Characterization of a *cytokinesis defective* (*cyd1*) mutant of *Arabidopsis*

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Abstract

Although several mutations and genes affecting plant cytokinesis have been identified, mutant screens are not yet saturated and knowledge about gene function is still limited. A novel *Arabidopsis* mutation, *cytokinesis defective1* (*cyd1*), was identified by partial or missing cell walls in stomata. Stomata with incomplete or no cytokinesis still differentiate and some contain swellings of the outer wall not found in the wild type. The incomplete walls are correctly placed opposite stomatal wall thickenings suggesting that the mutation interferes with the execution of cytokinesis rather than with the placement of the division site. Cytokinesis defects are also detectable in other cell types throughout the plant, defects which include cell wall protrusions, two or more nuclei in one cell, and reduced cell number. The extent of cytokinetic partitioning correlates with nuclear number in abnormal stomata. Many *cyd1* epidermal cells, stomata and pollen are larger, and trichomes have more branches. *cyd1* is partially lethal with poor seed set and some defective ovules, but many plants are fertile despite abnormalities in vegetative and reproductive development such as missing, reduced, fused or misshapen leaves and floral organs. *cyd1* appears to be the only cytokinesis mutant described where defects are known to occur in both mature vegetative and reproductive organs. Thus, the *CYD1* gene product appears to be necessary for the execution of cytokinesis throughout the shoot. The examination of stomata by microscopy may be a useful screen for the directed isolation of additional cytokinesis mutations that are not embryo or seedling lethal.

Key words: Cytokinesis, stomata, cell division.

Introduction

Cell division is a fundamental process involving division of the nucleus (karyokinesis) and cytoplasm (cytokinesis). This process functions at all levels of development, such as in producing new cells for growth and reproduction, and in allocating separate cell fates in asymmetric divisions.

Cytokinesis in plants encompasses events unique among eukaryotes, including the functioning of a plant-specific cytoskeletal array, the phragmoplast, and the centrifugal development of the cell plate which forms the new cell wall (Mineyuki and Gunning, 1990; Samuels *et al.*, 1995; Staehelin and Hepler, 1996; Assaad *et al.*, 1997; Heese *et al.*, 1998). Stages of cytokinesis include the transport and fusion of exocytic Golgi vesicles, the assembly and subsequent fusion of a network of tubules, the outward growth of the cell plate, and the fusion of the cell plate with the parental cell wall at the division site.

A few proteins are known to be associated with cytokinesis in plants (Heese *et al.*, 1998). Phragmoplastin, a dynamin-like protein, has been shown to localize to the cell plate and may be involved in vesicle trafficking (Gu and Verma, 1996). A syntaxin-like protein was identified by the *knolle* mutation in *Arabidopsis* in which cytokinesis is defective perhaps through impaired vesicle fusion in the nascent cell plate (Lauber *et al.*, 1997; Lukowitz *et al.*, 1996). Many other proteins must participate, but little is known about the genetic and cellular regulation of cytokinesis in plants.

Several plant mutations have been described that affect cytokinesis and are likely candidates for genes encoding other components of the cytokinetic machinery. These include the *cyd* (*cytokinesis defective*) mutant of pea, and the *knolle*, *keule* and *cyt1* mutants of *Arabidopsis* (Liu *et al.* 1995; Lukowitz *et al.*, 1996; Assaad *et al.*, 1996;

Nickle and Meinke, 1998). These were identified indirectly by screening for abnormalities in the morphology or vitality of the embryo which proved to exhibit perturbations in cytokinesis. In other cytokinesis mutations in *Arabidopsis* the phenotype seems restricted to specific domains of the mature plant. For example, the *tso1* mutation affects the inflorescence meristem but not vegetative tissues (Liu *et al.*, 1997), and the multinucleic (*mun*) mutations may affect only root tissues (Hauser *et al.*, 1997). Cytokinesis mutants in pollen development have also been described in *Arabidopsis* (*stud*, *tetraspore*) and alfalfa (McCoy and Smith, 1983; Hülskamp *et al.*, 1997; Spielman *et al.*, 1997).

Here a new locus is reported that affects cytokinesis in *Arabidopsis*, *cytokinesis defective1* (*cyd1*), that was isolated in a microscopy-based screen for stomatal mutants (Yang and Sack, 1995). Because the division of *Arabidopsis* guard mother cells is stereotyped and symmetric (Larkin *et al.*, 1997), cytokinesis defects were readily detected in stomata. Although cytokinesis is also disrupted throughout the shoot and appears to result in defective organogenesis, this mutation is not fully lethal.

Materials and methods

Plant material and culture

M_2 seeds of *Arabidopsis thaliana* Columbia ecotype (ethyl methane sulphonate mutagenesis; Lehle Seeds, Round Rock, Texas, USA) were used for screening as in Yang and Sack (1995). The seeds were also homozygous for the *glabrous1* (*gl1*) mutation (Larkin *et al.*, 1997). Seeds were sown on 1% nutrient agar plus 2% sucrose. Seedlings were grown at 22 °C under continuous 50–100 μmol m^{-2} s^{-1} white light.

To detect abnormalities in vegetative organ development, *cyd1* seeds were sown on agar and then grown under four different cultural conditions: (1) 30 μmol m^{-2}s^{-1} light intensity and 22 °C, (2) 100 μmol m^{-2} s^{-1} and 22 °C, (3) 30 μmol m^{-2} s^{-1} and 30 °C, and (4) 100 μmol m^{-2} s^{-1} and 30 °C. For each cultural condition the phenotypes of 108–122 seeds or seedlings were evaluated. Although there was a slight influence of culture condition on abnormality frequency (Yang, 1996), plants from all four conditions were pooled for collective analysis. Class 3 and 4 *cyd1* seedlings (see Results) were transferred to a soil mix (peat, perlite, and vermiculite) for further examination. These plants were maintained at their original temperatures (22 °C or 30 °C) with a light intensity of 50 μmol m^{-2} s^{-1} near the top of the pots. A total of 75–99 flowers from 20–22 plants for each of the four different growing conditions was examined under a dissecting microscope. For crosses and mapping, etc, plants were grown in pots as above.

Genetic analysis and mapping

cyd1 was backcrossed to its wild-type (except for *gl1*) Columbia parent twice and then to wild-type (*GL1*) Columbia for the third backcross. For mapping, *cyd1* (Col) was crossed to Landsberg erecta (Ler) and the segregating F_2 plants were used for linkage analysis. Genomic DNA was prepared from individual F_2 plants and SSLPs analysed (Dellaporta *et al.*, 1983; Bell and Ecker, 1994). Primer pairs were obtained from Research Genetics (Huntsville, AL, USA).

Quantitative characterization of phenotypes

The numbers and densities of stomata and non-stomatal epidermal cells (ECs) in the abaxial epidermis of mature cotyledons from 18-d-old pot-grown plants were determined (Yang and Sack, 1995). Guard cell length (along the ventral wall) and width, and stomatal pore length were measured from the abaxial epidermis of cotyledons of 18–21-d-old agar-grown plants, using an ocular reticule at a magnification of 400×. Pollen size was determined as the product of the polar and equatorial diameters (Altmann *et al.*, 1994). Pollen grains from newly opened flowers were measured within 10–20 min after wetting, since dimensions change as the pollen hydrates ($n=60$ wild type and 120 *cyd1* grains). All quantitative differences reported were statistically significant at the 0.05 level (*t*-test).

Microscopy

Cells were observed with differential interference contrast and fluorescence optics (Zeiss IM35 microscope). Whole cotyledons or roots and separated mesophyll cells were stained with 10 μg ml^{-1} 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Melaragno *et al.*, 1993). To determine nuclear number in individual cells, mesophyll cells were separated according to Pyke and Leech (Pyke and Leech, 1991). Ovules and embryos were cleared as described (Yadegari *et al.*, 1994).

Roots of both normal and abnormal morphology were examined for possible cell wall protrusions and for multiple or abnormal nuclei using the propidium iodide method (van den Berg *et al.*, 1995). Plants were cultivated on sterile nutrient agar for 10–15 d, and then immersed in 0.5 mg ml^{-1} propidium iodide for 2–15 min. Whole roots were destained briefly in water and examined using a Nikon Optiphot fluorescence microscope attached to a BioRad MRC-600 confocal laser scanning system. Over 3000 cells of the epidermis, cortex and meristem were examined for each genotype (*cyd1* and wild-type Columbia) from many primary and lateral roots.

Material for light microscopy was also fixed in 3% glutaraldehyde, 1.3% formaldehyde and 1% acrolein in 0.05 M phosphate buffer at pH 6.8 for 10 h at room temperature. The tissue was embedded in Spurr's epoxy resin, sectioned at 1.5–4 μm, stained with 0.05% toluidine blue at 58 °F, and photographed using Kodak Ektachrome 100 film. For electron microscopy, leaf pieces were fixed in 3% glutaraldehyde in 75 mM potassium/sodium phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in an acetone series, and infiltrated with Spurr's epoxy resin. Sections were stained with uranyl acetate and lead acetate and examined with a CM-12 Phillips electron microscope at 60 kV.

Results

Cytokinesis is incomplete or absent in many cyd1 stomata

The *cyd1* mutant was identified by the presence of abnormal stomata that were larger or that had incomplete cytokinesis. Normally during stomatal development, the guard mother cell divides symmetrically and the new cell wall formed (ventral wall) then develops a pore along its mid-length. The cytokinesis defects found in *cyd1* stomata were grouped into categories for cytological analysis and quantification (Fig. 1). Stomata which lack any cytokinesis defect were scored as 'Type 1' stomata (Fig. 2A) and these comprised 63% of all stomata sampled. Type 2
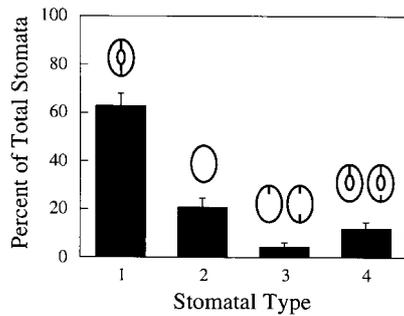
Fig. 1. The frequencies of occurrence of four different stomatal types in the abaxial epidermis of *cyd1* cotyledons (*n*=20; 18-d-old, pot-grown plants).
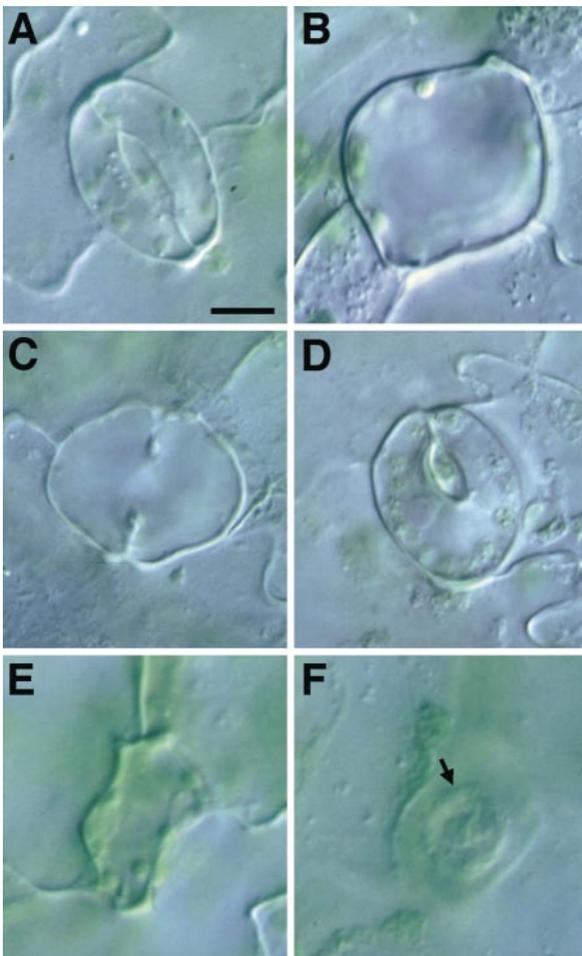

Fig. 2. Abnormal guard mother cell cytokinesis in *cyd1*. Abaxial epidermis from 15-d-old leaves. Differential interference contrast optics. Bar in (A)=10 µm for all micrographs. (A) *cyd1* Type 1 stomate with normal cytokinesis and pore. (B) Type 2 stomate with defective cytokinesis lacking any ventral wall or pore. Chloroplasts are typical of normal stomata. (C) Type 3 stomate with two wall protrusions at opposite ends of the cell with no detectible pore. (D) Type 4 stomate with incomplete wall that has a pore. (E, F) Two different planes of focus of the same defective Type 2 stomate showing a swelling on the outer tangential wall (arrow in F).

stomata (21%) entirely lack a ventral wall and a pore (Fig. 2B). Type 3 stomata (4%) have one or two ventral wall protrusions at the ends of the cell, but the wall is incomplete and no pore was detected using light microscopy (Fig. 2C). Type 4 stomata (12%) have a pore that is attached to one wall protrusion; if a second protrusion is present on the other side, it does not extend to the pore (Fig. 2D). Overall, 37% of all stomata showed some type of defective cytokinesis. While data for the frequency of occurrence of cytokinesis defects in stomata were obtained from examination of cotyledons, these defects were also regularly observed in stomata located in all organs of the plant, including rosette leaves, cauline leaves and sepals. Abnormal stomata with incomplete cytokinesis were never seen in wild-type plants.

Types 2 and 3 stomata develop an irregular papillate thickening of the outer cell wall (Fig. 2E, F) that is often elliptical to circular in the paradermal plane. In Type 2 stomata it is usually centred along the mid-length of the cell where the outer ledges of the stomatal pore would normally form. In Type 3 stomata, the swelling is associated with an incomplete protrusion of the ventral wall, closer to one end of the cell.

Incomplete walls are present in other cell types

To assess whether the defect was confined to the division of guard mother cells, other cell types were examined for incomplete cell walls. Wall protrusions were found in mature epidermal cells (ECs; Fig. 5). These protrusions usually extended from only one side of the cell and were of varying lengths. Examination of whole mounts typically revealed about three ECs with incomplete walls per *cyd1* cotyledon. Protrusions were also seen in ECs in rosette leaves, cauline leaves and sepals, but not in wild-type plants.

Wall protrusions were also found in developing and mature leaf mesophyll cells (Fig. 3A, B) and in the ground tissue of mature hypocotyls, stems and petioles (Fig. 3C). Stubs could also be found in successive serial sections through the shoot apical meristem (Fig. 3D, E) and more commonly, in meristematic cells of leaf primordia (Fig. 3F). Thus, incomplete cytokinesis occurs throughout the shoot, and some defects in cytokinesis that occur early in development are not resolved during cell maturation.

Roots were also examined for cell wall stubs using differential interference contrast microscopy of cleared roots, by optical sectioning using confocal scanning laser microscopy, and in longitudinal sections of fixed and embedded tissue. Most *cyd1* roots exhibited wild-type tissue organization (Fig. 3I) and growth rates. No wall protrusions were detected in any roots, even in those few *cyd1* roots that displayed irregular cell files, cell size and architecture (Fig. 3J). These abnormal roots were always
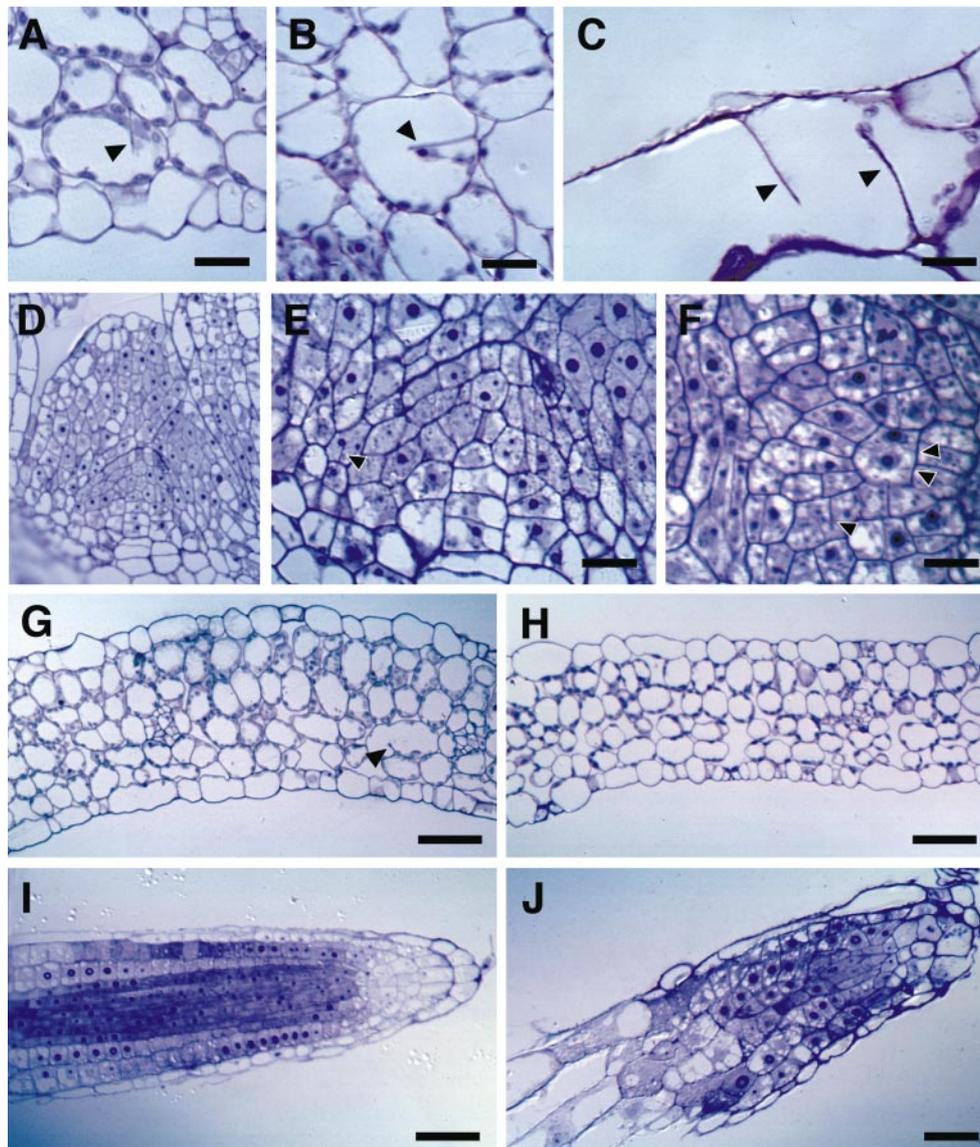
Fig. 3. Light micrographs showing anatomical phenotypes in vegetative organs of *cyd1* (except H which is wild-type). Epoxy sections stained with toluidine blue. Bars = 10 μm (A–F) or 30 μm (G–J). (A, B) Incomplete walls (arrowheads) in mesophyll cells from mature leaves. (C) Two incomplete walls in the same hypocotyl cell. (D, E) Adjacent serial sections of the same shoot apical meristem at two different magnifications. The arrowhead in (E) indicates a wall protrusion in a cell on the flank of the meristem. (F) Incomplete walls (arrowheads) in two different cells of a leaf primordium. (G, H) Cross sections of *cyd1* (G) and wild-type (H) leaves showing that *cyd1* leaves have a more or less normal organization of tissues despite the presence of incomplete cytokinesis (arrowhead). (I) *cyd1* root that displays an essentially wild-type tissue architecture. (J) *cyd1* root with disorganized cell files but no detectible incomplete walls.

found in plants exhibiting the most severe defects in overall seedling organogenesis.

cyd1 *has fewer and larger cells*

Since defects in cytokinesis would be expected to affect cell number, the numbers of cells of different types in the epidermis were quantified in fully expanded cotyledons. *cyd1* cotyledons have fewer cells per unit area and reduced absolute numbers of stomata and epidermal cells (Fig. 4). The reduction in cell number is much greater than the number of cells containing wall protrusions. The majority of *cyd1* ECs are slightly larger than the wild-type, but since there are fewer cells, *cyd1* leaf area is somewhat reduced.

cyd1 guard cells and stomatal pores are, on average, 147% and 156% longer than those of the wild-type. *cyd1* stomata also have a much broader range of sizes than the wild type (Yang, 1996). The ratio of ECs to stomata is 2.6 ± 0.1 ($\pm$ SE) for the wild-type and 3.6 ± 0.2 for *cyd1*. *cyd1* pollen grains are about 25% larger than those of the wild type (754 ± 15 versus 608 ± 7 μm^2) and also exhibit a greater range of sizes.
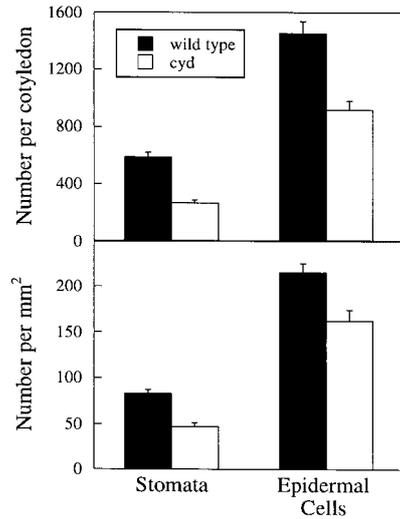
Fig. 4. Number and density of stomata and epidermal cells in mature wild-type and *cyd1* cotyledons. The *cyd1* abaxial epidermis contains fewer stomata (of all types) and epidermal cells than the wild type on both an absolute number and density basis. Same plants as in Fig. 1.

Fig. 5. Nuclei in *cyd1* cells. Fluorescence micrographs (A, C) of DAPI-stained abaxial epidermis of 14–15-d-old whole-mounted cotyledons from agar-grown plants. Corresponding bright-field images of same regions shown in (B) and (D). Bar in (A) = 10 μm for all micrographs. (A, B) Elongate, two-lobed nucleus (arrows) in a single epidermal cell with an undulate cell wall protrusion (arrowhead in B). (C, D) Two nuclei (arrows) in a single-celled, Type 2 stomate.

The *cyd1* mutation was crossed into a GL1 (Columbia) background so that the effect on trichomes could be assessed. The number of trichome branches in *cyd1* was higher than in the wild-type in the adaxial epidermis of the first leaf. Most (96%) wild-type trichomes had three branches, whereas only 3% and 1% had four and two branches, respectively, and none had five branches. In contrast, 65% of *cyd1* trichomes had four branches, 26% had three, 1% had two, and 8% had five branches. Thus, *cyd1* has fewer and larger epidermal cells and stomata, larger pollen, and trichomes with more branches than the wild type.

Two or more nuclei in the same cell

Nuclear number and morphology were studied using DAPI staining in the cotyledon epidermis, in isolated leaf mesophyll cells, and in roots. While most cells lack cell wall protrusions and contain only one nucleus per cell, each cotyledon usually contains a few ECs without wall protrusions that had two or three nuclei per cell. ECs with cell wall protrusions had either one nucleus, which was frequently abnormally large, or two nuclei. When two nuclei were present, their position in the cell was variable. Nuclei were often found in contact, or connected by irregular extensions of DAPI-staining material (Fig. 5A, B). Double nuclei were also observed in some abnormal stomata (Fig. 5C, D) and in some mesophyll cells, but not in cortical or epidermal cells of *cyd1* roots or in any wild-type cells.

Examination of sectioned tissues confirmed the presence of multiple nuclei in some leaf epidermal and mesophyll cells as well as in cells of developing leaf primordia
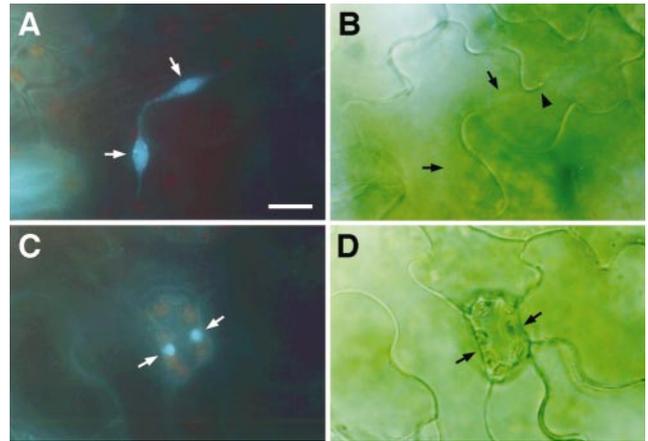
(Fig. 3F). Multiple nuclei were not observed in cells of sectioned root tissue.

Nuclear number correlates with the extent of cytokinetic partitioning in stomata

Because division of the guard mother cell is stereotyped and even stomata without a pore or wall protrusions are easily recognized, a complete failure of cytokinesis is readily apparent. It is more difficult to identify an EC which failed to undergo cytokinesis because the plane of EC division is not predictable and EC size is variable. Thus, a possible relationship between defective cytokinesis and nuclear number was evaluated quantitatively in stomata (Fig. 6). All stomata of normal morphology (Type 1) contained one nucleus per guard cell. In contrast all Type 4 stomata contained two nuclei. Types 2 and 3 stomata were intermediate in nuclear number. These data indicate that the binucleate phenotype is positively correlated with the extent of cell partitioning, so that the greater
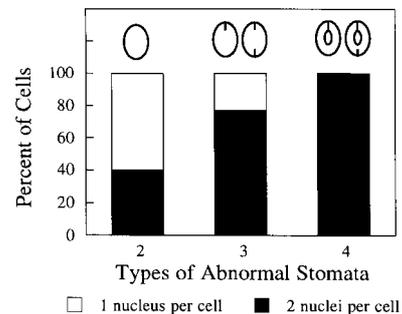

Fig. 6. Percentage of Types 2–4 *cyd1* stomata with one or two nuclei obtained using DAPI fluorescence of cotyledons. The proportion of cells containing two nuclei increases with greater cell partitioning, e.g. all Type 4 stomata had two nuclei; $n = 25$–70.

the degree of ventral wall formation in *cyd1* stomata with incomplete cytokinesis, the higher the percentage of binucleate cells.

cyd1 *wall protrusions are correctly positioned in stomata*

The ultrastructure of mature Type 1 *cyd1* stomata is roughly comparable to those of the wild-type, for example, in chloroplast differentiation and positioning (Fig. 7A). In *cyd1* stomata with defective cytokinesis, electron microscopy confirms the presence of two nuclei in some single cells, and of nuclei with abnormal morphology and position (Fig. 7B, C).

Electron microscopy also reveals that some short wall protrusions form small pore openings, although only one pore has been found per cell, even in cells with two wall stubs (Fig. 7C). *cyd1* wall protrusions are composed of material that closely resembles that of the parent wall in staining intensity and texture (Fig. 7B, C). These protrusions appear to be located where the ventral wall would normally attach to the parent wall during division of the guard mother cell. In wild-type *Arabidopsis* guard mother cells, the division site becomes thickened at opposing ends of the cell prior to division, and this thickening can still be distinguished once cytokinesis is complete (Zhao and Sack, 1999). In *cyd1* stomata, protrusions are also associated with wall thickenings, and when two protrusions are present, the underlying wall thickenings are located opposite each other as in the wild-type. *cyd1* stomatal wall stubs never appear to be associated with thinner portions of the wall. Thus, the placement of the new cell wall appears normal in *cyd1* stomata with incomplete cytokinesis.

cyd1 *is a nuclear recessive and partially lethal mutation*

cyd1 was backcrossed to wild-type plants for genetic analysis. *cyd1* is a recessive mutation since all F_1 plants ($n=17$) from two independent crosses had a wild-type cellular phenotype. After selfing of F_1 plants, F_2 plants ($n=247$) displayed a segregation ratio (wild type:*cyd1*) of 4.4:1 instead of 3:1, suggesting that *cyd1* is a partially lethal nuclear mutation. Similar segregation ratios were obtained regardless of whether *cyd1* was the pollen donor or acceptor in the original backcross. The *cyd1* phenotype was stable after three successive backcrosses. The *cyd1* gene was mapped to the interval between nga225 and nga249 in the top arm of chromosome 5 using simple sequence length polymorphisms (SSLPs), a position that distinguishes it from other mapped cytokinesis mutants (Lukowitz *et al.*, 1996; Assaad *et al.*, 1996; Liu *et al.*, 1997; Hülskamp *et al.*, 1997; Nickle and Meinke, 1998).

Abnormalities in cyd1 vegetative development

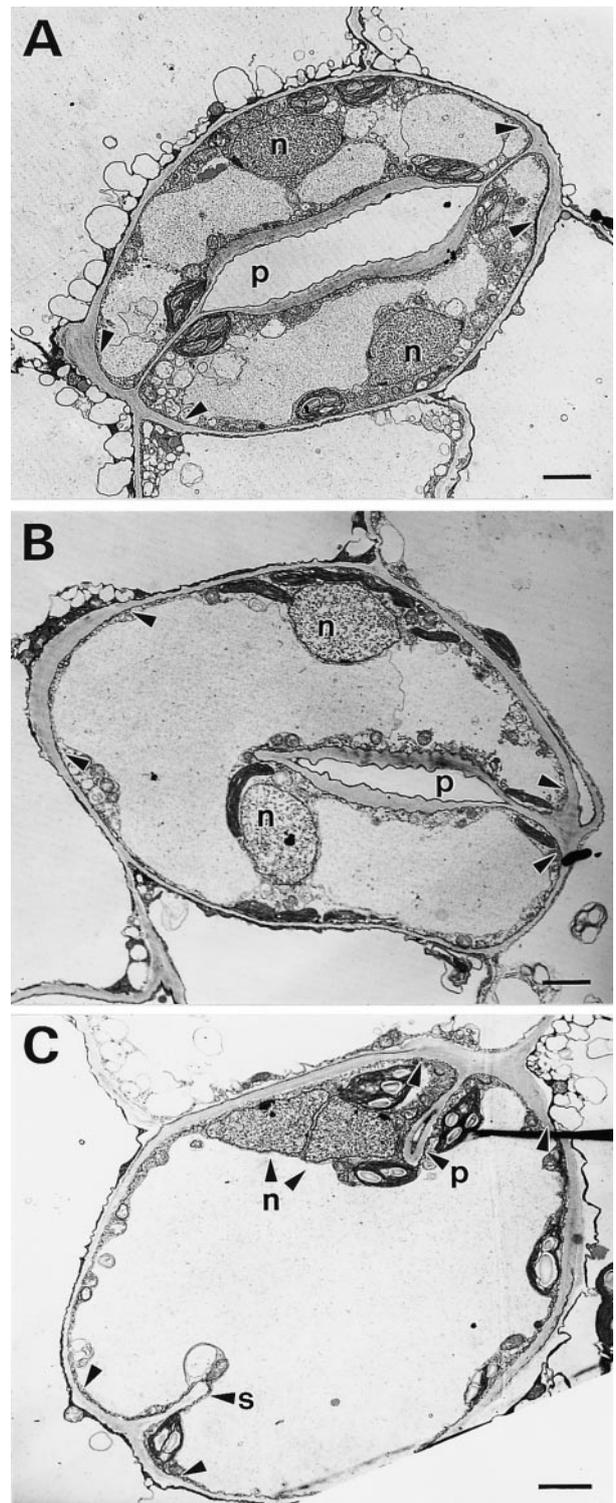To evaluate possible effects of the *cyd1* mutation on organogenesis, individual plants were analysed through-

Fig. 7. Transmission electron micrographs of *cyd1* stomata. Wall thickenings (arrowheads) underlie the point of attachment of the ventral wall in both the wild-type and in *cyd1* stomata: n, nucleus; p, stomatal pore; s, wall stub. Bars=2 μm. (A).*cyd1* stomate with complete cytokinesis. (B) Binucleate, Type 4 stomate with incomplete cytokinesis. One wall protrusion is present and it has developed a pore. (C) Stomate with two wall protrusions, one of which contains a small pore. Either two nuclei or two lobes of the same nucleus are present to the left of the pore, and are abnormally located at the end of the stomate.

out their development, first as seedlings grown on agar for 8–10 d and then after transfer from agar to pots. All *cyd1* plants showed at least some alterations in vegetative growth relative to wild-type seedlings (Fig. 8A–E), but the severity of the phenotype was highly variable. The same range of abnormalities was seen under four different cultural conditions (see Materials and methods). Of 447 seeds sown, about 25% did not germinate. 19% of all seeds germinated and developed four or fewer abnormal leaves and an abnormal root, and then the development of these seedlings arrested. For example, some of these seedlings had only one cotyledon and no apparent root, or they had only limited root development (Fig. 8B). 20% of all seeds developed into mature, fertile plants, but contained gross deformities such as missing, reduced, fused or abnormally lobed cotyledons or leaves, or the production of unidentifiable, teratological structures. The remaining 36% of seeds produced mature plants that at a gross level appeared normal. But almost all of these plants contained abnormalities such as unequal sizes of young leaves especially those of the first pair, or leaves that were irregular and asymmetric in shape with larger marginal teeth (Fig. 8C, E).

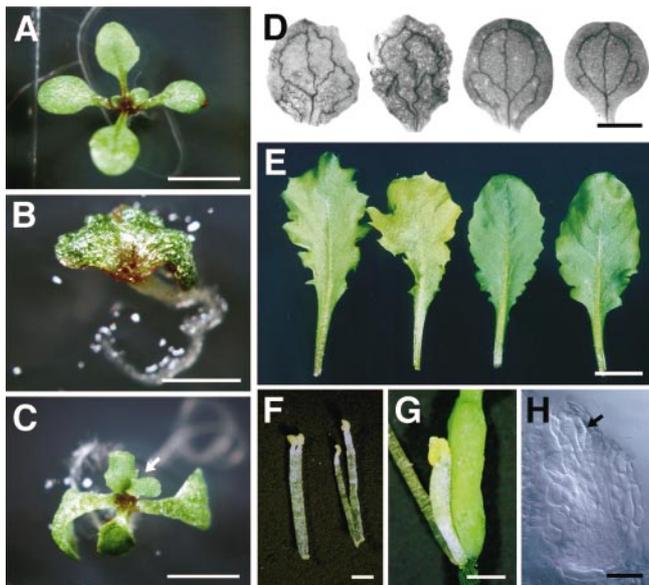All *cyd1* leaves, regardless of the severity of the overall

Fig. 8. Vegetative (B–E) and reproductive (F–H) abnormalities in *cyd1* plants. Plants were grown on agar (A–C) or in pots (D–H) for 8–10 d. (A) 10-d-old wild-type seedling. Bar = 10 mm. (B) Abnormal *cyd1* seedling with a single fan-shaped organ, perhaps a cotyledon, and a root. Bar = 3 mm. (C) Leaf lobing or possible fusion between two leaves (arrow). Bar = 5 mm. (D) Clearings of *cyd1* (left) and wild-type (right) cotyledons illustrating wavy venation and irregular margin in *cyd1*. Bar = 1 mm. (E) Leaves of *cyd1* (left) and wild-type (right) showing more pronounced dentation in *cyd1*. Bar = 1 cm. (F) Two pairs of laterally fused stamens from the same flower with different degrees of fusion in each pair. Bar = 0.4 mm. (G) A stamen fused with a carpel along the entire length of the filament. Bar = 1 mm. (H) Abnormal *cyd1* ovule with tracheids (arrow) that developed in place of the gametophyte. Bar = 30 μm.

plant phenotype, exhibited a roughened and undulate blade. *cyd1* cotyledons and leaves have wavy and distorted veins, but the overall pattern is comparable to the wild type (Fig. 8D). The pattern of epidermal cell expansion is also disrupted so that the long axis of many epidermal cells parallels the veins. However, the overall anatomical organization and differentiation of tissue layers in *cyd1* leaves is comparable to the wild type (Fig. 3G, H).

Embryo morphology was evaluated in cleared green seeds. A range of phenotypes was found in *cyd1* seeds from the same silique, from embyros that exhibited severe defects in organization to those that were normal morphologically. Defects appeared as early as the pre-globular stage. Thus, *cyd1* plants often have missing, reduced, fused or abnormally-shaped leaves, cotyledons, and roots, defects which are rarely seen in wild-type plants.

Abnormalities in cyd1 reproductive development

A sample of 354 flowers from 84 *cyd1* plants were classified and scored for defective development and 55% of all flowers contained abnormalities. The most frequent defects were, in order, (1) flowers with five (rather than six) stamens, (2) lateral fusion of two sepals, (3) fusion of a stamen and a carpel (Fig. 8G), (4) fusion of a stamen and a petal, (5) fusion of two stamens (Fig. 8F), and (6) flowers with one reduced and five normal stamens. Other types of abnormalities were found at much lower frequencies such as fusion between more than two organs in a whorl including between three or four stamens or between three sepals. Stamens were the organ most likely to show a reduction in number or size, but the minimum number of stamens observed was four. Thus, the most frequent defects in *cyd1* floral organogenesis were a loss of an organ, fusion of organs within a whorl and fusion of organs in adjacent whorls.

In addition, each carpel contained at least some defective ovules in *cyd1* plants. Abnormalities included ovules with altered patterns of cell division, and mature ovules that were distorted and composed of irregular cell files. Ovule morphology ranged from wild-type to severely distorted. In some cases tracheids developed where a gametophyte would normally be found (Fig. 8H).

The seed set of self-fertilized *cyd1* plants is poor, for example, in one experiment, *cyd1* siliques had only 11.2 ± 1.1 (mean $\pm$ SE) relatively normal-looking seeds, compared to 34 ± 0.8 in the wild type. Unlike the wild type, *cyd1* siliques also contained some collapsed seeds (4.0 ± 0.6 seeds per silique). Seed set was also poor when *cyd1* plants were used as the female parent in backcrosses with wild-type plants although it was better when *cyd1* was the pollen donor. This suggests that ovule or female gametophyte production in *cyd1* is sometimes defective.

Thus, almost all reproductive and vegetative organs of *cyd1* showed morphological abnormalities, but there was

great variation in which organ was affected and how it was affected between individual plants.

Discussion

The *cytokinesis defective1* mutant of *Arabidopsis* identifies a novel locus that appears to be necessary for the execution of cytokinesis and for the normal development of the vegetative and reproductive organs of the shoot.

cyd1 *and other plant cytokinesis mutations*

cyd1 shares some of the cellular defects described in other cytokinesis-defective mutants in plants such as the presence of several nuclei in one cell, larger and fewer cells, and incomplete cell walls (Assaad *et al.*, 1996; Kitada *et al.*, 1983; Liu *et al.*, 1995, 1997; Lukowitz *et al.*, 1996; Hauser *et al.*, 1997; Nickle and Meinke, 1998). *cyd1* is also similar to many of these mutations in that defective cytokinesis is correlated with the abnormal development of embryos and organs.

However, *cyd1* maps to a position that differs from that of published cytokinesis mutations in *Arabidopsis*, i.e. *keule*, *knolle*, *tso1*, *stud*, *tetraspore*, and *cyt1* (Assaad *et al.*, 1996; Lukowitz *et al.*, 1996; Liu *et al.*, 1997; Hülskamp *et al.*, 1997; Spielman *et al.*, 1997; Nickle and Meinke, 1998).

The *cyd1* phenotype also differs in cytology and/or in the locations of the cytokinesis defects. Most other cytokinesis mutants have more nuclei and wall protrusions in one cell than *cyd1* and may have branched protrusions (Liu *et al.*, 1995) which are absent in *cyd1*. Similarly, *cyd1* lacks the irregular wall thickenings found in cyt1 (Nickle and Meinke, 1998). In *cyd1*, wall protrusions are present both in meristematic and in mature vacuolated cells. In contrast, in other mutants, incomplete walls have been found only in embryonic, meristematic and dividing cells (Assaad, *et al.*, 1996; Lukowitz, *et al.*, 1996; Nickle and Meinke, 1998), or only in mature vacuolated cells (Liu *et al.*, 1995), but not in both categories of cells.

Finally, *cyd1* is the only plant cytokinesis mutant described where defects are known to occur throughout the vegetative and reproductive shoot. In some mutants the defect is confined to a specific set of organs or cell types such as flowers (*tso1*), roots (*mun*), or to specific stages of pollen development, for example, *stud* and *tetraspore* (McCoy and Smith, 1983; Hauser *et al.*, 1997; Hülskamp *et al.*, 1997; Liu *et al.*, 1997; Spielman *et al.*, 1997). Other mutants are either embryo or seedling lethal (*cyt1*, *knolle*, *keule*) or the embryos can be rescued in culture but do not progress to maturity (pea *cyd*; Liu *et al.*, 1995; Assaad *et al.*, 1996; Lukowitz *et al.*, 1996; Nickle and Meinke, 1998).

Stomatal division site placement and differentiation are normal

The *CYD1* gene product appears to be necessary for the execution of cytokinesis rather than for its placement, at least in stomata. In *Arabidopsis* guard mother cells, the division site—the eventual location of fusion of the cell plate with the parental cell wall—is marked by wall thickenings at opposite ends of the cell (Zhao and Sack, 1999). Incomplete walls in *cyd1* stomata are attached to division site thickenings indicating that the site of cell plate fusion is normal. This contrasts with the *fass* or *ton* mutant in which the placement rather than the execution of cytokinesis is defective (Torres-Ruiz and Jürgens, 1994; Traas *et al.*, 1995).

Cytokinesis defects in stomata have been described in wild-type plants that were treated with cytoskeletal inhibitors and with caffeine (Galatis, 1977; Galatis and Apostolakos, 1991; Terryn *et al.*, 1993). However, *cyd1* appears to be the first mutation documented to display such defects.

Data from both *cyd1* and from inhibitor treatment show that aspects of stomatal differentiation do not require cytokinesis. For example, stomata without any dividing wall can still be recognized by cell shape and chloroplast differentiation. Pore differentiation requires the presence of at least a part of a ventral wall because *cyd1* stomata without any dividing wall do not form a pore. In cells with short stubs, the pore forms at the end of the stub, suggesting that the site of pore targeting is correct, i.e. as close as possible to the middle of the cell. Pore number also seems correctly regulated, because even cells with two stubs have only one pore. Thus, pore differentiation requires the presence of some new cell wall, but not full cytokinesis, a conclusion reinforced by the observation that pores form naturally on incomplete walls in wild-type stomata of several genera (Sack, 1987).

cyd1 stomata with short stubs or with no ventral wall have an abnormal, papillate swelling on the outer wall. Because the location of this swelling might be a default target for the fusion of exocytic Golgi vesicles, further analysis could provide an insight into the mechanisms that regulate the site of vesicle targeting.

Nature of cytokinesis defect

The *CYD1* gene product appears necessary for the execution of cytokinesis. Caffeine, which also causes wall stubs, may guide vesicle fusion to the cell plate, or block plate stabilization (Hepler and Bonsignore, 1990; Liu *et al.*, 1995; Valets and Hepler, 1997). The presence of wall protrusions, rather than central 'islands', in both cytokinesis mutants and in caffeine-treated plants could imply that cell plates are more stable where they join the parent wall than in the cell centre. Evidence suggests that 'wall maturation and insertion factors' originate from the

parent wall and spread into the new wall to stabilize it (Mineyuki and Gunning, 1990). Thus, the *CYD1* gene product might function in stabilizing the first-formed regions of the cell plate.

Other aspects of the *cyd1* phenotype suggest that cellular processes in addition to cytokinesis might be affected. For example, the finding that the extent of cytokinetic partitioning in stomata correlates with nuclear number could indicate that nuclear division, or post-divisional nuclear reassembly or fusion are affected as well. Thus, it cannot be ruled out that *CYD1* functions only indirectly in cytokinesis and directly in mitosis (e.g. as in Mackay *et al.*, 1998) or in karyokinesis. Obviously, knowledge of the identity and the sub-cellular localization of the *CYD1* gene product could prove helpful in analysing gene function.

Increase in cell size and trichome branching

Larger stomata, pollen and epidermal cells are common indicators of polyploidy, and trichome branch number correlates with the degree of endoreduplication in *Arabidopsis* (Altmann *et al.*, 1994; Hülskamp *et al.*, 1994; Melaragno *et al.*, 1993). While *cyd1* exhibits these phenotypes, *cyd1* is not simply a polyploid plant. In 4n and 6n *Arabidopsis*, all stomata and pollen are larger, and all stomata have a normal morphology, whereas in *cyd1*, almost half of stomata and pollen grains are the same size as in the wild type (Yang, 1996). Moreover, *cyd1* appears to be a recessive, single-locus mutation.

Larger cells might result from selected progenitor cells becoming polyploid. Alternatively, increased size could be a consequence of slower or fewer cell cycles. For example, overexpression of a dominant negative mutant form of Cdc2 kinase, a cell cycle regulator, slowed cell division and produced larger cells without increasing DNA content (Hemerly *et al.*, 1995).

Cytokinesis and development

Because other cytokinesis mutants are embryo/seedling lethal or phenotypes only occur in specific organs, the *cyd1* mutation provides a novel opportunity to analyse the relationships between cytokinesis defects and shoot organogenesis. Although this *cyd1* allele is fully penetrant, the extent to which different *cyd1* plants exhibit organ abnormalities is variable. This could be explained if the cytokinesis defect affected all cells randomly and if the consequences of a defect depended on how essential that cell was for development. A lack of division in strategically located cells in primordia and during organogenesis could produce the organ abnormalities described. The observation that cytokinesis defects can be observed in developing primordia and meristems supports this hypothesis. Defects during embryonic development may be of

more consequence and more deleterious than those that occur after germination.

Altered leaf morphogenesis in *cyd1* might be related to reduced cell number. Cell deficits may restrict local expansion and cause differential stress–strain relationships resulting in vascular distortions, exaggerated dentation, a roughened leaf surface, and leaf asymmetry. Although veins are wavy, the anatomy of *cyd1* leaves is essentially normal. This contrasts with the knolle and keule mutants where non-epidermal cells differentiate in the embryonic epidermis (Assaad *et al.*, 1996; Lukowitz *et al.*, 1996).

Conclusions

The *cyd1* mutant of *Arabidopsis* identifies a new locus affecting cytokinesis. A fraction of cells throughout the shoot exhibits phenotypes shared by other cytokinesis mutants in plants such as multiple nuclei, wall protrusions, and larger and fewer cells. Cytokinesis defects in *cyd1* stomata were readily detected because guard mother cell divisions are stereotyped and because differentiation continues in the absence of cytokinesis. Since many *cyd1* plants are fertile and produce seeds, this mutant provides the opportunity to evaluate the relationships between a cytokinesis defect and organ development. Based on phenotype, the *CYD1* gene product would be expected to encode a novel component of the cytokinetic machinery.

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