The *Arabidopsis KNOLLE* and *KEULE* genes interact to promote vesicle fusion during cytokinesis

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Partitioning of the cytoplasm during cytokinesis or cellularisation requires syntaxin-mediated membrane fusion [1–3]. Whereas in animals, membrane fusion promotes ingression of a cleavage furrow from the plasma membrane [4,5], somatic cells of higher plants form *de novo* a transient membrane compartment, the cell plate, which is initiated in the centre of the division plane and matures into a new cell wall and its flanking plasma membranes [6,7]. Cell plate formation results from the fusion of Golgi-derived vesicles delivered by a dynamic cytoskeletal array, the phragmoplast. Mutations in two *Arabidopsis* genes, *KNOLLE* (*KN*) and *KEULE* (*KEU*), cause abnormal seedlings with multinucleate cells and incomplete cell walls [1,8]. The *KN* gene encodes a cytokinesis-specific syntaxin which localises to the cell plate [9]. Here, we show that KN protein localisation is unaffected in *keu* mutant cells, which, like *kn*, display phragmoplast microtubules and accumulate ADL1 protein in the plane of cell division but vesicles fail to fuse with one another. Genetic interactions between KN and KEU were analysed in double mutant embryos. Whereas the haploid gametophytes gave rise to functional gametes, the embryos behaved like single cells displaying multiple, synchronously cycling nuclei, cell cycle-dependent microtubule arrays and ADL1 accumulation between pairs of daughter nuclei. This complete inhibition of cytokinesis from fertilisation indicates that *KN* and *KEU*, have partially redundant functions and interact specifically in vesicle fusion during cytokinesis of somatic cells.

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Received: **28 July 2000**
Revised: **31 August 2000**
Accepted: **31 August 2000**

Published: **20 October 2000**

Current Biology 2000, 10:1371–1374

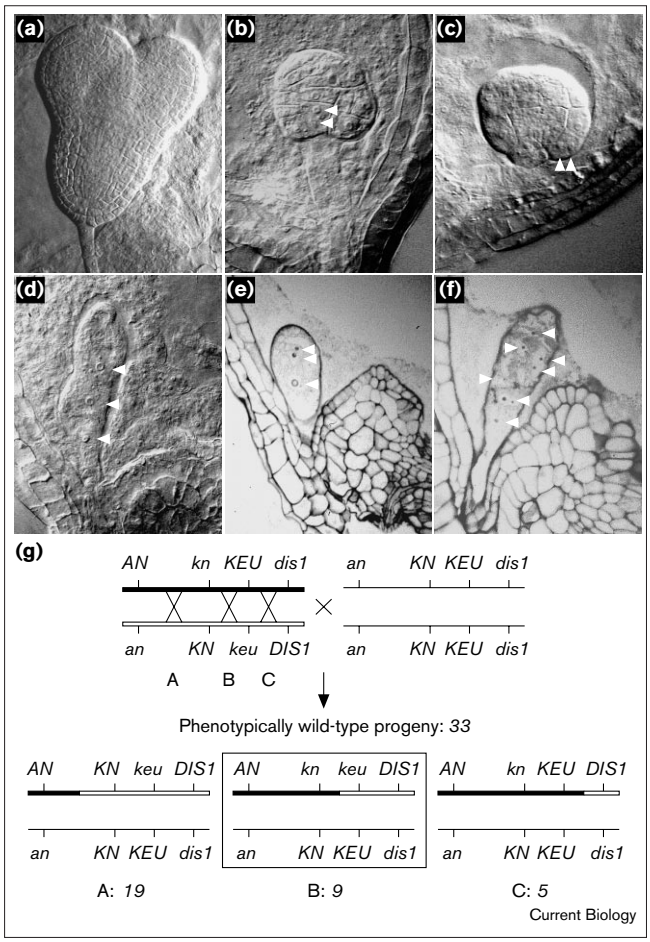
0960-9822/00/$ – see front matter

Results and discussion

Cytokinesis of plant somatic cells requires two components, a specific cytoskeletal array for vesicle delivery and a vesicle fusion machinery. Genetic inactivation of either component is expected to block cytokinesis. Mutations in the *Arabidopsis PILZ* group of genes can give rise to single-celled embryos that lack microtubules, including those of the phragmoplast, and that fail to localise KNOLLE (KN) protein [10]. By contrast, attempts to isolate mutants that completely abolish cytokinesis by interfering with vesicle fusion were unsuccessful. The plant cytokinesis mutants described previously, *cyd* [11] in pea as well as *kn* [1] and (*keule*) *keu* [8] in *Arabidopsis*, all impair but do not block cytokinesis. Both *kn* and *keu* mutant embryos show phenotypes typically associated with defects in cytokinesis, such as incomplete cell walls and supernumerary and polyploid nuclei (Figure 1). Examination of the cell-cycle dependent changes in the microtubular cytoskeleton did not reveal a dramatic difference to the wild type. All specific arrays, such as the preprophase band, mitotic spindle and phragmoplast, were present albeit slightly enlarged in *keu* and *kn* mutants, correlating with an increased cell size (Figure 2i–k; data not shown). To monitor membrane transport to the cell plate, we stained dividing *keu* mutant cells for KN protein and ADL1, a dynamin-like protein from *Arabidopsis* [12]. Both proteins are membrane-associated and accumulate at the plane of cell division during cytokinesis [9]. *keu* mutant cells showed essentially the same localisation pattern for these proteins as the wild type. KN and ADL1 accumulated between pairs of daughter nuclei (Figure 2l,m), which indicates that *keu* mutants are not impaired in the production, transport or spatial alignment of cytokinetic vesicles. Similar results have previously been obtained for *kn* mutants [9].

To examine the genetic interactions between *KN* and *KEU*, we analyzed the phenotype of double mutant embryos. The close genetic linkage of 8 cM between *kn* and *keu* enabled us to generate, by recombination, heterozygous plants carrying the two mutations on the same chromosome and the two wild-type alleles on the homologous chromosome (Figure 1g; see Supplementary material for details). Upon selfing, these plants segregated ~21% *kn keu* double mutants and, due to meiotic recombination between *kn* and *keu*, ~8% *kn* or *keu* single mutants (Table 1). The *kn keu* embryos showed a dramatically enhanced cytokinesis defect. They were rod- or club-shaped with multiple nuclei that were not separated by internal cell walls (Figure 1d–f). Their apical tips often

Figure 1



Overview of **(a)** wild-type, **(b)** *knolle*, **(c)** *keule* and **(d–f)** *knolle keule* embryos. Note enlarged cells and supernumerary nuclei (arrowheads) in (b,c) and lack of internal cell walls and multiple nuclei (arrowheads) in (d–f). (a–d) Whole-mount preparations of heart-stage embryos using Nomarski optics; by focusing through the embryo in (d), we counted 14 nuclei of which three are visible in this focal plane. (e,f) Histological sections of embryos at the globular and torpedo stages, respectively. The embryos shown in (e) and (f) contained 11 and 21 nuclei, respectively, as determined by the inspection of serial sections; three (e) and seven (f) nuclei (arrowheads) are visible in the sections shown. Apical end up. **(g)** Construction of the *kn keu* double mutant chromosome (boxed) by crossover in interval B of the *an–dis1* region (see Supplementary material).

Table 1

Quantitative analysis of mutant embryos: phenotypes of progeny of *AN kn keu DIS1/an KN KEU dis1* plants.

Seed*		Early embryo†		Expected‡	
Normal	76%	Normal	68%	Wild type	71%
Collapsed	19%	No internal cell walls	24%	*knolle keule*	21%
knolle or *keule*	5%	*knolle* or *keule*	8%	*knolle* or *keule*	8%

*Normal seeds contained mature embryos; no. analysed: 1,723. †Normal embryos from the same silique were at the globular or heart stage; no. analysed: 633. A small proportion of the embryos (about 1%) had one or two internal cell walls but clearly differed from *kn* or *keu* embryos (see Table 2). ‡From meiotic recombination frequency based on a genetic distance of 8 cM between *KNOLLE* and *KEULE* (see Supplementary material).

contained a large vacuole and the nuclei were confined to a stalk-like portion. The number of nuclei increased during embryogenesis to ~30 in the most advanced stages, indicating that nuclear division cycles occurred in the absence of cytokinesis. The double mutant embryos collapsed when their normal siblings had almost completed embryogenesis.

Among the progeny of *kn keu/KN KEU* plants, ~1% of the embryos consisted of 2 or 3 cells. To determine the likely genotype of these exceptional embryos, we performed reciprocal test crosses with heterozygous plants bearing one or the other mutant allele or both (Table 2). The same minority of exceptional embryos were observed among progeny of crosses between *kn keu/KN KEU* and *keu/KEU* plants, suggesting that the exceptional embryos from selfed *kn keu/KN KEU* plants were generated by meiotic recombination and had the genotype *kn keu/KN keu*. Only a minority of the *kn keu/KN keu* embryos showed the exceptional phenotype, suggesting that in *keu* mutants cytokinesis becomes sensitive to the reduced amount of *KN* activity. By contrast, reduction of *KEU* activity in *kn* mutant embryos had little effect.

Whereas wild-type embryos displayed asynchronous cell cycles from early on (Figure 2a,e), all nuclei of *kn keu* double mutant embryos were synchronised during the cell cycle (Figure 2b–d,f–h). Hoops of cortical microtubules normally found in interphase cells girdled the entire *kn keu* embryo (Figure 2b,f). The multiple nuclei of individual embryos progressed synchronously through the mitotic cycle. Mitotic embryos had multiple metaphase plates associated with spindles (Figure 2c,g). Metaphase plates differed in size, due to different chromosome numbers (Figure 2g). At the end of mitosis, double mutant embryos contained multiple phragmoplasts that formed a nearly continuous band separating multiple pairs of daughter nuclei (Figure 2d,h). To examine whether cytokinetic vesicles were produced and transported to the plane of cell division, *kn keu* double mutant embryos were immunostained for ADL1 (Figure 2n). ADL1 accumulated in a long, band-like structure between multiple pairs of daughter cells. Taken together, *kn keu* embryos were able to form a microtubular phragmoplast and to accumulate membrane material at the plane of cell division. However, they completely lacked internal cell walls, indicating that cell plate formation was blocked. Consistent with this finding, they behaved physiologically like single, multinucleated cells in which all nuclei were subject to the same cell-cycle controls.

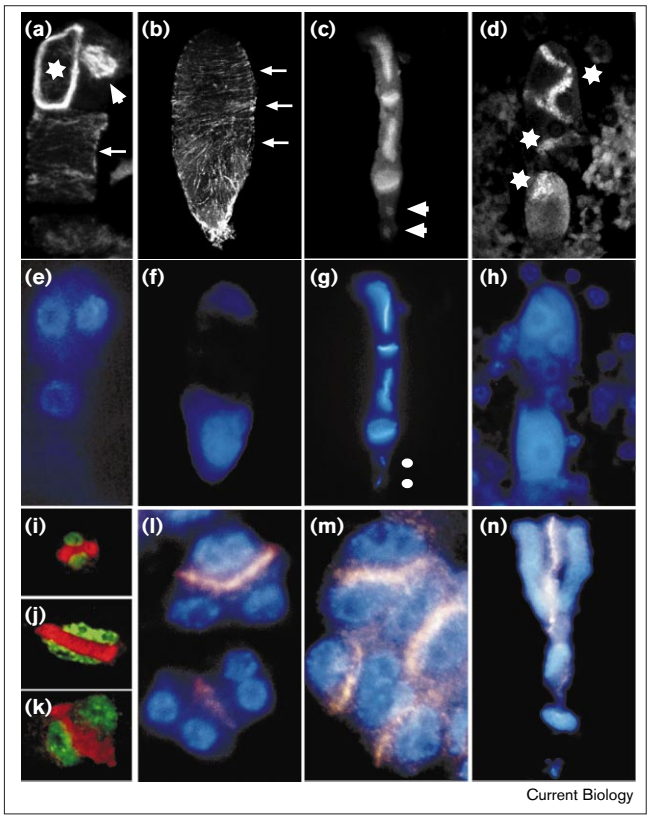
Table 2

Quantitative analysis of mutant embryos: origin of exceptional embryo progeny as revealed by test crosses.

Cross*	Embryos (siliques) analysed†	Embryos with 2 or 3 cells‡
kn/KN × *kn/KN*	440 (10)	0
keu/KEU × *keu/KEU*	725 (15)	0
kn keu/KN KEU × *kn keu/KN KEU*	282 (8)	4 (1%)
kn/KN × *kn keu/KN KEU*	517 (11)	0
kn keu/KN KEU × *kn/KN*	429 (11)	1 (< 1%)
keu/KEU × *kn keu/KN KEU*	508 (11)	5 (1%)
kn keu/KN KEU × *keu/KEU*	492 (12)	9 (2%)

*Alleles used: *kn*, AP6-16; keu, AP77-18; *kn keu*, AP6-16 AP77-18.
†Normal embryos were at the early-heart to torpedo stages.
‡Excluding suspensor.

Figure 2



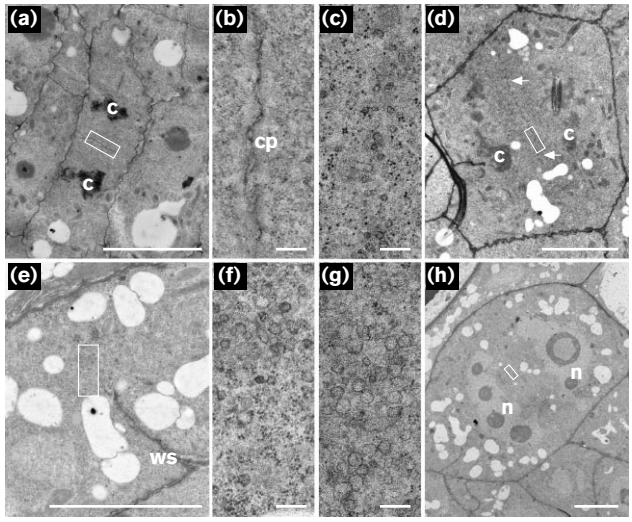
Cell-cycle-specific features of wild-type, *knolle*, *keule* and *knolle keule* embryos. **(a–h)** Whole-mount preparations of (a,e) wild-type and (b–d,f–h) *kn keu* double mutant embryos stained for microtubules (panels a–d, confocal images) and chromatin (panels e–h, epifluorescence): (a,e) wild-type two-cell stage embryo with asynchronous cell cycles (preprophase band, asterisk; mitotic spindle, arrowhead; interphase microtubules, arrow); (b,f) *kn keu* interphase with hoops of cortical microtubules (arrows) girdling the entire embryo; (c) *kn keu* mitotic spindles and (g) metaphase plates, the two smallest are marked by arrowheads or dots; (d,h) microtubular phragmoplasts (d; asterisks) separating pairs of daughter nuclei (h). **(i–k)** Confocal images of cells from squashed late-heart stage embryos showing phragmoplast microtubules (red) and chromatin (green): (i) wild-type, (j) *kn* and (k) *keu* cells. **(l–n)** Accumulation of cytokinesis marker proteins (pink) between pairs of daughter nuclei (blue) in (l,m) dividing *keu* cells and in (n) *kn keu* embryo: (l) KN protein; (m,n) ADL1 protein; epifluorescence microscopy.

The embryonic phenotype of the *kn keu* double mutant implied that the *KEU* gene is required for the fusion of cytokinetic vesicles. For confirmation, we performed transmission electron microscopy on embryo sections (Figure 3). In wild-type cells, the newly formed cell plate grows from the centre to the periphery during telophase (Figure 3a,b). By contrast, a band of unfused membrane vesicles was observed in the division plane of the *keu* telophase cell (Figure 3c,d). In the *keu* interphase cell, most of the cytokinetic vesicles were still separate and located near the tip of a cell wall stub extending from the parental cell wall (Figure 3e,f). Very similar defects were found in the *kn* interphase cell (Figure 3g,h; [9]). The defects observed in *keu* mutant cells were therefore indistinguishable from those of *kn* mutant cells, which lack a cytokinesis-specific syntaxin [9].

Our analysis of the *kn keu* double mutant indicates that both KN and KEU are specifically involved in vesicle fusion during somatic cytokinesis. Although cytokinesis was completely blocked from fertilisation on, the *kn keu* embryos enlarged and underwent several rounds of synchronised nuclear divisions, suggesting that non-cytokinetic vesicle fusion is not impaired. Furthermore, the double-mutant embryos originated from *kn keu* gametophytes of the parental heterozygous plant, indicating that cytokinesis in the haploid phase of the plant life cycle does not require KN and KEU but involves different, as yet unidentified, genetic components [7].

The genetic interaction between *knolle* and *keule* suggests that the two gene products, KN and KEU, contribute different functions to promote vesicle fusion during cytokinesis. In the absence of either gene product, cytokinesis is compromised but can occur, although inefficiently. One possible explanation is that the two proteins are part of a complex that would still function to some extent in the absence of one or another component. However, such a scenario has to account for the role of KN protein as a cytokinesis-specific syntaxin. If KEU were to interact exclusively with KN, thus modifying its activity, this could not account for the complete inhibition of cytokinesis in the *kn keu* double mutant embryo. The only case known in which inactivation of a syntaxin still allows for some residual membrane fusion is the homotypic vacuolar fusion in yeast [13] but it is currently not known whether cell-plate formation also results from homotypic vesicle fusion. Another possible explanation for the apparent redundancy of cytokinetic vesicle fusion may be sought in protein families with partially overlapping functions. For example, promiscuity of syntaxin–synaptobrevin interaction in vesicle fusion has been demonstrated *in vitro*

Figure 3



Cytokinetic vesicles and cell-wall fragments in embryonic cells. **(a,b)** Wild-type telophase: a cell plate (box; cp) has formed between the two sets of daughter chromosomes (labelled 'c'); (b) higher magnification of area boxed in (a). **(d)** *keu* telophase: vesicles are lined up (arrowheads) between the two sets of daughter chromosomes; **(c)** higher magnification of area boxed in (d); **(e)** *keu* interphase: aggregate of vesicles (box) near cell wall stub (ws); **(f)** higher magnification of area boxed in (e); **(h)** *kn* interphase: aggregate of vesicles (box) between nuclei (labelled 'n'); **(g)** higher magnification of area boxed in (h). Scale bars, 5 μm (a,d,e,h); 200 nm (b,c,f,g).

[14,15]. If both KN and KEU were members of different protein families and each of them could interact with additional members of the other family to bring about vesicle fusion, some active complexes would still form in the single mutants but not in the *kn keu* double mutant. Genetic interactions similar to KN and KEU have not been reported for membrane fusion in animal cytokinesis, which may indicate that plant cytokinesis has to be executed very efficiently, involving *de novo* formation of a membrane compartment by vesicle fusion.

Materials and methods

The *knolle* [1] and *keule* [8] mutant lines, plant growth conditions [16], procedure for immunofluorescence [9] and electron microscopy [9] have been described previously.

Supplementary material

Supplementary material including additional methodological details, and construction of the *knolle keule* double mutant chromosome is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

I.W. and W.L. contributed equally to this work. We thank I. Zimmermann for preparing the electron microscopy sections, I. Hwang for a gift of the anti-ADL1 antiserum, and J. Mathur and K. Schrick for critical reading of the manuscript. This work was supported by a DFG Leibniz award to G.J. and funded in part by the European Communities' Biotechnology Program Framework IV.

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