Functional diversification of *Arabidopsis* SEC1-related SM proteins in cytokinetic and secretory membrane fusion

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Sec1/Munc18 (SM) proteins contribute to membrane fusion by interacting with Qa-SNAREs or nascent *trans*-SNARE complexes. Gymnosperms and the basal angiosperm *Amborella* have only a single *SEC1* gene related to the *KEULE* gene in *Arabidopsis*. However, the genomes of most angiosperms including *Arabidopsis* encode three SEC1-related SM proteins of which only KEULE has been functionally characterized as interacting with the cytokinesis-specific Qa-SNARE KNOLLE during cell-plate formation. Here we analyze the closest paralog of KEULE named SEC1B. In contrast to the cytokinesis defects of *keule* mutants, *sec1b* mutants are homozygous viable. However, the *keule sec1b* double mutant was nearly gametophytically lethal, displaying collapsed pollen grains, which suggests substantial overlap between SEC1B and KEULE functions in secretion-dependent growth. SEC1B had a strong preference for interaction with the evolutionarily ancient Qa-SNARE SYP132 involved in secretion and cytokinesis, whereas KEULE interacted with both KNOLLE and SYP132. This differential interaction with Qa-SNAREs is likely conferred by domains 1 and 2a of the two SM proteins. Comparative analysis of all four possible combinations of the relevant SEC1 Qa-SNARE double mutants revealed that in cytokinesis, the interaction of SEC1B with KNOLLE plays no role, whereas the interaction of KEULE with KNOLLE is prevalent and functionally as important as the interactions of both SEC1B and KEU with SYP132 together. Our results suggest that functional diversification of the two SEC1-related SM proteins during angiosperm evolution resulted in enhanced interaction of SEC1B with Qa-SNARE SYP132, and thus a predominant role of SEC1B in secretion.

membrane traffic | cell-plate formation | secretion | Qa-SNAREs | SEC1/Munc18

Membrane fusion in eukaryotes is mediated by the formation of *trans*-complexes between SNARE proteins C-terminally anchored in adjacent membranes. Sec1/Munc18 (SM) proteins assist in SNARE complex formation (1). The family of SM proteins comprises four members that act at different subcellular locations [SEC1, plasma membrane; SLY1, endoplasmic reticulum (ER), and Golgi; VPS45, *trans*-Golgi network (TGN)-early endosome; VPS33, late endosome-lysosome/vacuole] and are evolutionarily conserved across the eukaryotes (2). VPS45 and VPS33 have also been studied in the flowering plant *Arabidopsis* (3–6). The plasma membrane-localized Sec1p of yeast has counterparts in multicellular eukaryotes, with the latter often occurring in several isoforms. For example, Munc18 isoforms in mammals are specialized toward specific tasks or are expressed in tissue-specific ways (7). The *Arabidopsis* genome codes for three variants of Sec1p-related SM proteins, named KEULE (KEU), SEC1A, and SEC1B (8). KEULE has been studied in some detail, whereas SEC1A and SEC1B have been barely touched. KEULE is essential for vesicle fusion by which the partitioning membrane named "cell plate" is formed in cytokinesis (8–12). KEULE has recently been proposed also to coordinate that membrane fusion with the dynamics of phragmoplast microtubules supporting cell plate formation (13). KEULE (named SEC11) has also been implicated in trafficking to the plasma membrane in interphase by interacting with PEN1 (also known as SYR1 or SYP121), a Qa-SNARE protein involved in ABA response, pathogen attack, and programmed stomatal closure (14, 15). It is not known whether the two other SEC1-related proteins play different roles than KEULE or whether there is substantial functional overlap between the three SEC1 isoforms.

Here we analyze the *Arabidopsis* SEC1 isoform SEC1B in regard to functional requirement, subcellular localization, and Qa-SNARE interaction, in comparison with the cytokinesis-essential isoform KEULE. SEC1B overlapped functionally with KEULE in general secretion and, to a lesser extent, in cytokinesis. However, unlike KEULE, SEC1B almost failed to interact with Qa-SNARE KNOLLE (KN) in cytokinesis but interacted

Significance

Membrane fusion is a fundamental process of eukaryotic cells required for subcellular organization and cell–cell communication, involving SNARE proteins and regulatory Sec1/Munc18 (SM) proteins. In plant cytokinesis, membrane vesicles delivered to the cell-division plane fuse with one another to form the partitioning membrane, which requires cytokinesis-specific Qa-SNARE KNOLLE and interacting SM protein KEULE in *Arabidopsis*. KEULE has a paralog named SEC1B, which originated through gene duplication and subsequent functional diversification during angiosperm evolution. Biochemical interaction studies and analysis of relevant double mutants reveal that a predominant interaction of SEC1B with Qa-SNARE SYP132 entailed its preferential role in secretion, whereas KEULE acquired a unique role in cytokinesis through its interaction with cytokinesis-specific Qa-SNARE KNOLLE.

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strongly with the ancient Qa-SNARE SYP132, indicating a prominent role for SEC1B in secretory traffic to the plasma membrane.

Results

SM Protein SEC1B Is Nonessential Because of Functional Overlap with Its Paralog KEULE. From an evolutionary perspective, gymnosperms like Norway spruce (*Picea abies*), the basal angiosperm *Amborella*, the basal dicot *Aquilegia*, and the secondarily simplified monocot *Spirodela* all encode only a single SEC1 isoform (Fig. 1 and *SI Appendix*, Fig. S1) (16–23). Most dicot and monocot angiosperms have three SEC1 isoforms. Two copies of KEULE/SEC1B appear to have arisen by gene duplication independently in the two angiosperm lineages, whereas the gene duplication giving rise to *SEC1A* and *KEULE*/*SEC1B* may have occurred before the monocot–dicot split some 140 million y ago (24). The two copies of the *KEULE*/*SEC1B* gene are most strongly diverged by sequence in the Brassicaceae and allied species.

In *Arabidopsis*, the two SM proteins, KEULE and SEC1B, are closely related by sequence (69% identity, 82% similarity) (*SI Appendix*, Fig. S2) and might thus perform similar functions. One criterion is the subcellular localization of the protein. HA-tagged KEULE accumulates at the forming cell plate of dividing seedling root cells, in addition to cytosolic staining in both dividing and nondividing root cells (Fig. 2*A*) (25). mRFP-tagged SEC1B was detected in the cytosol, at the plasma membrane, and the forming cell plate in seedling root cells (Fig. 2 and *SI Appendix*, Fig. S3*A*). SEC1B colocalized with cytokinesis-specific Qa-SNARE KNOLLE at the forming cell plate (Fig. 2*B*). At the plasma membrane, SEC1B also colocalized with the Qa-SNARE SYP132 (Fig. 2*C*). SEC1B localization was insensitive to brefeldin A (BFA), a fungal toxin interfering with exchange factors for ARF small GTPases (ARF-GEFs) (*SI Appendix*, Fig. S3*B*) (26). This observation suggests that SEC1B, like KEULE, localizes to the cell plate and the plasma membrane independently

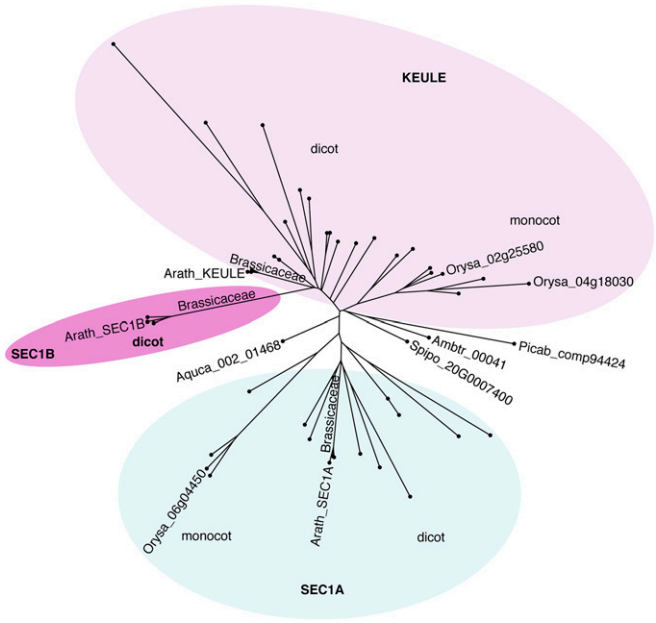

Fig. 1. Simplified phylogenetic tree of plant SEC1 proteins. The phylogenetic tree was generated using the neighbor-joining method in the CLC workbench program (abridged). Note that KEULE and SEC1B evolved differentially from SEC1A and that SEC1B is only present in dicot plants. Ambtr, *Amborella trichopoda* (basal dicot); Aquca, *Aquilegia caerulea* (basal angiosperm); Arath, *Arabidopsis thaliana* (Brassicaceae); Orysa, *Oryza sativa* (Monocot); Picab, *Picea abies* (gynmosperm); Spipo, *Spirodela polyrhiza* (basal monocot). See *SI Appendix*, Fig. S1 for a detailed phylogenetic tree.

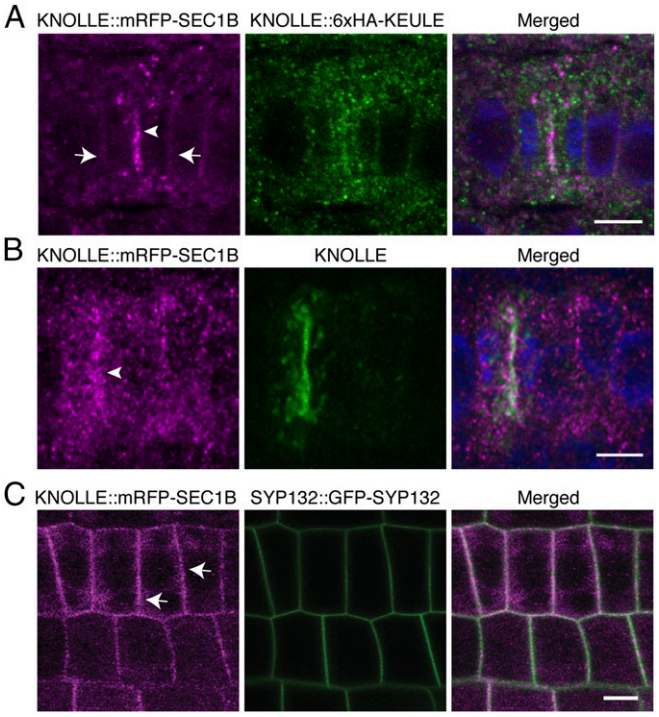

Fig. 2. Subcellular localization of KNOLLE::mRFP-SEC1B. Immunofluorescence (*A* and *B*) and live-imaging (*C*) of KNOLLE::mRFP-SEC1B in seedling roots: (*A*) KNOLLE::mRFP-SEC1B (magenta) and KNOLLE::6xHA-KEULE (green) labeled with anti-HA antibody in fixed seedling root. (*B*) KNOLLE::mRFP-SEC1B (magenta) and KNOLLE (green) labeled with anti-KNOLLE antiserum in fixed seedling root. (*C*) Live imaging of KNOLLE::mRFP-SEC1B (magenta) and SYP132::GFP-SYP132 (green) in seedling roots. Note that mRFP-SEC1B locates at the cell division plane (arrowheads in *A* and *B*) and plasma membrane (arrows in *A* and *C*) (see also *SI Appendix*, Fig. S3*A*). Note also that the punctate signal of mRFP-SEC1B becomes more prominent after fixation (compare also *Right* and *Left* panels in *SI Appendix*, Fig. S3*A*). DAPI was used for staining nuclei (blue). (Scale bars: 10 μm.)

of membrane traffic from the Golgi/TGN (25). In contrast, the more distantly related SEC1A was only detected in the cytosol of seedling root cells (*SI Appendix*, Fig. S3*C*).

The functional relatedness of SEC1B to KEULE was also revealed by the ability of SEC1B when strongly expressed from the *KNOLLE* cassette (*KNOLLE::mRFP-SEC1B*) to rescue the *KEULE* deletion mutant *keule*^{MM125} (Fig. 3*A* and *SI Appendix*, Fig. S3 *D* and *F* and Table S1) (8). The same rescuing effect had been demonstrated for *KNOLLE::6xHA-KEULE* (25). In contrast, *KNOLLE::mRFP-SEC1A* failed to rescue *keule*^{MM125} (*SI Appendix*, Fig. S3 *E* and *G* and Table S1).

To reveal functional requirements of the *SEC1B* gene, we analyzed a knockout allele caused by T-DNA insertion (GABI-KAT_601G09) (*SI Appendix*, Fig. S4 *A* and *B*). The level of mRNA accumulation for both *SEC1A* and *KEULE* appeared not to be altered in the *sec1b* knockout mutant (*SI Appendix*, Fig. S4*B*). Thus, there was no compensatory up-regulation of *KEULE* expression in response to the loss of SEC1B protein. The *sec1b* mutant plants were homozygous viable, fertile, and phenotypically normal and thus indistinguishable from wild-type plants (Fig. 3*A*). Moreover, *sec1b* mutant seedlings displayed normal root hairs (*SI Appendix*, Fig. S4*C*), in contrast to *keule* mutant seedlings (8). Thus, *SEC1B* appeared not to play an essential role in *Arabidopsis* development. It should be noted, however, that in all tissues and developmental stages analyzed, *KEULE* was expressed about 10-fold more strongly than *SEC1B* (*SI Appendix*, Fig. S4*D*) (27).

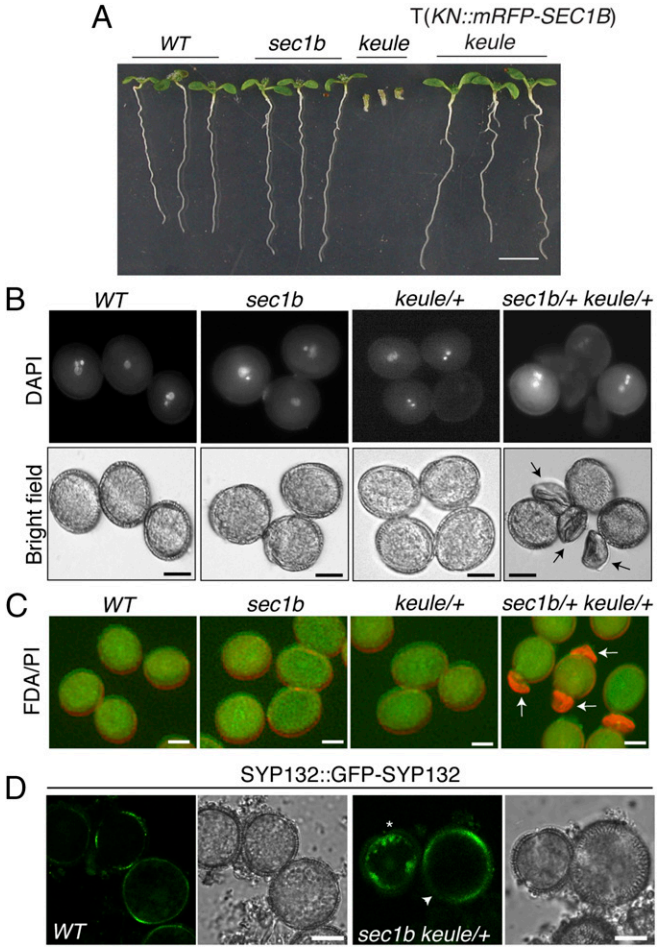

Fig. 3. Analysis of *sec1b* single and *sec1b keule* double mutant. (*A*) Mutant phenotype. Note that the *sec1b* single mutant is indistinguishable from wild-type (*WT*), unlike the *keule* single mutant, which is fully rescued with a *KNOLLE(KN)::mRFP-SEC1B* transgene (T) (see *SI Appendix*, Table S1 for genetic analysis). (*B* and *C*) Mature pollen of *sec1b keule* double mutant. Pollen were stained with DAPI (*B*) or with fluorescein diacetate (FDA) (green) and propidium iodide (PI) (red) (*C*). Note that *sec1b keule* double-mutant pollen are collapsed and not labeled with DAPI or FDA (arrows in *B* and *C*; see *SI Appendix*, Fig. S5*B* for images in separate channels; *SI Appendix*, Table S2*C* for quantification). (*D*) SYP132::GFP-SYP132 expression in developing pollen of *sec1b keule*/+ plants. Note the aggregates of GFP-SYP132 in the *sec1b keule* double mutant (asterisk, *Right*) compared with the accumulation of GFP-SYP132 in a *sec1b* single mutant at the plasma membrane (arrowhead, *Right*) as in wild type (*Left*). See also *SI Appendix*, Fig. S7 for overview and line intensity profiles and *SI Appendix*, Table S3 for quantitative measurement. (Scale bars: 2 mm in *A* and 10 μm in *B*–*D*.)

Unlike the *sec1b* knockout mutant, elimination of *KEULE* gene function is zygotically lethal, resulting in morphologically abnormal seedlings (Fig. 3*A*) (8). The *keule* mutant embryos display characteristic cytokinesis defects, including cell-wall stubs and unfused vesicles in the plane of cell division (10). Interestingly, no *sec1b keule* double homozygotes were detected among the seedling progeny of the selfed double heterozygotes. Instead, many unfertilized ovules were observed in the siliques of selfed plants (*SI Appendix*, Fig. S5*A* and Table S2*A*), suggesting that reproduction was compromised. Reciprocal crosses with wild type revealed that both embryo sac and pollen were affected. Strongly reduced transmission through pollen (2%) and the embryo sac (7%) indicated that the *sec1b keule* double mutant was nearly gametophytically lethal (*SI Appendix*, Table S2*B*). Moreover, ∼25% of the pollen produced by doubly heterozygous plants were collapsed (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S5*B* and Table S2*C*). Because the asymmetric division of the microspore was not obviously impaired (*SI Appendix*, Fig. S6 *K* and *L*), the pollen phenotype might rather reflect defects during the growth phase of the pollen. To analyze this further, we examined the subcellular localization of Qa-SNARE SYP132 fused to GFP. While GFP-SYP132 was detected at the plasma membrane in pollen of wild type as well as *sec1b* mutant or *keule*-heterozygous plants, aggregates were detected in the cytoplasm in ∼47% of the microspores produced by *sec1b keule*/+ plants (Fig. 3*D*; see *SI Appendix*, Fig. S7 for line intensity profiles, *SI Appendix*, Table S3 for quantitative measurement, and Movie S1). In conclusion, KEULE and SEC1B were functionally sufficiently similar to one another to largely compensate for each other's absence in the single mutants, except for the main role of KEULE in cytokinesis.

Physical Interactions of SEC1-Like SM Proteins with SYP1 Qa-SNAREs. SM proteins exert their effects by interacting with Qa-SNAREs or incipient *trans*-SNARE complexes (1). KEULE has been shown to interact with the open form of monomeric KNOLLE, which requires the sequence-specific linker separating the N-terminal helices from the SNARE domain (25). We performed several assays to determine whether and to what extent KEULE and SEC1B can interact with the two SYP1 Qa-SNAREs involved in membrane fusion during cytokinesis: KNOLLE and SYP132 (25, 28, 29).

In semiquantitative yeast two-hybrid assays, KEULE interacted with both KNOLLE and SYP132 in their constitutively open forms to similar extents (*SI Appendix*, Fig. S9 *A*, *E*, and *F*). In contrast, SEC1B interacted with SYP132 very strongly but only 10-fold less with KNOLLE (*SI Appendix*, Fig. S9 *B*, *E*, and *F*). SEC1B also tended to interact with the constitutively open form of SYP132 more strongly than with the normal form. Thus, unlike KEULE, SEC1B had a clear preference to interact with Qa-SNARE SYP132 involved in both cytokinesis and secretion (29).

To assess the interaction between SYP1 Qa-SNAREs and SEC1-related SM proteins *in planta*, we performed coimmunoprecipitation assays with extracts from seedlings expressing *KNOLLE::vYFP-KEULE* or *KNOLLE::GFP-SEC1B* (Fig. 4) (25, 30). The anti-GFP immunoprecipitates were examined for the presence of the two Qa-SNAREs, KNOLLE and SYP132. In addition, the presence of their SNARE partners VAMP721/722, SNAP33, SYP71, and NPSN11 was also analyzed to clarify whether SEC1B interacted with the monomeric Qa-SNARE, as shown for the KEULE–KNOLLE interaction, or with the assembled SNARE complex, as shown for the interaction of Munc18-1 with the mammalian neuronal SNARE complex (25, 31). Endogenous SYP132 was coimmunoprecipitated with SEC1B. In contrast, virtually no KNOLLE, nor any of their SNARE partners, were detected in the immunoprecipitate (Fig. 4). KEULE interacted with both Qa-SNAREs but not with any of their SNARE partners VAMP721/722, SNAP33, SYP71, and NPSN11 in seedlings (Fig. 4) (25, 32). The interaction of KEULE with SYP132 was comparatively weak, as evidenced by the fact that in another coimmunoprecipitation assay with *KNOLLE::6xHA-KEULE*, only Myc-tagged SYP132, but not endogenous SYP132, was detected in the precipitate (*SI Appendix*, Fig. S8). In conclusion, both KEULE and SEC1B interact with monomeric Qa-SNAREs but apparently not with assembled SNARE complexes. Furthermore, only KEULE but not SEC1B showed detectable interaction with KNOLLE. Conversely, SEC1B interacted more strongly than KEULE with endogenous SYP132, taking their relative protein levels into account. These results suggest that the yeast two-hybrid interaction assays reflect the in situ conditions. Thus, SEC1B might only make a small contribution to cytokinesis compared with KEULE.

Domains of SEC1-Related SM Proteins Conferring Differential Interaction with Qa-SNAREs. SEC1 proteins comprise five domains—1, 2a, 3a, 3b, and 2b—as revealed by crystal structure analysis (33). In

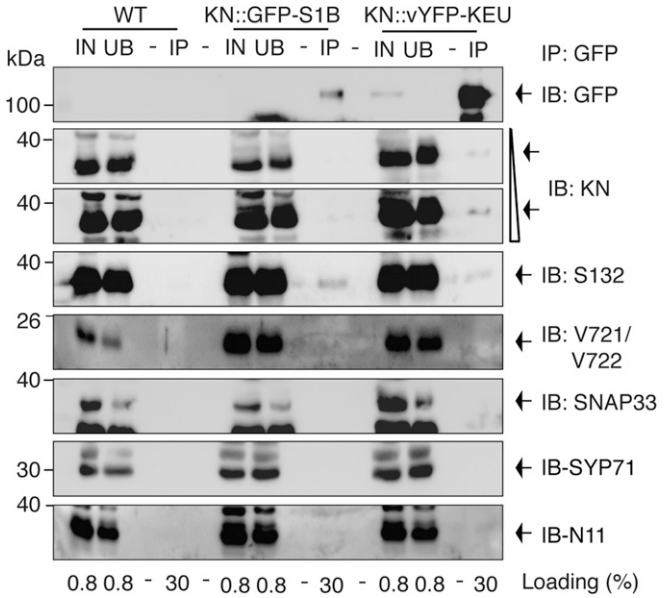

Fig. 4. Coimmunoprecipitation analysis of SEC1-like SM proteins and SYP1 Qa-SNAREs. Protein extracts from *KNOLLE::GFP-SEC1B* (KN::GFP-S1B) and *KNOLLE::vYFP-KEULE* (KN::vYFP-KEU) seedlings were subjected to immunoprecipitation with anti-GFP beads. Wild-type (WT, Col) seedlings were used as control. Immunoprecipitates (IP) were immunoblotted (IB) with the antisera indicated: GFP, anti-GFP; KN, anti-KNOLLE; S132, anti-SYP132; V721/V722, anti-VAMP721/V722; S33, anti-SNAP33; S71, anti-SYP71; N11, anti-NPSN11. Note that KNOLLE is barely detected in GFP-SEC1B precipitates in longer exposure, but similar to the background level of control. IN, input; M, molecular markers; UB, unbound (size in kilodaltons on the *Left*). Loading (%), relative loading volume to total volume.

neuronal SEC1 (nSEC1), largely conserved amino acid residues in domains 1 and 3a make contact with specific amino acid residues of the Qa-SNARE syntaxin 1a (33). To examine whether the differential interaction of the two closely related SM proteins with the Qa-SNAREs KNOLLE and SYP132 is mediated by specific domains, we generated chimeric proteins by swapping domains 1 and 2a between KEULE and SEC1B: one chimera comprised domains 1 and 2a of KEULE and the other domains of SEC1B ($KEULE_{1–2a}SEC1B_{3a–2b}$, KS), whereas the complementary chimera comprised domains 1 and 2a of SEC1B and the other domains of KEULE ($SEC1B_{1–2a}KELE_{3a–2b}$, SK) (*SI Appendix*, Fig. S9*C*; see *SI Appendix*, Fig. S2 for the sequences). Semiquantitative yeast two-hybrid interaction assay revealed that the SK chimera interacted strongly with SYP132 but not with KNOLLE, whereas the complementary KS chimera weakly interacted with both SYP132 and KNOLLE as did KEULE (*SI Appendix*, Fig. S9 *D* and *E*). These results suggest that domains 1 and 2a of the closely related SM proteins KEULE and SEC1B might contain features conferring differential interaction with Qa-SNAREs.

Genetic Interactions of SYP1 Qa-SNAREs and SEC1-Like SM Proteins. In addition to the physical interaction assays, all possible double mutants of SYP1 Qa-SNAREs and SEC1-like SM proteins were generated and analyzed phenotypically (Fig. 5). The *knolle keule* double mutant clearly showed an embryo-lethal phenotype in which cytokinesis was completely blocked from the zygote stage of embryogenesis, as reported previously (10). Consequently, the mutant embryo was a single growing cell in which the number of nuclei increased over time (Fig. 5*E*). This result suggested that the single mutants, *knolle* and *keule*, were not embryo-lethal but displayed abnormal seedling phenotypes because functionally overlapping related genes enabled completion of embryogenesis. The easiest explanation would be that functionally overlapping SYP1 Qa-SNARE and SEC1-like SM proteins might interact with KEULE and KNOLLE, respectively, such that in the *knolle keule* double mutant, *keule* knockout would render a KNOLLE-redundant SYP1 Qa-SNARE inactive and *knolle* knockout would render a KEULE-redundant SEC1-like SM protein inactive. Consistent with this idea, our recent study has revealed that SYP132 is the relevant redundant SYP1 Qa-SNARE and that $syp132^{tam}$ mutant, a combined mutant of two alleles of $syp132^{T\text{-}DNA}$ and *RPS5A>>amiR(SYP132)*, in a *knolle* mutant background results in lethal embryos nearly resembling the *knolle keule* double mutant (29). Furthermore, a $syp132^{tam}$ *keule* double mutant also showed a strong embryo-lethal phenotype, approaching the *knolle keule* phenotype (Fig. 5*H*; compare with the $syp132^{tam}$ mutant alone in Fig. 5*G* and *SI Appendix*, Table S4*A*). Thus, *KEULE* interacts genetically with both *KNOLLE* and *SYP132* in cytokinesis. Regarding the genetic interactions of *SEC1B*, a *knolle sec1b* double mutant also gave a very similar embryo-lethal phenotype to *knolle keule* (Fig. 5*F*; compare with Fig. 5*E* and *SI Appendix*, Table S4*B*). These data suggested that both KEULE and SEC1B interact with SYP132 in cytokinesis. In contrast to $syp132^{tam}$ *keule*, however, a $syp132^{tam}$ *sec1b* double mutant did not die as an embryo that completely failed to undergo cytokinesis. Instead, the $syp132^{tam}$ *sec1b* double mutant completed embryogenesis, displaying a seedling-lethal phenotype that closely resembled the $syp132^{tam}$ single-mutant phenotype (Fig. 5 *I* and *K*; compare with Fig. 5 *G* and *J* and *SI Appendix*, Table S4*C*). Additionally, reciprocal crosses of $syp132^{T\text{-}DNA}$/*SYP132 s1b*/*SEC1B* doubly heterozygous plants with wild-type plants revealed that the transmission frequency of the $syp132^{T\text{-}DNA}$ *sec1b* double mutant was reduced via the pollen (*SI Appendix*, Table S5*B*). This result supported the conclusion that the interaction of SEC1B with SYP132 is involved in a general secretory pathway. Thus, unlike KEULE, SEC1B did not play an essential role in KNOLLE activation during embryogenesis, which suggests functional diversification among the SEC1-like SM proteins.

Discussion

The two paralogs of *Arabidopsis* SEC1 protein, KEULE and SEC1B, appear to be closely related to each other, displaying substantial functional overlap. This is clearly demonstrated by the *keule sec1b* double mutant being nearly gametophytically lethal, whereas *sec1b* mutant plants are homozygous viable and *keule* knockout mutants are only seedling-lethal. Our data did not give any evidence for a primary defect in pollen cytokinesis, whereas the growth defect and the eventual collapse of the developing pollen grain suggested that secretory trafficking might be seriously impaired in the *keule sec1b* double mutant, which is consistent with the compromised delivery of GFP-SYP132 to the plasma membrane. The simplest interpretation of the data would be that KEULE and SEC1B are stable proteins such that carryover from the meiocyte would suffice for the asymmetric division of the microspore (and possibly for the division of the generative cell as well) but not for the subsequent substantial growth of the tricellular pollen before maturation.

Although the *keule* single mutant displays seemingly specific cytokinesis defects, the root hairs are stunted or absent in *keule* seedlings, in contrast to *knolle* and *sec1b* seedlings, suggesting an additional, noncytokinetic function for KEULE (8). Interestingly, estradiol-inducible *amiRNA*(*SYP132*) expression in root hair cells caused reduced root hair growth (34), which might be KEULE-dependent. Nonetheless, KEULE appears to be primarily involved in cytokinesis, whereas SEC1B makes its major contribution to secretory traffic. Thus, like their interacting SYP1 Qa-SNAREs, KNOLLE, and SYP132, these SM proteins appear to have specialized to some extent. However, the single mutants of *keule* and *sec1b* suggested a nonreciprocal relationship between the two proteins: KEULE could replace SEC1B

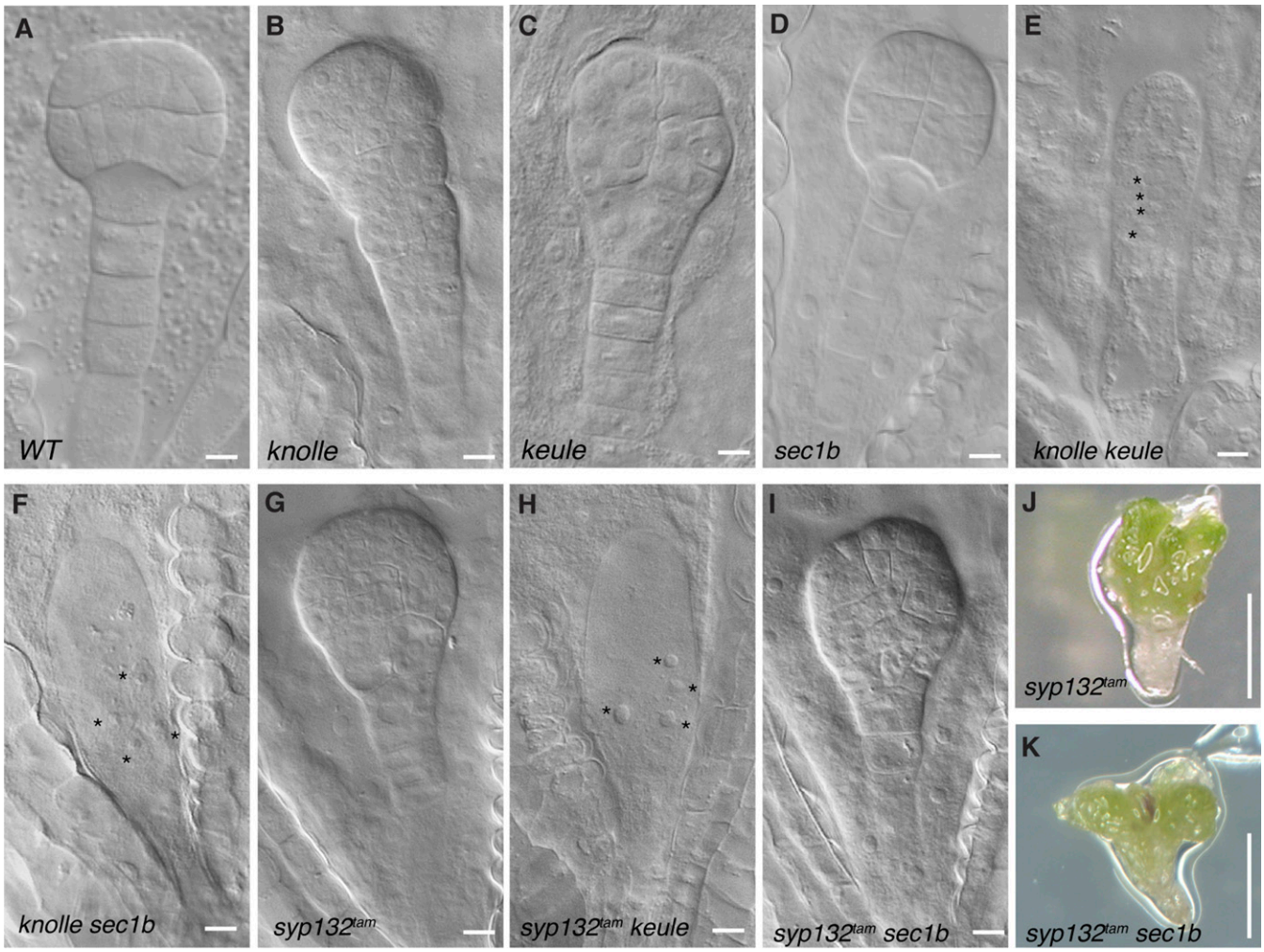

Fig. 5. Analysis of double mutants revealing interaction between SEC1-like SM proteins and SYP1 Qa-SNARE proteins. (*A–I*) Embryo images of wild type (*WT*; *A*), *knolle* (*B*), *keule* (*C*), *sec1b* (*D*), *knolle keule* (*E*), *knolle sec1b* (*F*), *syp132*^{tam} (*G*), *syp132*^{tam} *keule* (*H*), and *syp132*^{tam} *sec1b* (*I*). Note that *knolle keule*, *knolle sec1b* and *syp132*^{tam} *keule* double mutants form a single-celled embryo with multiple nuclei, due to almost completely blocking cytokinesis (asterisks in *E*, *F*, and *H*). In contrast, the *syp132*^{tam} *sec1b* double mutant is phenotypically indistinguishable from the *syp132*^{tam} single mutant at both embryo (*I*, compare with *G*) and seedling (*K*, compare with *J*) stages. See *SI Appendix*, Table S4 for genetic analysis. (Scale bars: 10 μm in *A–I*; 5 mm in *J* and *K*.)

completely, whereas SEC1B could not replace KEULE in cytokinesis even though strongly overexpressed SEC1B from the *KNOLLE* promoter can fulfill the function of KEULE in the *keule* knockout mutant by interacting with KNOLLE, which in turn mediates membrane fusion in cytokinesis. This was clearly demonstrated by the analysis of Qa-SNARE SM-protein double mutants. In combination with either *knolle* or *syp132*, the *keule* mutant blocked cytokinesis completely, revealing the ability of KEULE to interact with both Qa-SNAREs. In contrast, *sec1b* inhibited cytokinesis completely only in the *knolle* mutant background, whereas the *sec1b syp132* double mutant essentially resembled the *syp132* single mutant seedling, suggesting preferential interaction of SEC1B with the ancient Qa-SNARE SYP132 in both secretion and cytokinesis. This conclusion is also supported by the physical interaction analyses involving yeast two-hybrid and coimmunoprecipitation assays.

It is important to note that strong overexpression of SEC1B from the *KNOLLE* promoter in cytokinesis can rescue *keule* mutant seedlings, suggesting that the two proteins, KEULE and SEC1B, are functionally sufficiently similar to replace each other. The yeast two-hybrid data suggest that both KEULE and SEC1B can interact with KNOLLE, although SEC1B interacts much more strongly with SYP132 compared with KNOLLE and the interaction of KEULE with SYP132. This difference, together with the 10-fold lower level of SEC1B expression compared with KEULE, might explain why the lack of SEC1B is not deleterious, whereas absence of KEULE impairs cytokinesis profoundly. In a highly simplified view, KNOLLE–KEULE interaction might account for most of membrane-fusion activity in cytokinesis, with SYP132–KEULE, SYP132–SEC1B and, possibly, KNOLLE–SEC1B interactions each making minor contributions (*SI Appendix*, Fig. S10). In contrast, fusion of secretory vesicles with the plasma membrane involving SYP132 appears to be equally well supported by both KEULE and SEC1B.

The yeast two-hybrid assay revealed that domains 1 and 2a of the two SM proteins KEULE and SEC1B influence the way the SM proteins interact with the Qa-SNAREs KNOLLE and SYP132. Our results are consistent with the structural analysis of nSec1–syntaxin 1a interaction, which identified several conserved amino acid residues in the domain 1 of nSec1 that play a prominent role in the interaction with the N-terminal helices (Habc domain) or the SNARE domain (H3 domain) of syntaxin 1a (33).

Our results suggest that functional diversification of KEULE and SEC1B only started in early angiosperm evolution. Considering that SEC1B predominantly interacted with the ancient Qa-SNARE SYP132, KEULE (or its precursor, the not yet duplicated

SEC1-related protein) might have acquired an additional function after KNOLLE had arisen in the early angiosperms. Nonetheless, the retention of a *KNOLLE* gene in the secondarily simplified duckweed *Spirodela polyrhiza* suggests that the single remaining SEC1-related protein is not only related to KEULE by sequence but also able to interact with KNOLLE like KEULE in *Arabidopsis*. This raises the possibility that membrane fusion in angiosperm cytokinesis was made more efficient by the coevolution of a cytokinesis-specific Qa-SNARE KNOLLE, as opposed to its nonspecialized precursor SYP132, and an interacting SM protein KEULE, as opposed to the mainly secretory SM protein SEC1B.

Materials and Methods

Plant Material and Growth Conditions. *Arabidopsis thaliana* plants were grown either on soil or on agar plates with MS medium (2.15 g/L Murashige and Skoog, 0.5 g/L MES, 1% sucrose, pH 5.6) at 23 °C in continuous light. Transgenic plants were generated with the floral-dip method of *Agrobacterium tumefaciens*-mediated transformation (35). T1 plants were selected by spraying with 1:1,000 diluted BASTA (183 g/L glufosinate; AgrEvo) or hygromycin (20 μg/mL; Duchefa). T-DNA insertional *sec1b* (GABI-KAT_601G09, sulfonamide-resistant) mutant seed was purchased from the European *Arabidopsis* Stock Centre (NASC).

The following transgenic lines were used: KNOLLE::6xHA-KEULE (25), KNOLLE::Myc-SYP132 (28), SYP132::GFP-SYP132 (29, 36), KNOLLE::Myc-KNOLLE (25), and VHA-a1-GFP (37).

In Silico Analysis. SEC1-related sequences were obtained from genome database sources, such as Phytozome v12 (https://phytozome.jgi.doe.gov/pz/portal.html) and others (https://www.cacaogenomedb.org; spirodelagenome.org/jgi_csp; congenie.org; banana-genome-hub.southgreen.fr/organism/Musa/acuminata). Peptide sequences were aligned in the CLC workbench (v7.8.1) program. The unrooted phylogenetic tree was generated using the neighbor-joining method together with the bootstrap test (1,000 replicates) in the CLC workbench (v7.8.1) program.

Statistical Analysis. The dataset was analyzed using R software (https://www.r-project.org/) and performing ANOVA (single-factor for *SI Appendix*, Fig. S9 *A* and *B*; two-way for *SI Appendix*, Fig. S9*D*) and a posteriori Tukey test. F value = variance of the group means/mean of the within group variances. P = the significance probability associated with the F value.

See *SI Appendix, Materials and Methods*, for details on molecular cloning, genetic and transcript analysis, chemical treatment, pollen staining, coimmunoprecipitation and immunoblot analyses, immunofluorescence analysis, and yeast analysis.

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