph1-2 mutants. Anti-HSP82 was used as a loading control.
structures colocalized with the microtubule and arranged in order along the microtubule filaments (Supplemental Figure S8, B–D). These observations together indicate that OsSCD2-GFP was somehow colocalized with microtubule.

Since endomembrane system markers have not been well established in rice, we next investigated the nature of OsSCD2-labeled punctate organelles in protoplasts prepared from N. benthamiana leaves. Colocalization with known organellar markers used to identify structures labeled with OsSCD2-GFP showed that except for a few associated puncta, OsSCD2-GFP rarely colocalized with the Golgi (GmMan1-mRFP) and TGN (mRFP-AtSYP61) (Supplemental Figure S9, A and B). Consistent with the immunogold labeling experiment, except for localization of OsSCD2-GFP near the PVC, colocalization with the PVC marker mRFP-AtVSR2 was also observed in some puncta (Supplemental Figure S9C). We found that a considerable subpopulation of OsSCD2-GFP colocalized with the CCV marker (mCherry-AtCLC2) (Supplemental Figure S9D). Quantification of these colocalization experiments further validated the partial overlapping with PVC and CCV (Supplemental Figure S9E).

To further determine the possible colocalization with CCV, we performed immunofluorescence labeling using antibodies against clathrin heavy chain (anti-CHC; Wang et al., 2013) in OsSCD2-GFP complemented root cells. As shown in Figure 4E, immunofluorescence staining showed that OsSCD2-GFP-labeled puncta colocalized with a subpopulation of CHC-labeled puncta in root cells. To directly evaluate the co-localization of OsSCD2 with CHC, the fluorescence intensity profile of these two proteins was quantified along a specific linear distance (Figure 4F). The substantial overlapping between two fluorescence signals indicating that OsSCD2 colocalized with clathrin in vivo. As a control, immunofluorescence labeling without primary antibodies detected few signals (Supplemental Figure S7B). In addition, we performed immuno-EM studies using anti-CHC antibodies in ultrathin sections prepared from high-pressure frozen/freeze-substituted samples of root cells. Consistent with the distribution pattern of OsSCD2-GFP in rice root cells (Figure 4, C and D), quantitative immuno-EM analysis showed that CHC-labeled gold particles exhibited a major distribution at or near the PM and TGN compared with control group (Supplemental Figure S7, C–E). Together, these results suggest that OsSCD2 associates with clathrin at or near the PM and TGN.

The dph1-1 mutation affects endocytosis and post-Golgi trafficking

Based on the subcellular localization profile of OsSCD2, we hypothesized that OsSCD2 might function in endocytosis and/or post-Golgi trafficking. We treated WT and dph1-1 mutant roots with FM4–64 to determine the endocytic activity. Uptake and intracellular accumulation of FM4–64 were pronounced after 30 min in both WT and dph1-1 mutant roots with endosomes clearly discernable (Figure 5, A and B). Quantification of FM4–64 internalization, expressed as the average ratio of intracellular and PM fluorescence intensities, revealed that the relative internalization of FM4–64 was dramatically decreased in dph1-1 mutant roots compared with the WT (Figure 5C). We also expressed FLS2-GFP as an endocytosis marker in the WT and the dph1-1 mutant to monitor ligand-induced FLS2 endocytosis. Upon treatment with flg22, increasing abundance of FLS2-GFP in intracellular endosomal puncta was internalized from the PM in the WT, as evidenced by the presence of numerous GFP fluorescence puncta (Supplemental Figure S10, A and C). In contrast, the number of FLS2-GFP puncta was significantly fewer in the dph1-1 mutant (Supplemental Figure S10, B and C). These results suggest that loss of OsSCD2 function impaired endocytosis in the dph1-1 mutant.

PIN-FORMED2 fused to green fluorescent protein (PIN2-GFP), a genomic fusion construct of PIN2 that functions in polar distribution of auxin, has been widely used as a marker protein to monitor post-Golgi trafficking in Arabidopsis and rice (Wang et al., 2013; Wu et al., 2015). To examine whether post-Golgi trafficking was altered in the dph1-1 mutant, we transformed PIN2-GFP construct into the WT and dph1-1 mutant. As expected, PIN2-GFP was localized on the PM in both genotypes (Figure 5D). To monitor PIN2-GFP trafficking, Brefeldin A (BFA, a reversible vesicle trafficking inhibitor; Nebenführ et al., 2002; Kleine-Vehn and Friml, 2008) treatment experiments were performed in the presence of cycloheximide (CHX, a protein synthesis inhibitor; Schneider-Poetsch et al., 2010). After CHX and BFA treatment, the number of PIN2-GFP-labeled BFA bodies per cell in the dph1-1 mutant was fewer than that in the WT at 0 min (Figure 5, E and F), confirming the endocytosis defect in the dph1-1 mutant. Strikingly, we observed a significant delay in the disappearance rate of PIN2-GFP-labeled BFA bodies in the dph1-1 mutant after BFA washout 30–90 min (Figure 5, E and F), suggesting that exocytosis and endosomal recycling of PIN2-GFP from BFA bodies were altered in the dph1-1 mutant. Similar trafficking defects were observed in Arabidopsis scd1 and scd2 mutants (Mayers et al., 2017). Taken together, these results suggest that OsSCD2 functions in both endocytosis and post-Golgi trafficking in rice.

OsSCD2 physically interacts with OsSCD1

To search for putative interactors of OsSCD2 protein, we performed co-immunoprecipitation (Co-IP) assays with lysates from the 1-week-old seedlings expressing either the empty GFP or OsSCD2-GFP. The immune-precipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to mass spectrometry assays (Figure 6A). We detected OsSCD1 protein in the OsSCD2-GFP precipitate but not in the empty GFP precipitate (Figure 6B; Supplemental Data Set 1), suggesting a possible interaction between OsSCD2 and OsSCD1. OsSCD1 protein is a homolog of AtSCD1 but had not been functionally characterized in rice. To visualize the interaction between OsSCD2 and OsSCD1, we next performed a bimolecular fluorescence complementation (BiFC) assay using leaf epidermal cells of N. benthamiana. While OsSCD2-eYNE and
Figure 4 Subcellular localization of OsSCD2 protein. A, Confocal microscopy images focused on the optical section of PM showing the subcellular localization of OsSCD2 near PM in rice protoplasts. PIP2-mCherry was used as a PM marker. Yellow arrowheads indicate the colocalization signals at the PM. Bars, 10 μm. B, Confocal microscopy images focused on optical section of cytoplasm showing the punctate localization pattern of OsSCD2-GFP in transgenic rice root tip cells combined with FM4–64 staining. Yellow arrowheads indicate colocalization signals. Bar, 5 μm. C, Immunogold localization of OsSCD2 in transgenic rice root tip cells expressing OsSCD2-GFP. Ultrathin sections samples were prepared from high pressure frozen/freeze substitution followed by single immunogold labeling using anti-GFP polyclonal antibodies from rabbit in combination with 15-nm gold-coupled secondary antibodies. Red dots outline the distribution of OsSCD2. Bars, 200 nm. D, Quantification of gold particles in immunogold labeling experiments (C). Cyto, cytoplasm; Mito, mitochondria; Vac, vacuole; CW, cell wall. Data are means ± s.e. (n ≥ 15). E, Confocal microscopy images focused on optical section of cytoplasm showing immunofluorescence staining of CHC in root cells expressing OsSCD2-GFP. Yellow arrowheads indicate colocalized puncta structures. Bar, 5 μm. F, Line scan measurement (red dotted line) of confocal microscopy image in (E) plotted to show overlapping intensity profiles.
OsSCD1-eYCE were co-expressed with CCVs marker mCherry-AtCLC2, strong eYFP signals were observed in the clathrin-associated punctate structures (Figure 6C; Supplemental Figure S11), consistent with the localization pattern of OsSCD2 alone (Supplemental Figure S8, A and B). Furthermore, we verified the interaction between

Figure 5  Endocytosis and post-Golgi trafficking defects in dph1-1 mutant. A and B, Confocal microscopy images showing the internalization of FM4–64 in root cells of WT and dph1-1 at 0 min (A) and 30 min (B). Bars, 10 μm. C, Quantitative analyses of FM4–64 fluorescence intensity ratio of intracellular and PM in (B) (n > 60). D, Subcellular localization of PIN2-GFP in WT and dph1-1. Bars, 10 μm. E, Time-course analysis of the number of PIN2-GFP-labeled BFA bodies after BFA washout in the WT and dph1-1 mutant root cells. Seedling roots were pretreated with 50 mM CHX in 1/2 liquid MS medium for 30 min and then treated together with 50-mM BFA for 90 min prior to washout. Root cells were imaged at four different time points (0, 30, 60, and 90 min). White arrows indicate PIN2-GFP-labeled BFA bodies. Bars, 10 μm. F, Quantitative analyses of numbers of PIN2-GFP-labeled BFA bodies per cell after BFA washout in (E) (n = 15). All data are means ± se. *P < 0.01; **P < 0.05 (t test, compared with the WT value).
OsSCD2 and OsSCD1 using an in vivo firefly luciferase complementation imaging (LCI) assay in leaf epidermal cells of *N. benthamiana* (Figure 6D) and a coimmunoprecipitation assay in rice protoplasts (Figure 6E). These results together suggest that OsSCD2 and OsSCD1 might function as a complex to regulate the clathrin-mediated vesicular trafficking.

**Cellulose abundance and distribution were altered in the dph1-1 mutant**

Plant cell walls play a fundamental role in determining plant growth (Wolf et al., 2012; Zhang et al., 2021a). Cellulose is the main polymer in most plant cell walls. Studies in Arabidopsis demonstrated that cellulose synthase (CESA) complexes are assembled in the Golgi apparatus and then transported to the PM by the vesicle-mediated post-Golgi trafficking pathways (Bashline et al., 2014; Polko and Kieber, 2019). To test whether OsSCD2 is involved in CESA transport to the PM, we performed a colocalization analysis of OsSCD2 with OsCESA3 and OsCESA8 in leaf epidermal cells of *N. benthamiana*. OsCESA3 and OsCESA8 share 82% and 86% amino acid sequence similarity with Arabidopsis CESA6 and CESA3, respectively (Carroll et al., 2012). OsSCD2 exhibited colocalization with both OsCESA3 and OsCESA8 at the

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**Figure 6** OsSCD2 physically interacts with OsSCD1. A and B, Identification of OsSCD2 protein interactors by IP–MS. Lysates from 1-week-old rice seedlings expressing either free GFP or OsSCD2-GFP were subjected to immunoprecipitation using anti-GFP beads, precipitated proteins with GFP or OsSCD2-GFP were separately loaded and shown as CBB stained SDS–PAGE gel (A). Summary of proteins that coprecipitated with OsSCD2-GFP and identified by mass spectrometry in three independent experiments (B). Protein scores were calculated by Mascot (Matrix Science). Listed proteins were not detected in the GFP control group. “--” indicate that the protein was not detected within the sample. C, BiFC assay showing that OsSCD2 physically interacts with OsSCD1 in AtCLC2-labeled punctate structures in leaf epidermal cells of *N. benthamiana*. White arrowheads indicate overlapping signals near the PM. Bars, 10 μm. D, Firefly LCI assay confirming interaction between OsSCD2 and OsSCD1 in *N. benthamiana* leaves. nLUC, cLUC, and OsCNGC9 were used as controls. Colored scale bar indicates the luminescence intensity in counts per second. E, Co-IP assay of interaction between OsSCD2 and OsSCD1 in rice protoplasts. Flag-OsSCD1 coimmunoprecipitated with OsSCD2-GFP but not with GFP alone. Marker size in kDa is on the right. It is noted that the slowly migrated bands at and above 100 kDa may represent modified OsSCD2 proteins, while bands at lower molecular weight may indicate degraded OsSCD2-GFP products.
PM and in a subpopulation of punctate compartments (Figure 7A), suggesting the possible involvement of OsSCD2 in the trafficking of OsCESAs.

To further investigate the effect of the OsSCD2 mutation on OsCESAs, we examined the subcellular distribution of OsCESA4 using anti-OsCESA4 antibodies (Xiong et al., 2010). Quantitative immunoblot analysis showed that total OsCESA4 protein levels were reduced in dph1-1 (Figure 7B).

Interestingly, a subcellular fractionation assay to separate the PM and endomembrane fractions detected a significantly higher proportion of OsCESA4 distributed to the endomembrane fraction in dph1-1 compared to the WT (Figure 7C), suggesting that the trafficking of OsCESAs in the dph1-1 mutant is partially blocked.
Consistent with the decreased accumulation of OsCESA4 at the protein level, RT-qPCR analysis showed that including OsCESA4, the expression levels of several main OsCESA genes were reduced in the mutant (Figure 7D). The expression levels of OsCESA3 and OsCESA8, which are required for primarily cell wall cellulose synthesis (Wang et al., 2010) as well as OsCESA4, OsCESA7, and OsCESA9, which are required for secondary wall cell wall cellulose synthesis (Tanaka et al., 2003), were significantly lower in the dph1-1 mutant compared with the WT (Figure 7D). We next observed the cell walls of sclerenchyma and parenchymal cells and found that they are significantly thinner in dph1-1 than those in WT and dph1-1 rescued line pG-DPH1 (Supplemental Figure S12). These phenotypic differences prompted us to postulate that cellulose synthesis was changed in the dph1-1 mutant. Supporting this hypothesis, the dph1-1 mutant was more sensitive to isoxaben (a cellulose synthesis inhibitor) treatment, as evidenced by decreased root length compared with WT (Supplemental Figure S13). Cell wall component determination showed that cellulose content decreased by ~8% in dph1-1 and cri-Osscd2 mutant plants relative to the WT (Figure 7E). A consequence was that the level of glucose (Glc), the major monosaccharide of cellulose, was also reduced. However, several primary monosaccharide components of hemicellulose or pectin, including arabinose (Ara), xylose (Xyl), rhamnose (Rha), and galactose (Gal) were significantly increased in the dph1-1 mutant compared with the WT (Figure 7F). These results collectively suggest that loss of OsSCD2 function affected cell wall biosynthesis.

Discussion

**DPH1 encodes a homolog of Arabidopsis SCD2, but its mutation leads to a different outcome compared to the Arabidopsis scd2 mutant**

We identified the rice dph1-1 mutant as a plant architecture mutant exhibiting pleiotropic defects in plant height, panicle morphology, and grain size (Figure 1). Reduced growth occurred throughout the entire life cycle, including its effect on seed size. Molecular cloning demonstrated that DPH1 encodes a protein homolog of AtSCD2 (Figure 3).

Like the Arabidopsis scd2 mutant (McMichael et al., 2013), the rice dph1-1 mutant had restricted cell growth causing a generalized semi-dwarfing phenotype (Figure 1). A signature phenotype of the Arabidopsis scd2 mutant, but not evident in the rice dph1-1 mutant (Supplemental Figure S14, A and B), was the asymmetrically dividing stomata and epidermal pavement cells, indicating an important role of AtSCD2 in cytokinesis (McMichael et al., 2013). In many plants, such as Arabidopsis, the stomata undergo asymmetrical division and always oriented in the same direction on leaves (Väthén and Bergmann, 2012); conversely, in grasses, such as rice, the stomata undergo asymmetrical division and always oriented in the same direction on leaves (Hepworth et al., 2018). Additionally, incongruous spaces or gaps between leaf pavement cells and clusters of small cells were also readily observed in the Arabidopsis scd2 mutant. Such phenotypic defects were not observed in leaf epidermal cells of the dph1-1 mutant (Supplemental Figure S14C). Therefore, it seems that OsSCD2 plays a minor role, if any, in stomatal division and leaf cell arrangement in rice, probably due to the distinctive differences in stomatal and leaf cell formation between Arabidopsis and rice.

**OsSCD2 may function in clathrin-dependent vesicular trafficking pathways**

Earlier studies in Arabidopsis showed that SCD1 and SCD2 cofractionate with CCVs (McMichael et al., 2013). In agreement with Arabidopsis, we identified OsSCD2 as a clathrin-associated protein (Figure 4). In eukaryotic cells, CCVs mediate vesicular traffic of cargos such as proteins between organelles in endocytosis and various post-Golgi trafficking pathways (McMahon and Boucrot, 2011). Our Immunoprecipitation and mass spectrometry (IP–MS) experiment identified several dynamin-related proteins (DRPs) and adaptor proteins in the OsSCD2 precipitate (Figure 6B). These proteins were reported to function as core clathrin-related proteins in plants (Fujimoto and Tsutsumi, 2014; Wang et al., 2016). Supporting the postulated role of OsSCD2 in vesicular trafficking, the dph1-1 mutant displayed defects in internalization of FM4–64 and FLS2-GFP from the PM in root cells (Figure 5C and Supplemental Figure S10), features previously demonstrated to be in part dependent upon CME (Dhonukshe et al., 2007; Smith et al., 2014; Mbengue et al., 2016). In addition to endocytosis abnormalities, recycling of PIN2-GFP from BFA bodies was delayed in the dph1-1 mutant (Figure 5, E and F), indicating that OsSCD2 might be involved in post-Golgi trafficking. It is possible that the abnormal internalization in the dph1-1 mutant caused the partial functioning of OsSCD2 in exocytosis. Supporting this notion, studies from yeast, metazoans, and Trypanosoma brucei demonstrated that defective exocytosis indeed affects endocytosis (Hirata et al., 2015; Jose et al., 2015; Boehm et al., 2017).

**OsSCD2 could be involved in cell wall biosynthesis**

Sturdy plant architecture requires relevant materials to build strong cell walls for support (Polko and Kieber, 2019). Cellulose is the predominant component of plant cell walls and are synthesized at the PM by CESA complexes (CSCs) (Cosgrove, 2005; Li et al., 2014; McFarlane et al., 2014). CESA proteins are initially synthesized in the endoplasmic reticulum (ER) and then assembled into the CSC in the ER and the Golgi apparatus. CSCs follow the secretory pathway to the PM, and thus intracellular trafficking of the CESAs is crucial in modulating cellulose synthesis in plants (Crowell et al., 2010; Worden et al., 2012; Bashline et al., 2014). Studies in stl1 stl2 double mutants showed reduced CSC insertion rates and motility at the PM, resulting in cellulose deficiency, and indicated that Golgi-localized STELLO (STL) proteins function in CSC assembly and exocytosis (Zhang et al., 2016). Inhibition of V-ATPase activity in the det3 mutant reduced the ability to acidify the TGN/EE, leading to impaired secretion and recycling of a brassinosteroid.
receptor and CSCs to the PM (Luo et al., 2015). Interestingly, the shou1 shou4 double mutant had enhanced exocytosis, resulting in increased accumulation of PM-localized CESAs and elevated levels of amorphous instead of crystalline cellulose (Polko et al., 2018).

In addition to the involvement of exocytosis in the trafficking of CESAs, it has been proposed that CESAs are also regulated by the CME pathway. In endocytosis-defective mutants, such as ap2m, twd40-2, and amiTIR-TML line, PM-located CESAs were over-accumulated and their movements slowed down, leading to reduced efficiency in cellulose biosynthesis (Bashline et al., 2013, 2015; Sánchez-Rodríguez et al., 2018). However, the exocytosis routes of CESAs are not fully understood and the role of CCVs in this process remains ambiguous. Our results showed that OsSCD2 colocalized with OsCESA3 and OsCESA8 (Figure 7A), suggesting a possible role of OsSCD2-involved clathrin-associated vesicular trafficking pathways in the trafficking of OsCESAs. Supporting this notion, subcellular fraction assay showed the increased ratio of OsCESA4 in the endomembrane fraction (Figure 7C), indicating that loss of OsSCD2 function affected the trafficking efficiency of CESAs.

In addition to the subcellular fractionation difference of OsCESA4, we found that OsCESAs were significantly reduced at the transcriptional and translational levels in the dph1-1 mutant (Figure 7B and D). Furthermore, we found that the dph1-1 mutant is more sensitive to isoosben treatment (Supplemental Figure S13), and confirmed the significant reduced cellulose content in the dph1-1 mutant (Figure 7E). Cellulose plays a critical role in plant growth, disruption of cellulose synthesis usually caused defects in cell expansion, evidenced by various phenotypic defects including reduced plant height (Williamson et al., 2001; Tanaka et al., 2003; Zhang and Zhou, 2011; McFarlane et al., 2014; Polko and Kieber, 2019). A recent study reported that loss of function of VPS18, which encoding a core component of vacuolar trafficking pathway, enhanced the secretion of pectin in the cell wall, accompanied by the increased level of pectin and higher expression level of pectin methyltransferase gene PME1 (Hou et al., 2021). Therefore, it is possible that the reduced level of OsCESAs may be due to an indirect feedback regulation of the disrupted CESA trafficking route. Monosaccharide composition analysis of cell wall revealed that Glc content was reduced, while Rha, Ara, and Gal content were significantly increased in the dph1-1 mutant (Figure 7F). Rha, Ara, and Gal act as the major raw materials of rhamnogalacturonan-I, which is the main component of pectin. These results suggested that the mutation in OsSCD2 not only affected cellulose deposition but also altered noncellulosic carbohydrates such as pectin, probably by unidentified compensatory effects. Future efforts in characterizing CESAs and pectin synthesis-related proteins motility in the dph1-1 mutant would facilitate our understanding of whether OsSCD2 is directly involved in cell wall biosynthesis.

Rice DRP OsDRP2B was also proposed to function in clathrin-related membrane trafficking pathways; its mutation affects the distribution of certain proteins essential for cellulose biosynthesis in secondary cell walls (Xiong et al., 2010). It will be interesting to investigate the biochemical and genetic relationships of both the OsDRP2B and OsSCD2 proteins in regulating cell wall component trafficking in rice.

It is noted that in addition to clathrin association, we also provided several lines of evidence that OsSCD2 together with OsSCD1 and clathrin were associated with intracellular structures that colocalize with microtubule (Supplemental Figure S58). Consistent with our results, it has been reported that CSC-containing vesicles move along microtubule in plant cells (Paredes et al., 2006; Lei et al., 2015). Myosin motors can drive vesicle movement along actin filaments and thus play essential roles in secretory pathways and exocytosis in eukaryotic cells (Hammer and Sellers, 2011). Very recent studies in Arabidopsis demonstrated that myosin XI is a key regulator in CSC delivery to the PM and myosin XI interacts with the exocyst complex via its globular tail domain to facilitate vesicle tethering during exocytosis (Zhang et al., 2019b, 2021b). A myosin-TH1 domain-containing protein (LOC_Os01g43360) was identified as a putative OsSCD2 interactor in our proteomic analysis (Figure 6B), although it lacks the typical ATPase motor domain of myosin. It will be interesting to investigate how the SCD complex coordinates microtubule and actin in vesicle trafficking.

Materials and methods

Plant materials and growth conditions

The dph1-1 mutant was isolated from a tissue culture-derived population of the rice (O. sativa) japonica cultivar Kitaake. Rice plants were grown in greenhouses or paddy fields at the Chinese Academy of Agricultural Sciences in Beijing during the natural growing season. For seedling work, seeds were germinated and grown on 1/2 Murashige and Skoog (MS) solid or liquid media in a growth chamber (14-h/30°C light and 10-h/25°C darkness at 70% relative humidity).

Microscopy observation

For observation of internode cells, sections of internode tissues were prepared by a modified pseudo-Schiff propidium iodide staining technique as previously described (Ogawa et al., 2011). Resin-embedded root tissues were sectioned (5 mm in thickness) using a Leica RM2265 microtome, stained in 0.05% (w/v) toluidine blue for 5 min, and examined under a Leica CTR5000B microscope. Immunogold labeling for scanning and transmission electron microscopy was performed as described previously (Ren et al., 2014). ImageJ software (http://rsb.info.nih.gov/ij/) was used in quantification of data.

Flow cytometry

Rapidly growing 1-week-old seedlings were chopped into very fine slices in ice-cold Galbraith’s buffer containing 45-
mM MgCl₂, 20-mM MOPS, 30-mM sodium citrate, and 0.1% (v/v) Triton X-100, pH 7.0. Crude extract containing isolated cells was filtered through a 42-mm nylon mesh, followed by 4 μg/mL 4′,6-diamino-phenylindole staining and immediately analyzed using FACS Aria II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of 10,000 events were recorded and three independent experiments were performed. Data were analyzed using the ModFit LT (Verity Software House Inc., Topsham, ME, USA) and FlowJo V10 software (Tree Star, Inc., Ashland, OR, USA).

**Map-based cloning of the DPH1 allele**

To map the DPH1 locus, more than 1,100 mutant individuals were selected from an F₂ population derived from a cross between the dph1-1 mutant and japonica cultivar IRAT129. Molecular markers used for fine mapping were designed based on nucleotide polymorphisms in the candidate regions between japonica cultivar Kitaake and IRAT129. Primers used for generating molecular markers are listed in Supplemental Table S1. Fine mapping delineated the DPH1 locus to a 73-kb genomic region between markers 1–40 and 1–25. Annotations of nine predicted open reading frames (ORFs) were from the rice genome database (O. sativa v7.0), followed by subsequent genomic sequencing analysis.

**Vector construction and rice transformation**

For genetic complementation tests, a 6,623-bp genomic fragment covering the entire coding region of LOC_Os01g70320, plus 3,032-bp upstream and 818-bp downstream sequences, was amplified from WT DNA. PCR products were cloned into the binary vector pCAMBIA1305.1 and confirmed by plasmid sequencing.

To generate an OsSCD2 RNAi construct, two inverted repeats of 400 bp were amplified from the WT and the PCR products were sequentially cloned into the pCUbi1390-DFAD2 vector followed by sequencing for confirmation. To create OsSCD2 CRISPR lines, a 20-bp gene-specific spacer sequence of OsSCD2 was cloned into the CRISPR–Cas9 expression vector followed by sequencing confirmation. CRISPR transgenic plants were confirmed by DNA sequencing combined with phenotyping. To generate GFP-tagged OsSCD2 transgenic plants, the WT OsSCD2 coding sequence was amplified, and PCR products were cloned into the pCAMBIA1390 vector under the control of the CaMV35S cauliflower mosaic virus promoter to generate translationally fused p35S:OsSCD2-GFP constructs. Transgenic plants were confirmed by PCR amplification, western blots, and fluorescence. Different constructs were introduced into Agrobacterium tumefaciens strain EHA105 to infect WT or dph1-1 mutant calli. The primers for creating the above constructs are listed in Supplemental Table S1.

**RT-qPCR analysis and immunoblot analyses**

RT-qPCR analyses were performed on an Applied Biosystems 7500 real-time PCR system using SYBR Premix Ex Taq Kit (Takara Clontech, Shiga, Japan). The rice UBIQUITIN gene was used as an internal control. Data from three independent biological replicates were analyzed; each replicate sample was extracted from a pool of tissues collected from at least three individual plants. Primers used in this assay are listed in Supplemental Table S1.

For immunoblot analyses, proteins extracted from tissues were subjected to SDS–PAGE gels followed transferred to nitrocellulose membranes. Antibody–antigen reactions were detected with the ECL detection reagent (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by the ECL detection system (Odyssey-Fc, LI-COR). Anti-GFP (dilution 1:5,000; Roche, Basel, Switzerland), anti-Flag (dilution 1:5,000; Sigma-Aldrich, St Louis, Missouri, USA), and anti-UGPase (dilution 1:5,000; Agrisera) antibodies are commercially available. Anti-γTip, Anti-OsCESA4 antibodies were previously described (Xiong et al., 2010; Wang et al., 2016b).

**Phylogenetic analysis**

Homologs of OsSCD2 were identified using the BlastP tool of the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments of homologs were performed using ClustalX version 2.0 and GeneDOC software. The phylogenetic tree was constructed using the neighbor-joining method in MEGAX software.

**Subcellular localization**

The OsSCD2 coding sequence for subcellular localization of OsSCD2 in rice protoplasts was cloned into pANS80 vector with a GFP tag and driven by the d35S promoter. Protoplast transformation followed previously described procedures (Wang et al., 2019).

For transient expression analysis in N. benthamiana leaf epidermal cells or protoplasts, the OsSCD2 coding sequence was cloned into pCAMBIA1305 vector with a GFP tag and driven by the mannopine synthase TR2’ promoter. The construct was introduced into A. tumefaciens strain EHA105 and infiltrated into N. benthamiana leaves. Leaf epidermal cells or protoplasts lysed from leaves were observed two days after infiltration.

For the colocalization analysis, The Pearson–Spearman Correlation plugin for ImageJ was used to analyze images acquired from confocal laser microscopy and obtain the Pearson correlation coefficients as colocalization readouts (French et al., 2008; Ren et al., 2014). A complete protoplasts region was defined as regions of interest and threshold was set to 10. Colocalization data pooled from three independent biological replicates, each replicate sample was extracted from a pool of tissues collected from three individual plants, at least eight protoplast cells were analyzed in each sample.

For immunofluorescence staining assays, N. benthamiana leaves transiently transformed with OsSCD2-GFP construct or rice roots and leaf sheath of OsSCD2-GFP transgenic plants were collected and fixed in 4% (v/v) paraformaldehyde in PEM buffer (0.1-M PIPES, 2-mM EGTA, and 1-mM MgSO₄, pH 7.0) with vacuum infiltration for 20 min. After
twice rinsing in PEM buffer, the samples were treated with 1% (v/v) Triton X-100 for 1 h and blocked with 2% (w/v) Bovine serum albumin (BSA) for 1 h in PBS buffer. Samples were incubated with monoclonal mouse anti- tubulin antibody (1:200; Sigma-Aldrich) or anti-CHC antibodies (1:100; Wang et al., 2013) at 4°C overnight. After three PBST washes, samples were incubated with the secondary antibody Texas Red 555p-conjugated goat anti-mouse IgG (1:300; Invitrogen, Waltham, Massachusetts, USA) for 40 min at 37°C in darkness and washed. The samples were observed using LSM700 or LSM980 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). GFP signals with white light laser at emission wavelength of 510–550 nm were recorded with the excitation wavelength at 488 nm, Red signals with white light laser at emission wavelength of 575–630 nm were recorded with the excitation wavelength at 555 nm.

Chemical treatments
The FM4–64 internalization assay was performed in root epidermal cells. Roots were immersed in 0.5 × MS liquid media with 5-μM FM4–64 (Life Technologies, Carlsbad, California, USA) for 5 min on ice, washed once, and internalization assays were conducted at room temperature for 30 min. For BFA trafficking inhibition experiments, PIN2-GFP transgenic plants roots were pre-treated with 50-μM BFA (Sigma-Aldrich) for 90 min in 1/2 MS liquid media and followed by a single washout. After BFA washout, roots were imaged in different times (0, 30, 60, and 90 min). For cellulose synthesis inhibitor isoxaben treatment, seeds were germinated and placed in 96-well plates, water-based 0/5/10/lose synthesis inhibitor isoxaben treatment, seeds were germinated and placed in 96-well plates, water-based 0/5/10/15/20% isoxaben (Sigma-Aldrich) solutions were applied and assays were conducted after 3 d.

Immunoprecipitation and mass spectrometry (IP–MS)
IP–MS analyses were performed as described previously (Ren et al., 2020). Briefly, ~10 g of 1-week-old transgenic seedling tissue expressing free GFP or OsSCD2-GFP were separately homogenized in ice-cold protein extraction buffer containing 50 mM Tris-MES, pH 7.5, 1 mM MgCl2, 0.5 M sucrose, 10 mM EDTA, 5 mM DTT, 0.1% (v/v) Nonidet P-40, and 1 × Complete Protease Inhibitor Cocktail (Roche). The samples were centrifuged at 20,000 g for 15 min at 4°C to remove the cell debris and supernatants were incubated with GFP-Trap magnetic beads (ChromoTek, Planegg, Germany). Immunoprecipitation samples were loaded to SDS–PAGE gels and stained with coomassie brilliant blue (CBB), followed by mass spectrometry analysis (Huada Gene Research, Beijing, China).

For mass spectrometry, sample proteins in lanes were cut from the gel followed by dehydration and digestion with trypsin. Digested peptides were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using the Q Exactive Orbitrap mass spectrometer system (Thermo Fisher Scientific). The system delivered solvents A [0.1% (v/v) formic acid] and solvents B [99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid] at 0.4-μL/min flow rate to load the peptides and 0.2-μL/min to elute peptides. Eluted peptides loaded into gradual gradient from 3% (v/v) B to 40% (v/v) B followed by fast gradient from 40% (v/v) B to 90% (v/v) B. The mass spectrometer was set with positive ion mode and the main mass spectrometry parameters were as follows: spray voltage of 1,800 V, capillary temperature of 360°C, mass spectral resolution 17,500, 375–1,400 m/z MS1 scan range. Mass spectra data were analyzed using Proteome Discoverer software (Thermo Fisher Scientific) and searched against O. sativa amino acid sequence database in The Institute for Genomic Research using the Mascot search engine server (http://www.matrixscience.com/) to finish protein identification.

In vivo protein interaction assays
The coding sequence of OsSCD2 for luciferase complementation assays was fused upstream of the N-terminus of LUC (nLUC) in the pCAMBIA-nLUC vector, and the coding sequences of OsSCD1 and OsCNGC9 were fused downstream of C-terminus of LUC (cLUC) in the pCAMBIA-cLUC vector. These constructs were transformed into A. tumefaciens strain EHA105 infiltrated into N. benthamiana leaves. Relative LUC activity was measured after 2 d as previously described (Wang et al., 2019).

Full-length coding sequences of OsSCD2 and OsSCD1 for BiFC assays were cloned into the pSPYNE173 and pSPYCE vector, respectively. These were then transformed into A. tumefaciens and infiltrated N. benthamiana leaves as previously described (Ren et al., 2014). Yellow fluorescent protein (YFP) signals were monitored and imaged by a LSM980 confocal laser scanning microscope (Carl Zeiss) after 2 d. YFP signals with white light laser at emission wavelength of 525–575 nm were recorded with the excitation wavelength at 510 nm.

The coding sequences of OsSCD2 and OsSCD1 for Co-IP assays were cloned into pCAMBIA1305-GFP and pCAMBIA1300-221-Flag vectors, respectively. Rice protoplasts isolated from WT plants were transfected with various combinations of constructs for transient expression. Total protein was extracted with protein extraction buffer (150 mM KCl, 50 mM HEPES, pH 7.5, 0.1% Triton-X 100, 1 mM DTT, and 1 × Complete Protease Inhibitor Cocktail) after incubation for 14 h, following guidelines for Co-IP assays. Primers for the constructs are listed in Supplementary Table S1.

Cell wall analysis
Second internodes of the WT, dph1-1 mutant and crip OsSCD2 plants were used for cell wall analysis. For monosaccharide analysis, alcohol-insoluble residues (AIRs) were prepared as previously described (Xiong et al., 2010). In brief, AIR samples were hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121°C for 90 min and centrifuged at 10,000 g for 10 min to collect supernatants that were air dried and reduced with sodium borohydride (2 mg/mL in 1 M ammonium hydroxide). After acetylation by acetic anhydride, the
generated alditol acetates were extracted in ethyl acetate and analyzed by an Agilent 7890 Series GC system equipped with a 5975B inert XL MSD. For crystalline cellulose analysis, the remains after TFA treatment were hydrolyzed in Updegoff reagent (acetic acid: nitric acid: water, 8:1:2 v/v) at 100°C for 30 min and the cellulose contents were quantified by anthrone assays.

Subcellular fractionation

One-week-old OsSCD2-GFP transgenic seedlings were used for subcellular fractionation as previously described (Wang et al., 2016b). Briefly, the homogenates of samples were filtered through millipore cloth and then centrifuged to remove cellular debris. The supernatant was centrifuged at 100,000g for 1 h at 4°C, the supernatant and pellet obtained after centrifuging were assigned as S100 and P100 fractions, respectively. The P100 fraction was placed in different resuspension solutions treatment (high salt buffer, alkaline buffer, Triton X-100 buffer and SDS buffer). After 20-min incubation on ice, the resuspension solutions were centrifuged to obtain S100 and P100 fractions for immunoblot analysis. For aqueous two-phase separation of the PM and endomembrane fractions, 2 g rice second internode samples were ground into fine flour and homogenized in extraction buffer (25-mM Tris–HCl, pH 7.5, 2-mM EDTA, 0.25-M sucrose, 2-mM DTT, 10% glycerol, 15-mM β-mercaptoethanol, and 1 × Complete Protease Inhibitor Cocktail) on ice. To separate the proteins into polyethylene glycol (PEG) and dextran (DEX) fractions the supernatant was centrifuged at 100,000 × g for 1 h at 4°C to obtain total membrane proteins. These were further resuspended in fractionation buffer (5-mM K2HPO4–KH2PO4, pH 6.8, 0.25-M sucrose, 1-mM DTT, 6.2% PEG3350, and 6.2% Dextran T500) and mixed thoroughly. Two-phase separation will be observed after centrifugation at 5,000g for 10 min at 4°C. The PEG and DEX fractions were separately collected and centrifuged at 100,000g for 1 h, followed by dissolving in suspension buffer (2-mM Tris–HCl, pH 6.5, 1-mM DTT, and 0.25-M sucrose). Different protein fractions were identified with anti-OsCESA4 polyclonal antibody (Xiong et al., 2010). Anti-OsCNGC9 (1:1,000; Wang et al., 2019) and Anti-Arf1 (1:1,000, Agrisera) antibodies were used as markers for the PM and endomembrane, respectively.

Statistical analysis

For statistical analysis, two-tailed Student’s t test was used to analyze the significance between two noted samples (**P < 0.01 and *P < 0.05).

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: OsSCD2 (Os01g0928100), OsSCD1 (Os01g0575500), and OsPIN2 (Os06g0660200).

Supplemental data

The following materials are available in the online version of this article.

- Supplemental Figure S1. Comparisons of root cells between the WT and dph1-1 mutant.
- Supplemental Figure S2. Phenotypic characterization of the dph1-1 allelic mutant.
- Supplemental Figure S3. Phenotypic characterization of RNAi and CRISPR transgenic lines of OsSCD2.
- Supplemental Figure S4. Phylogenetic tree and amino acid sequence alignment of OsSCD2 and its homologs.
- Supplemental Figure S5. Expression pattern of the OsSCD2 gene and phenotypic characterization of OsSCD2-GFP complemented line.
- Supplemental Figure S6. Membrane association assay of OsSCD2 protein.
- Supplemental Figure S7. Antibodies specificity evaluation and localization distribution of clathrin in rice root tip cells.
- Supplemental Figure S8. OsSCD2 colocalized with microtubule.
- Supplemental Figure S9. Colocalization analysis of OsSCD2 and different organelle markers in leaf protoplasts of N. benthamiana.
- Supplemental Figure S10. FLS2 endocytosis was impaired in the dph1-1 mutant.
- Supplemental Figure S11. BiFC assay of OsSCD2 and OsSCD1.
- Supplemental Figure S12. Comparison of cell wall thickness between the WT, dph1-1 mutant, and complementation plant pG-DPH1.
- Supplemental Figure S13. The dph1-1 mutant is sensitive to isoxaben treatment.
- Supplemental Figure S14. Stomata morphology and leaf epidermal cell arrangement in WT and dph1-1.
- Supplemental Table S1. Primers used in this study.
- Supplemental Data Set S1. Detailed LC–MS/MS Datasheet of proteins identified with OsSCD2-GFP in this study.

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Conflict of interest statement. The authors declare that they have no competing interests.

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