The *Arabidopsis* KNOLLE Protein Is a Cytokinesis-specific Syntaxin

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Abstract. In higher plant cytokinesis, plasma membrane and cell wall originate by vesicle fusion in the plane of cell division. The *Arabidopsis KNOLLE* gene, which is required for cytokinesis, encodes a protein related to vesicle-docking syntaxins. We have raised specific rabbit antiserum against purified recombinant KNOLLE protein to show biochemically and by immunoelectron microscopy that KNOLLE protein is membrane associated. Using immunofluorescence microscopy, KNOLLE protein was found to be specifically expressed during mitosis and, unlike the plasma membrane H+-ATPase, to localize to the plane of division during cytokinesis. *Arabidopsis* dynamin-like protein ADL1 accumulates at the plane of cell plate formation in *knolle* mutant cells as in wild-type cells, suggesting that cytokinetic vesicle traffic is not affected. Furthermore, electron microscopic analysis indicates that vesicle fusion is impaired. KNOLLE protein was detected in mitotically dividing cells of various parts of the developing plant, including seedling root, inflorescence meristem, floral meristems and ovules, and the cellularizing endosperm, but not during cytokinesis after the male second meiotic division. Thus, KNOLLE is the first syntaxin-like protein that appears to be involved specifically in cytokinetic vesicle fusion.

Aᴛᴛᴇʀ nuclear division, cytokinesis partitions the cytoplasm of the dividing cell. Whereas mitotic segregation of chromosomes is highly conserved among eukaryotes, cytokinesis can be carried out in fundamentally different ways. In animal cells, the existing plasma membrane is pulled in by means of a contractile acto-myosin–based ring, and the daughter cells are eventually pinched off (Fishkind and Wang, 1995). During this process, the surface of the plasma membrane is extended by the fusion of large membranous vesicles at the flanks of the cleavage furrow (Byers and Armstrong, 1986). In higher plants, cytokinesis is initiated at the center of the division plane, and a new plasma membrane is formed by vesicle fusion (Staehelin and Hepler, 1996). Plant cell division is assisted by specific arrays of cytoskeletal elements. Between the separating anaphase chromosomes, two groups of microtubules interdigitate with their plus ends at the plane of division, forming a cylindrical structure. This so-called phragmoplast also contains two opposing sets of actin microfilaments that, however, do not overlap or directly abut (Zhang et al., 1993). The phragmoplast mediates the accumulation of Golgi-derived vesicles that fuse to form the cell plate, consisting of an immature cross-wall bounded by an incipient plasma membrane. As the phragmoplast moves out to the periphery, the disc-shaped cell plate expands laterally, eventually reaching the parental cell surface (Staehelin and Hepler, 1996). Cytokinesis is completed by the fusion of the cell plate with the parental wall at a site that was transiently marked by a cortical preprophase band of microtubules at the onset of mitosis (Wick, 1991).

Little is known about the mechanism of cell plate formation, although the sequence of events at the plane of division has been well described at the electron microscope level (Samuels et al., 1995). Biochemical assays have been used to identify relevant molecules, such as polypeptides with microtubule-translocating activity from phragmoplasts of tobacco cells, that may be involved in transporting membrane vesicles to the site of cell plate formation (Asada and Shibaoka, 1994), and one candidate motor molecule, the kinesin-related TKRP125 protein, has been isolated (Asada et al., 1997). Another approach involved the study of plant homologues of proteins with defined roles in other systems. For example, an *Arabidopsis* kinesin-like protein, KatAp, has been localized to the phragmoplast in dividing cells (Liu et al., 1996). Two other plant homologues, AtCdc48 (Feiler et al., 1995) and the dynamin-like

GTPase phragmoplastin (Gu and Verma, 1996, 1997), show cell cycle–dependent intracellular redistributions, accumulating at the plane of cell division during cytokinesis. AtCdc48 is related to the yeast *N*-ethylmaleimide–sensitive ATPase Cdc48p involved in homotypic ER–ER fusion events (Latterich et al., 1995). Phragmoplastin and its *Arabidopsis* homologue, ADL1 (Park et al., 1997), bear resemblance to animal dynamins that are required for the pinching off of vesicles from membranes (Baba et al., 1995). However, the functions that these plant homologues have during cytokinesis are not known.

A small number of mutants that show cytokinesis defects have been described in pea (*cyd*: Liu et al., 1995) and *Arabidopsis* (*knolle*: Lukowitz et al., 1996; *keule*: Assaad et al., 1996). The *KNOLLE* gene was isolated by map-based cloning, and its mRNA appears to accumulate in a cell cycle–dependent manner (Lukowitz et al., 1996). The predicted 34-kD KNOLLE protein is related to a family of membrane-anchored proteins, the syntaxins, which are implicated in directing intracellular vesicle trafficking, with specific syntaxins mediating fusion events in different pathways (Bennett and Scheller, 1993). Although syntaxins form a target-membrane component of the vesicle-docking complex, t-SNARE, in heterotypic membrane fusion (Ferro-Novick and Jahn, 1994; Pfeffer, 1996), a new syntaxin has been recently reported to mediate homotypic vacuolar fusion in yeast (Nichols et al., 1997). Plant cytokinesis resembles homotypic fusion because there is no target membrane, and vesicle fusion itself forms the de novo plasma membranes separating the daughter cells. To determine its role in cytokinesis, we raised specific antiserum against KNOLLE protein and analyzed its membrane association, tissue distribution, and intracellular localization. We also examined, by immunofluorescence and electron microscopy, the cytokinetic defect of *knolle* mutant cells. Our results suggest that KNOLLE, which is specifically expressed in mitotically dividing cells and in the cellularizing endosperm, mediates vesicle fusion in the plane of cell division.

Materials and Methods

Plant Material and Growth Conditions

The *Arabidopsis thaliana* ecotype Landsberg *erecta* used as wild-type was the nonmutagenized parental strain in which the *knolle* alleles X37-2, 3-496, and AP6-16 were induced (Lukowitz et al., 1996). Plants were grown as previously described (Mayer et al., 1991).

Generation of KNOLLE-specific Antiserum

A *KNOLLE* (*KN*)[1] cDNA fragment lacking the sequence for the hydrophobic COOH terminus of the protein (Lukowitz et al., 1996) was expressed in *Escherichia coli* from the His-tag expression vector pQE 60 (Qiagen, Chatsworth, CA). Recombinant protein was purified by Ni^{2+} affinity chromatography (Qiagen) according to the manufacturer's instructions. After preparative SDS-PAGE and electroelution (Schleicher & Schuell, Inc., Keene, NH), 300 μg of purified protein in complete Freund's adjuvant emulsion was injected into a rabbit. After boosting with 500 μg recombinant protein in incomplete Freund's adjuvant on day 28, serum was collected once or twice a week, starting on day 8 after boosting. The antibody was purified by affinity chromatography with purified KN pro-

tein coupled to cyanbromide-activated Sepharose and elution of bound antibodies with 2.5 M and 4 M $MgCl_2$ (Harlow and Lane, 1988). Purified antibody gave the same signals as the whole antiserum in immunolocalization experiments.

Preparation of Protein Extracts and Western Blot Analysis

Plant material as specified in Results was ground in liquid nitrogen. After boiling in sample buffer (Laemmli, 1970) at 95°C for 10 min, the homogenate was centrifuged at 13,000 *g* for 15 min to remove insoluble debris. For Western blot analysis, proteins were separated by 12% SDS-PAGE (Laemmli, 1970) and electroblotted onto a polyvinylidene difluoride membrane (Pharmacia Biotech, Piscataway, NJ). The filter was blocked with 5% (wt/vol) nonfat dry milk in Tris-buffered saline with 0.2% Tween 20 (TBST: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20), probed with primary antibody, washed with TBST plus 400 mM NaCl and 0.8% Tween 20, incubated with horseradish peroxidase–conjugated secondary antibody diluted 1:2,000 in blocking buffer, and washed again before detection with the BM chemiluminescence system (Boehringer Mannheim Corp., Indianapolis, IN). Primary antibodies were used at the following concentrations: anti-KNOLLE, 1:1,000 to 1:8,000; anti-ADL1, 1:1,000; anti–plasma membrane H+-ATPase (PM ATPase), 1:1,000.

Cell Fractionation

Inflorescences were cut into small pieces and ground with mortar and pestle in homogenization buffer (100 mM Hepes-Tris, pH 7.8, 300 mM sucrose, 5 mM EDTA, 2.5 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, 0.5% [wt/vol] BSA). The homogenate was passed through one layer of Miracloth™ (Calbiochem, La Jolla, CA) and centrifuged at 10,000 *g* at 4°C for 10 min. For density gradient centrifugation, the supernatant (S10) was stirred briefly with a glass homogenizer (Potter-Elvejhem) and centrifuged through a linear 15–45% sucrose gradient at 107,000 *g* at 4°C for 18 h. Fractions of 500 μl were checked by refractometry before Western blot analysis of aliquots. To test for membrane association of KN protein, aliquots of the supernatant (S10) were centrifuged at 100,000 *g* for 90 min to give soluble fractions (S100) and pellets (P100). The pellets were resuspended in the original volumes of homogenization buffer without additives (control), with 1% Triton X-100, with 1 M NaCl, or with 0.1 M Na_2CO_3 (pH 10.9), incubated on ice for 40 min, and again centrifuged at 100,000 *g* for 90 min, giving wash fractions S100′ and pellets. This procedure was repeated to give wash fractions S100″ and washed pellets P100″. All fractions were subjected to Western blot analysis.

Immunofluorescence Localization

The procedure for whole-mount preparations was modified from Webb and Gunning (1990) and Goodbody and Lloyd (1994). Ovules or anthers were fixed in 4% paraformaldehyde in microtubule-stabilizing buffer (MTSB: 50 mM Pipes, 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9–7.0) at room temperature under vacuum for 1 h. Embryos and endosperm were dissected from washed ovules and squashed onto gelatine-coated microscopic slides, and anthers were squashed directly to release sporogenous cells, meiocytes, and developing pollen. Coverslips were removed after dipping the slides into liquid nitrogen. After drying for at least 15 min, specimens were rehydrated in MTSB for 10 min. Cell walls were partially digested with 2% (wt/vol) driselase (Sigma Chemical Co., St. Louis, MO) for 30 min. The plasma membrane was permeabilized with 0.5 or 3% Nonident P40 in 10% DMSO-MTSB for ~1 h. Unspecific interactions were blocked with 1% (wt/vol) BSA in MTSB overnight at 4°C, and antibodies were diluted in 3% (wt/vol) BSA in MTSB and incubated at 37°C for ~3 h. In double-labeling experiments, the antibodies were concomitantly incubated after performing appropriate controls. For cryosections, material was fixed in 4% paraformaldehyde in MTSB overnight at 4°C, washed in buffer, embedded in 30% (wt/vol) sucrose in MTSB (inflorescences and flowers) or only in MTSB (root tips), and frozen in tissue-tek on dry ice or liquid nitrogen. Sections 5–7 μm thick were cut at −15°C to −20°C, using a cryomicrotome (model 2700-Frigocut; Reichert-Jung; Leica, Wetzlar, FRG), washed, and incubated in 5% (wt/vol) nonfat dry milk in PBT (PBS with 0.2% Triton X-100) to block nonspecific antibody binding. Primary antibodies were used at the following concentrations: anti-KNOLLE, 1:2,000; anti-ADL1 (Park et al., 1997), 1:500; anti–α-tubulin YOL 1/34 (Harlan Sera-Lab), 1:25 or 1:50; anti–α-tubulin N356 (Amersham Corp., Arlington Heights, IL), 1:600; anti-PM ATPase (a kind gift from W. Michalke, Bi-

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1. *Abbreviations used in this paper*: ADL1, Arabidopsis dynamin-like protein; DAPI, 4′,6-diamidino-2-phenylindole; KN, KNOLLE; MTSB, microtubule-stabilizing buffer; PM ATPase, plasma membrane H+-ATPase.

ologie III, University of Freiburg, FRG; Villalba et al., 1991), 1:500 or 1:1,000. Fluorochrome-conjugated secondary antibodies (Dianova, Hamburg, FRG) were used at the following concentrations: anti rabbit–FITC, 1:300; anti–rabbit-Cy3™, 1:500; anti–rat-TRITC, 1:300; anti–mouse-TRITC, 1:200; anti–mouse-Cy3™, 1:500. DNA was stained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.). Mounting was done in Citifluor (Amersham). Photographs were taken on Fujichrome Provia 400 color films (Tokyo, Japan), using appropriate filters for FITC, TRITC, Cy3™, and DAPI fitted to a microscope (model Axiophot; Carl Zeiss, Inc., Thornwood, NY). Confocal laser-scanning was performed with an inverse fluorescence microscope (Carl Zeiss, Inc.), using the Comos program (Bio-Rad Labs, Hercules, CA). Standard scanning conditions: 100× objective, twofold zoomed, 10× Kalman filter. Images were processed with Photoshop™ 3.0 (Adobe, Mountain View, CA) and Aldus Freehand 7.0 (Macromedia, San Francisco, CA) software.

Immunogold Labeling and Electron Microscopy

For immunolabeling of membrane fractions, aliquots from a sucrose density gradient were adsorbed to glow-discharged pioloform/carbon-coated grids, washed with blocking buffer (PBS containing 0.5% BSA and 0.2% gelatine), incubated with KNOLLE antiserum (diluted 1:200) for 15 min, washed five times with blocking buffer, and labeled with protein A–15-nm gold conjugates. After extensive washes with PBS and H_2O, grids were negatively stained with 1% uranyl acetate and viewed in a transmission electron microscope (model CM10; Philips Electronic Instruments Co., Mahwah, NJ) at 60 kV. For the ultrastructural characterization, embryos were fixed with 4% paraformaldehyde in MTSB at room temperature for 30 min and, after addition of 1% glutaraldehyde at 4°C for 17 h, postfixed with 1% OsO_4 in PBS on ice for 40 min and, after rinsing with aqua bidest, treated with 1% aqueous uranyl acetate at 4°C for 40 min. Samples were dehydrated through a graded series of ethanol, infiltrated with ethanol/resin mixtures, and finally embedded in Spurr's epoxy resin. Ultrathin 60-nm serial sections were stained with uranyl acetate and lead citrate and viewed as above.

Results

KNOLLE Protein Is Associated with Membranes in Dividing Cells

The nucleotide sequence of the *KNOLLE* cDNA predicted that the KN protein, like other syntaxins, consists of an NH_2-terminal variable region, a conserved region of 68 amino acids, and a COOH-terminal putative hydrophobic membrane anchor (Lukowitz et al., 1996). Recombinant KN protein without the hydrophobic COOH terminus was purified from *E. coli* for generating polyclonal antiserum (for details see Materials and Methods). The specificity of the antiserum was determined by Western blot analysis of recombinant protein (Fig. 1 *a*) and by immunostaining of *knolle* mutant embryos (described below). In Western blots of plant cell extracts, the KN-specific antiserum detected a protein of ~40 kD and, if used at higher concentration, an additional ~55-kD protein (Fig. 1 *a*).

KN mRNA was shown previously to accumulate preferentially in organs with actively dividing tissues, such as developing flowers and siliques (Lukowitz et al., 1996). Essentially the same organ distribution was observed for KN protein (Fig. 1 *b*). Developing flowers and immature siliques, which contain developing embryos, showed the highest levels of KN protein. Roots and callus cells contained less KN protein while almost none was detectable in young seedlings and leaves. This distribution suggested that KN protein, like its mRNA, is expressed in dividing cells.

The subcellular distribution of KN protein was determined by sucrose density gradient centrifugation of cell extracts (Fig. 1 *c*). KN protein was found in all gradient frac-
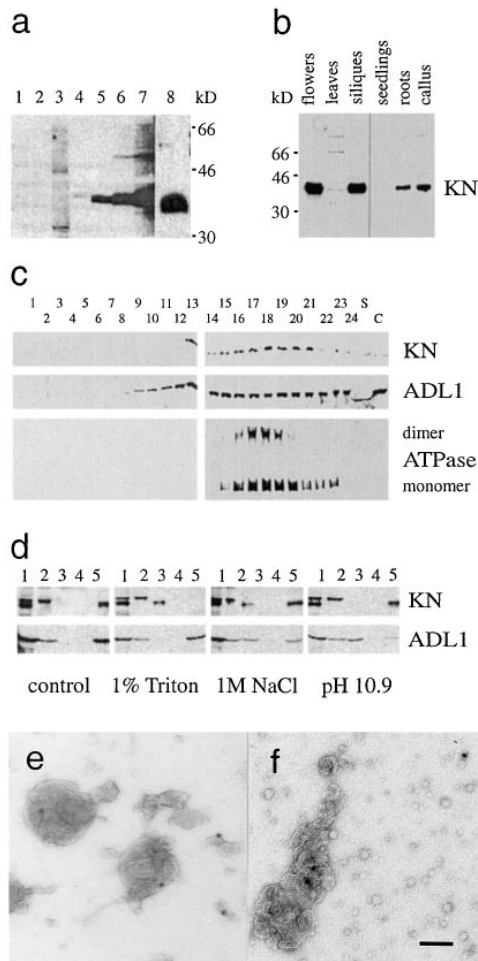

Figure 1. KNOLLE protein from plant cell extracts. (*a–d*) Western blots. (*a*) Specificity of anti-KN antiserum. Equal amounts of protein from inflorescences were loaded in lanes *1–7*. Lanes: *1*, primary antibody only; *2*, secondary antibody only; *3*, preimmune serum diluted 1:500; *4–7*, antiserum diluted 1:8,000, 1:4,000, 1:2,000, 1:1,000, respectively; *8*, 2.5 ng recombinant protein, antiserum 1:1,000. (*b*) Organ distribution of KN protein. Equal amounts of protein were loaded. (*c*) Subcellular distribution of KN protein as determined by 15 to 45% linear sucrose density gradient centrifugation. Gradient fractions (*1*, top; *24*, bottom) were probed with anti-KN, -ADL1, and -plasma membrane H^+-ATPase antibodies; *S*, supernatant of 10,000 g centrifugation; *C*, total cell extract. (*d*) Membrane association of KN protein (differential centrifugation, anti-KN, and anti-ADL1 antiserum at 1:1,000). Cell extract was centrifuged at 10,000 g, and aliquots of supernatant (S10, lanes *1*) were centrifuged at 100,000 g to give supernatants S100 (lanes *2*). Pellets (P100) were resuspended in homogenization buffer (*control*), supplemented with 1% Triton X-100, 1 M NaCl, or Na_2CO_3 (*pH 10.9*) and washed twice by centrifugation at 100,000 g (supernatants S100′, lanes *3*; S100′′, lanes *4*) to give the washed pellets (P100′′, lanes *5*). For details, see Materials and Methods. (*e* and *f*) Electron micrographs of KN protein immunogold localization on membranous material from (*e*) gradient fraction 18 (see Fig. 1 *c*) and (*f*) S100 supernatant (Fig. 1 *d*, *control*, lane 2). Note abundance of vesicles in *f*. Bar, 0.1 μm.

tions, although the highest levels were observed in denser fractions, some of which contained the PM ATPase (Villalba et al., 1991). The KN protein showed essentially the same distribution as the *Arabidopsis* dynamin-like (ADL1) pro-
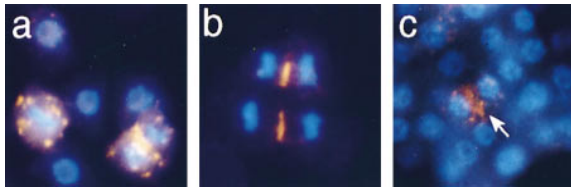
Figure 2. Cell cycle–dependent distribution of KN protein. Whole-mount preparations of single cells from squashed wild-type embryos were stained with DAPI (*blue*) to visualize the stage of the cell cycle and with KN-specific antiserum (Cy3, *orange*). (*a*) Mitotic cells show KN-positive patches; interphase cells are KN negative. (*b*) Two telophase cells with KN-positive material in plane of division. (*c*) Early interphase cell with diffuse KN staining near division plane (*arrow*). No KN signal in other interphase cells.

tein, which was recently shown to be localized to membranes (Park et al., 1997). Samples of several fractions were processed for electron microscopy and stained with KN antiserum and protein A–gold. KN-positive material was observed on the surface of membranous material (Fig. 1 *e*).

The predicted amino acid sequence of the *KNOLLE* gene product includes a COOH-terminal hydrophobic stretch that could serve as a membrane anchor (Lukowitz et al., 1996). To determine whether KN is indeed an integral membrane protein, we investigated its association with membranes under various experimental conditions, such as alkaline pH, high salt concentration, or presence of detergent (Fig. 1 *d*). Cell extract was centrifuged at 10,000 *g*. Aliquots of the supernatant fraction (S10, Fig. 1 *d*, lanes *1*) were centrifuged at 100,000 *g* to give supernatant (S100, lanes *2*) and pellet fractions. The pellets were resuspended in different buffers and recentrifuged at 100,000 *g*, yielding wash fractions (S100′, lanes *3*) and pellets. The pellets were washed as before, resulting in wash fractions (S100″, lanes *4*) and washed pellets (P100″, lanes *5*). Western blotting detected a KN double band in the S10 supernatant fraction (lanes *1*) but only the upper band in the S100 supernatant fractions (lanes *2*). By contrast, the lower band was detected in the washed pellets (lanes *5*) except after treatment of the pellet with 1% Triton X-100, which released KN protein into the wash fraction S100′ (lane *3*). The other two treatments, high salt concentration and alkaline pH, released little or no KN protein from the pellet, respectively, suggesting that KN is an integral membrane protein. For comparison, ADL1 protein was recovered from the washed pellets (lane *5*) after treatment with Triton X-100 but released into the wash fraction (lane *3*) when the pellet had been resuspended at pH 10.9, suggesting that ADL1 is a peripheral membrane protein. In the presence of Triton X-100, ADL1 appears to be released from the membrane but forms a pelletable complex of 400–600 kD (Park et al., 1997). To determine whether the nonpelletable KN protein was soluble or associated with a specific membrane fraction, we processed an aliquot of the S100 supernatant for electron microscopy and immunogold labeling. As shown in Fig. 1 *f*, KN protein was attached to the surface of small vesicles as well as to lumps of membranous material. These data suggest that KN protein is tightly associated with membranes.
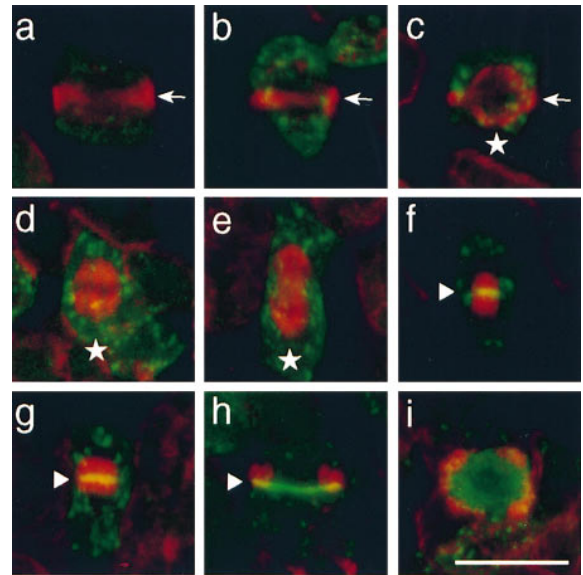

Figure 3. Confocal laser scanning microscopy of temporal sequence of KN protein redistribution in dividing embryonic cells. Whole-mount preparations of single cells from squashed wild-type embryos were stained with anti-KN antiserum (FITC, *green*) and anti–α-tubulin antibody (TRITC, *red*). (*a*) Onset of mitosis: early preprophase with preprophase band (*arrow*). KN signals are barely detectable. (*b*) Preprophase: KN-positive material in large patches away from preprophase band (*arrow*). (*c*) Late preprophase: only remnants of preprophase band are detectable (*arrow*), while mitotic spindles (*asterisk*) are forming. (*d*) Metaphase: mitotic spindles (*asterisk*) flank the metaphase plate. (*e*) Anaphase: mitotic spindles (*asterisk*) extend toward the poles; KN-positive material still scattered in large patches. (*f*) Early telophase: formation of phragmoplast (*red*; *arrowhead*) signals onset of cytokinesis; KN-positive material accumulates in the center (*yellow*) of the phragmoplast. (*g*) Mid-telophase: phragmoplast (*arrowhead*) and associated KN-positive material (*yellow*) coextend laterally toward the periphery of the cell. (*h* and *i*) Late telophase and end of cytokinesis: phragmoplast (*arrowhead*) is at lateral cell surface; KN-positive fuzzy disc extends from the center (*green*) to the edge of the phragmoplast (*yellow*); (*h*) side view; (*i*) face view. *Arrows*, preprophase bands; *asterisks*, mitotic spindles; *arrowheads*, phragmoplasts. Bar, 10 μm.

Intracellular Localization of KN Protein

To determine the intracellular localization of KN protein throughout the cell cycle, whole-mount preparations of single cells from squashed embryos were doubly labeled with DAPI and with KN-specific antiserum (Fig. 2). No KN signal was detected in interphase cells (Fig. 2 *a*). KN-positive material accumulated in large patches during early M phase (Fig. 2 *a*) and predominantly in the plane of division in telophase cells (Fig. 2 *b*). Cells exiting from mitosis showed more diffuse KN signals near the plane of division (Fig. 2 *c*). To determine the sequence of events more precisely, dividing embryonic cells were double-stained with KN-specific antiserum and with anti–α-tubulin antibody to visualize the microtubule cytoskeleton (Fig. 3). At the onset of mitosis, a preprophase band of cortical microtubules marks the future plane of cell division (Lambert, 1993). Some cells displaying the preprophase band contained large patches of KN-positive material while others did not, sug-

gesting that the expression of KN protein started at or soon after the onset of mitosis (Fig. 3, *a* and *b*). From prophase to anaphase, KN staining was detected only in large patches within the cytoplasm; during these stages, mitotic spindles formed and separated the sets of daughter chromosomes (Fig. 3, *c–e*). The phragmoplast forms during anaphase between the two sets of daughter chromosomes and narrows down in the center of the division plane during early telophase (Staehelin and Hepler, 1996). In early telophase cells, KN-positive material started to accumulate in the center of the phragmoplast (Fig. 3 *f*). As the phragmoplast was displaced toward the periphery of the cell, the KN-positive material extended from the center to the advancing edge of the phragmoplast (Fig. 3 *g*). When the phragmoplast reached the lateral cortex of the cell, the KN-positive disc extended across the entire plane of division, although the KN signal appeared weaker in the center than at the periphery (Fig. 3, *h* and *i*). In the early interphase, KN staining was rarely found, and if still present, it appeared fragmented (see Fig. 2 *c*). Thus, the time course of KN protein accumulation at the plane of cell division reflected the formation and lateral expansion of the cell plate.

To address whether the membrane of the cell plate is labeled by a marker for the plasma membrane, we examined the distribution of PM ATPase in cytokinetic cells. The PM ATPase did not accumulate in the forming cell plate as demonstrated by double labeling with KN (Fig. 4). Although the surface of the cytokinetic cell was decorated with PM ATPase, the developing cell plate only displayed the KN signal. Even when the KN signal was confined to the periphery of the nearly complete cell plate, PM ATPase was excluded (Fig. 4, *c* and *f*). Since the entire surface of surrounding interphase cells was labeled with PM ATPase, we presume that PM ATPase entered the newly formed plasma membrane only after the KN protein had disappeared from the cell plate. Thus, the developing cell plate appears to constitute a membrane compartment that is topologically distinct from the plasma membrane and is formed by a specialized set of cytokinetic transport vesicles (see Discussion).

Analysis of knolle Mutant Cells

We examined the expression of KN protein in whole-mount preparations of single cells from embryos mutant for each of the three *knolle* alleles described so far: 3-496 is a stop-codon mutation that truncates the KN protein at amino acid 70, AP6-16 is a frame-shift mutation, resulting in a KN protein without the conserved region and the COOH-terminal hydrophobic anchor, and X37-2 is a large deletion that removes about half the coding region and 3′-adjacent sequences (Lukowitz et al., 1996). Mutant cells were analyzed during telophase as visualized by DAPI staining. No KN-positive material was observed in kn^{X37-2} mutant cells (Fig. 5, *a* and *b*) and in mutant cells for the two other *kn* alleles (data not shown). Thus, all three mutations appear to interfere with the accumulation of stable mutant KN protein.

To address whether the lack of KN protein interfered with the transport of membrane vesicles to the plane of cell division, we analyzed the intracellular distribution of the membrane-associated *Arabidopsis* dynamin-like (ADL1)
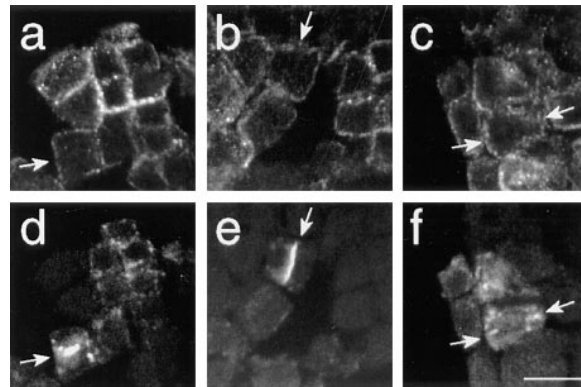

Figure 4. Confocal laser scanning microscopy (three Z layers each 0.5-μm thick) of KN protein and plasma membrane H⁺-ATPase distribution during cytokinesis. Double-immunofluorescence staining of cells from squashed wild-type embryos with anti-KN (FITC) and anti-PM ATPase (Cy3) antibodies. Cytokinetic cells are marked with arrows. (*a* and *d*) Early, (*b* and *e*) late, and (*c* and *f*) final stages of cytokinesis stained for PM ATPase (*a–c*) and KN protein (*d–f*). Note presence of PM ATPase in surrounding plasma membrane and its absence from the forming cell plate until the stage when only remnants of KN signal are present at the edges (*arrows* in *c* and *f*). Bar, 10 μm.

protein in cytokinetic cells, using an antiserum raised against recombinant ADL1 (Park et al., 1997). It had been shown previously that the dynamin-like phragmoplastin (PDL) from soybean was expressed in dividing as well as in nondividing cells and that a substantial fraction of PDL redistributed to the cell plate during cytokinesis (Gu and Verma, 1996). We made the same observation for ADL1, and Fig. 5 *c* shows a wild-type cell that accumulated ADL1 at the newly forming cell plate during cytokinesis (Fig. 5 *c*). We also detected ADL1 at the plane of cell division in kn^{X37-2} mutant cells, suggesting that membrane vesicles accumulated there in the absence of KN protein (Fig. 5 *d*).

To visualize the cytokinesis defect in more detail, we prepared serial sections of kn^{X37-2} mutant embryos for EM analysis. Fig. 6 *a* shows a diagram of an enlarged interphase cell displaying the characteristic features of incomplete cytokinesis, such as several nuclei and fragments of internal cell walls. In addition, bands of vesicles extend
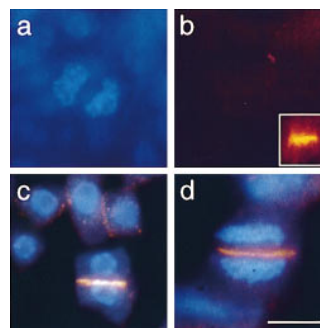

Figure 5. Cytokinetic *knolle* mutant cells. Cells from squashed embryos were stained with DAPI (*blue*) and with anti-KN antibody (*b*) or anti-ADL1 antibody (*c* and *d*) followed by Cy3-conjugated secondary antibody (*orange*). (*a* and *b*) Telophase cell from kn^{X37-2} mutant embryo: no KN-positive material. Inset in *b* shows KN signal from wild-type telophase cell photographed under identical conditions. (*c* and *d*) Cytokinetic cell from wild-type (*c*) and kn^{X37-2} mutant (*d*) embryo: ADL1 accumulates in the plane of cell division. Note enlarged mutant cell in *d*. Bar, 10 μm.
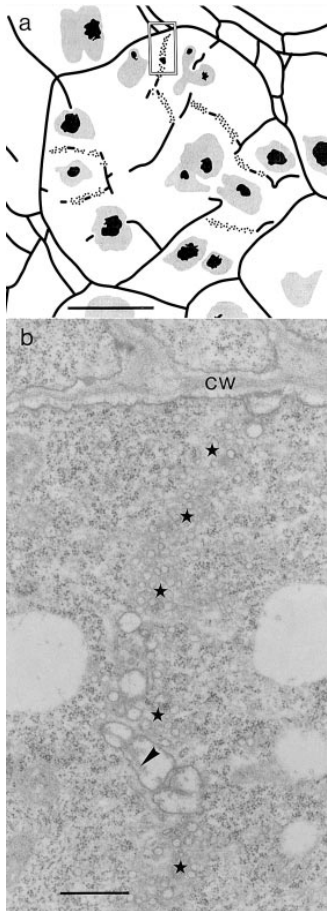
Figure 6. Ultrastructure of a *knolle* mutant cell. (*a*) Diagram of a single large interphase cell from a kn^{X37-2} mutant torpedo-stage embryo. Note multiple nuclei (*light shading*), nucleoli (*dark shading*), incomplete internal cell walls (*solid lines*), and bands of vesicles (*dotted lines*). (*b*) Higher-magnification electron micrograph of region boxed in *a*. A band of vesicles (*asterisks*) is interrupted by local aggregates of vesicles that enclose electron-dense material resembling the middle lamella of cell wall (*arrowhead*). Note nearly uniform size of vesicles. *cw*, outer cell wall of multinucleate cell. Bars: (*a*) 10 μm; (*b*) 0.5 μm.

some of the wall fragments. At higher magnification, a nearly 1-μm-wide band of vesicles appears to extend from the cell surface to the interior, interrupted by islands of vesicle aggregates that enclose stretches of material that resembles the middle lamella of the cell wall (Fig. 6 *b*). The vesicles are fairly uniform in size, measuring 60–80 nm in diameter, and appear to be coated by electron-dense material. The spaces between adjacent vesicles are free of ribosomes and paved with homogeneous material. We followed the extension of the vesicle band in adjacent sections and determined that the vesicles were actually arranged in a disc-shaped structure that eventually joined a mature cell wall (data not shown). These data imply that the lack of KN protein does not block vesicle transport but impairs vesicle fusion during the formation of the cell plate.

KN Protein Is Expressed in Dividing Somatic Cells and in the Cellularizing Endosperm but Not during Cytokinesis of Male Meiotic Cells

Most cell divisions during plant development involve the phragmoplast-assisted formation of a cell plate by vesicle fusion in the plane of division. However, there are exceptions to this type of cytokinesis. For example, the endosperm, which surrounds the developing embryo, passes through a syncytial stage of nuclear multiplication ("free nuclear endosperm") before cellularization by the simultaneous formation of cell walls between adjacent nuclei (Mansfield and Briarty, 1990*a*, *b*; for review see Olsen et al., 1995). The cell walls grow in from the surface, and masses of ves-
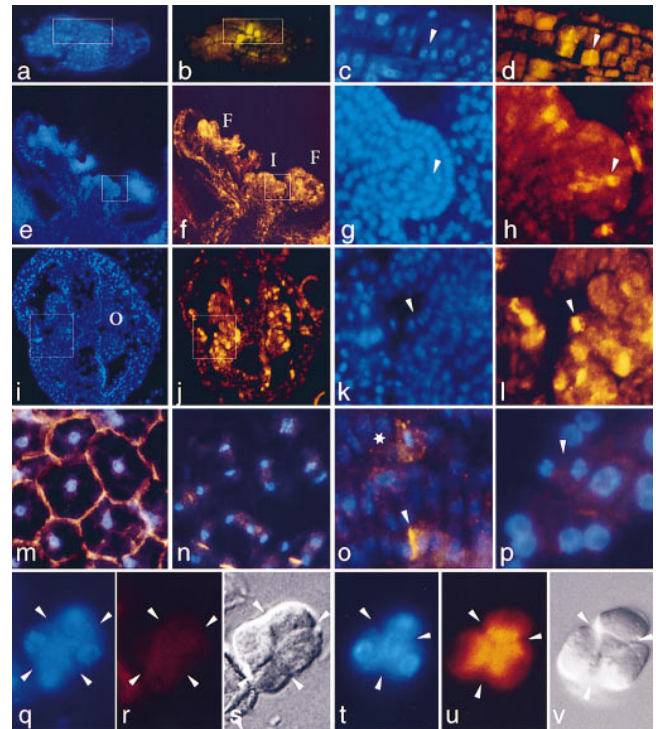

Figure 7. Accumulation of KN-positive material in dividing tissues. Cryosections (*a–l*) or squashed tissues (*m–v*) were stained with DAPI (*blue*) and with anti-KN antiserum (*b*, *d*, *f*, *h*, *j*, *l–p*, and *r*) or anti-tubulin antibody (*u*) followed by Cy3-conjugated secondary antibody (*yellow* or *orange*). (*a–d*) Seedling root tip; basal end to the right. Slightly oblique section. KN-positive mitotic cell (*arrowheads* in *c* and *d*) visible at higher magnification of region boxed in *a* and *b*. (*e–h*) Inflorescence apex with flower primordia. Longitudinal section. *I*, Inflorescence meristem; *F*, flower primordia. KN-positive mitotic cell (*arrowheads* in *g* and *h*) visible at higher magnification of region boxed in *e* and *f*. (*i–l*) Pistil of developing flower with ovules (*O*). Cross section. KN-positive mitotic cell (*arrowheads* in *k* and *l*) visible at higher magnification of region boxed in *i* and *j*. (*m*) Cellularizing endosperm. Note cages of KN-positive material (*orange*) surrounding nuclei (*blue*). (*n*) Cellular endosperm with KN-positive material in planes of cell division. (*o*) Male sporogenous tissue. *Asterisk*, metaphase; *arrowhead*, telophase. (*p*) Tapetum cell in early telophase (*arrowhead*) of endomitosis. No KN signal. (*q–v*) Cytokinesis after male second meiotic division. (*q* and *t*) DAPI staining. (*r*) No KN signal. (*u*) Phragmoplast-like arrangement of microtubules. (*s* and *v*) Corresponding Nomarski images. Note ingrowing cell walls (*arrowheads*).

icles ~70 nm in diameter and microtubules have been observed near the growing tips of cell walls (Mansfield and Briarty, 1990*b*). A special case of cytokinesis also occurs in male meiotic cells. After the two nuclear divisions, the new cell walls grow in from the cell surface, although microtubule arrays form a complex network of phragmoplast-like structures (Owen and Makaroff, 1995; Peirson et al., 1997).

To determine whether the KN protein is generally involved in cytokinesis or only in divisions that deploy the phragmoplast-assisted formation of a cell plate, we studied the accumulation of KN protein in various tissues undergoing cell division (Fig. 7). In addition to embryonic cells (see Fig. 3), KN-positive material was found in dividing cells

in the seedling root (Fig. 7, *a–d*), in the inflorescence apex (Fig. 7, *e–h*), and in developing flowers (Fig. 7 *i–l*). Thus, these somatic tissues appear to use KN protein in cytokinesis. Of the exceptional cases we analyzed, the endosperm expressed KN protein. During cellularization, hexagonal arrays of KN-positive material separated adjacent nuclei (Fig. 7 *m*), and later on, the asynchronously dividing endosperm cells accumulated KN-positive material in the plane of cell division in much the same way as dividing embryo cells (Fig. 7 *n*). The only truly exceptional case was the cytokinesis of the male meiotic cell. The sporogenous tissue of anthers, which generates the male meiotic cells, expressed KN protein essentially like somatic cells (Fig. 7 *o*). As expected, surrounding tapetum cells that were undergoing endomitosis, but not cytokinesis, did not give a KN signal (Fig. 7 *p*). Also, no KN-positive material accumulated during the cytokinesis of the male meiotic cell (Fig. 7, *q–s*) although phragmoplast-like microtubule arrays formed between the four nuclei of the tetrad after the second nuclear division (Fig. 7, *t–v*). Thus, the cytokinesis of the male meiotic cell appears to occur via a KN-independent mechanism.

Discussion

Current models of higher plant cytokinesis suggest that Golgi-derived vesicles are transported along the phragmoplast to the plane of cell division, where they fuse with one another to form an immature cross-wall, the cell plate, covered with a new plasma membrane (Samuels et al., 1995; Staehelin and Hepler, 1996). This mode of cytokinesis appears totally different from the pulling-in of the existing plasma membrane during animal cytokinesis and may thus involve a unique mechanism.

Mechanistic studies of plant cytokinesis require knowledge of the molecules involved. One way to identify relevant molecules is to isolate mutants with specific phenotypes, such as incomplete cell walls, and to clone the affected genes. The *KN* gene thus identified encodes a syntaxin-related protein and is expressed in a cell cycle–dependent manner (Lukowitz et al., 1996). Those findings raised the possibility that KN protein may be specifically involved in cytokinetic vesicle trafficking. In this study, we have made the following observations to determine the role of KN protein in cytokinesis: (*a*) KN protein is tightly associated with membranes. (*b*) KN protein is expressed only in dividing cells from the onset of mitosis, it accumulates at the plane of division in a centrifugal progression and disappears upon the completion of the cell plate. (*c*) In *kn* mutant cells, membrane vesicles accumulate at the plane of cell division, but their fusion appears to be impaired. (*d*) KN protein was not detected in male meiotic cells, which undergo a distinct mode of cytokinesis.

Role of KNOLLE in Membrane Fusion during Cell Plate Formation

The cytokinesis defect of *kn* mutant cells suggested that KN protein may be involved in vesicle trafficking to, or vesicle fusion at, the plane of cell division (Lukowitz et al., 1996). Our observation that ADL1 protein, the *Arabidopsis* homologue of the dynamin-like phragmoplastin, accumulates at the division plane of *kn* mutant cells makes it unlikely that KN protein is essential for the transport of Golgi-derived vesicles along the phragmoplast. This conclusion is supported by our electron microscopic study of *kn* mutant cells, which revealed bands of free vesicles interrupted by scattered patches of membrane-bounded material. What role ADL1/phragmoplastin plays in cell plate formation has yet to be established. However, arguments have been put forward to support the idea that these dynamin-like proteins act at an early step in cytokinesis. Their animal counterpart, dynamin, appears to attach to clathrin-coated vesicle buds of membranes, where it oligomerizes to form a collar-like structure or, under certain conditions, a tubular structure (Baba et al., 1995; Hinshaw and Schmid, 1995; Takei et al., 1995). These structures resemble the tubular extensions of vesicles observed in the early cell plate of dividing plant cells (Samuels et al., 1995; Gu and Verma, 1996). In addition, a GFP fusion of phragmoplastin, the soy bean homologue of ADL1, still accumulates in the division plane of transgenic tobacco cells treated with caffeine, a drug that inhibits maturation of the cell plate but has a less pronounced effect on the early steps in cell plate formation (Gu and Verma, 1997; see also Samuels and Staehelin, 1996).

In *kn* mutant cells, daughter nuclei are not or are incompletely separated by a cell wall (Lukowitz et al., 1996). To explain this phenotype as the consequence of a primary defect in cytokinetic vesicle fusion, two interpretations are conceivable. Considering its similarity to syntaxins that act as t-SNAREs, docking specific vesicles to target membranes (heterotypic fusion), KN protein might mediate heterotypic fusion of later-arriving vesicles with a short stretch of cell plate membrane that may have formed initially via a KN-independent mechanism. In this scenario, the same KN-independent mechanism may be used to form the incomplete cell walls found in *kn* mutant cells. However, there is no evidence for such a two-step formation of the cell plate during cytokinesis. Furthermore, KN protein accumulates in the plane of cell division essentially at the same time when the phragmoplast forms in the center of the early telophase cell. We thus favor the alternative interpretation that the entire cell plate arises by KN-mediated homotypic fusion of Golgi-derived vesicles. A recent study of the yeast vacuolar compartment has established that homotypic fusion can indeed be mediated by specific v- and t-SNAREs (Nichols et al., 1997). In that case, vesicle fusion was impaired but still occurred with low efficiency when the v-SNARE was absent and was almost abolished in the absence of the syntaxin-related t-SNARE (Nichols et al., 1997). A likely candidate for a v-SNARE complementary to the t-SNARE KNOLLE would be the product of the *KEULE* gene, which has not been isolated. Mutant *keule* cells display very similar defects in cytokinesis, although slightly weaker and more variable (Assaad et al., 1996).

Is the Cell Plate a Distinct Transient Membrane Compartment?

The expression of KN protein is tightly regulated in time and space. Not only is the *KN* mRNA made during a brief period of the cell cycle and turned over rapidly (Lukowitz

et al., 1996), but also the KN protein accumulates during M phase and disappears at the completion of cytokinesis. Topologically, the KN protein has only been localized to large cytoplasmic patches that resemble JIM 84–positive Golgi membranes in maize (Satiat-Jeunemaitre and Hawes, 1992) and to the developing cell plate. Our attempts to analyze these membrane compartments in more detail by immunogold labeling were not successful, presumably because the KN protein was no longer accessible after embedding of the tissue for electron microscopy; an alternative procedure, preembedding immunogold labeling of cytokinetic cells, gave distinct signals, but the necessary detergent treatment destroyed the ultrastructure (our unpublished observation). We attempted to identify the KN-positive cytoplasmic patches by double-labeling with the JIM 84 mAb. However, we did not observe cross-reaction signals in *Arabidopsis* cells, although we were successful in JIM 84 labeling of maize root cells (our unpublished observation). Nevertheless, the most likely interpretation seems that KN specifically localizes to post-Golgi vesicles destined to form the cell plate.

What is the biological significance of the tight regulation of KN protein expression? The Golgi apparatus is the site of departure for vesicle traffic to a variety of membrane compartments. During cytokinesis, much of the post-Golgi vesicle traffic has to be routed to the developing cell plate. Our results suggest that this is achieved by creating a specialized set of cytokinetic vesicles marked by KN protein: KN-positive vesicles are formed only before the onset of cytokinesis and are transported only along the phragmoplast to the plane of cell division. Vesicles normally destined to the plasma membrane are excluded from this transport route, as evidenced by the sharp boundary between the KN-positive cell plate and the PM ATPase–positive cell surface at the end of cytokinesis. Previous studies established that the newly formed cell plate differs in composition from the interphase cell wall, suggesting that cytokinetic vesicles also contain a specific cargo (Samuels et al., 1995, and references therein). The existence of a specific route for vesicle transport to the cell plate is also supported by the observation that *kn* mutant cells do not seem to be impaired in other aspects of membrane traffic, including increase in cell surface area as well as tip growth of root hairs and pollen tubes (Lukowitz et al., 1996). Although apparently not required for the transport of cytokinetic vesicles, KN protein may ensure that only vesicles with the correct membrane composition and cargo fuse with one another to form the cell plate and may thus help to establish and maintain this transient membrane compartment.

Different Modes of Plant Cell Division

The prevalent center-out mode of higher plant cell division involves a phragmoplast for directed vesicle transport to the plane of division, where the vesicles fuse to form a cell plate that expands laterally to reach the parental cell surface (Wick, 1991). Although cellularization of the endosperm involves ingrowth of cell wall from the surface, both microtubules and free vesicles have been observed near the growing cell wall tips (Mansfield and Briarty, 1990*b*). Subsequently, the center-out mode of division also operates in endosperm cells (Olsen et al., 1995). In all these cases, KN protein accumulates in the plane of division. The only exception we have found so far is the male meiotic cell, which undergoes cytokinesis by cell wall ingrowth from the periphery after meiosis II (Owen and Makaroff, 1995; Peirson et al., 1997) and does not express KN protein. Our observation is consistent with the recent report that cytokinesis of the male meiotic cell is specifically affected by mutations in a different *Arabidopsis* gene, *STUD* (Hülskamp et al., 1997). Thus, at least two distinct mechanisms of cell division appear to operate in higher plants.

In reality, the situation may be more complex because the fusion of the cell plate with the parental cell wall, which completes center-out cytokinesis, requires an active contribution of the cortical site that was marked earlier by the preprophase band (Mineyuki and Gunning, 1990, and references therein). Furthermore, when cell plate formation is disrupted by caffeine, cell wall stubs are formed, which may reflect a default mechanism for cytokinesis by ingrowth from the cell surface (Röper and Röper, 1977). From an evolutionary perspective, this may not be surprising since two separate mechanisms, actin-based cleavage initiated at the parental wall and microtubule-dependent formation of a cell plate in the center, combine to achieve cytokinesis in the alga *Spirogyra* (McIntosh et al., 1995; Sawitzky and Grolig, 1995). Considering the important role of the KN protein in cell plate formation, the isolation of KN homologues from lower plants may shed light on the evolution of the center-out mode of cytokinesis in higher plants.

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