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*arc*6, an extreme chloroplast division mutant of *Arabidopsis* also alters proplastid proliferation and morphology in shoot and root apices

E. J. Robertson, K. A. Pyke* and R. M. Leech†

Department of Biology, The University of York, PO Box No 373, York YO1 5YW, UK

*Present address: School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK
†Author for correspondence

SUMMARY

The *arc*6 (accumulation and replication of chloroplasts) mutant of *Arabidopsis* has only two greatly enlarged chloroplasts per mature leaf mesophyll cell compared with ninety chloroplasts per cell in the wild type. The mutation is a single nuclear gene and the plant phenotype is normal. Shoot and root apical meristems of *arc*6 plants have been examined to determine how early during plastid development the mutant *arc*6 phenotype can be recognised. In the cells of the *arc*6 apical meristem there are only two proplastids, which are larger than wild type with a highly variable morphology. In the cells of the leaf primordia where differentiation of proplastids to chloroplasts occurs *arc*6 plastids are larger and at a more advanced developmental stage than wild-type plastids. In *arc*6 root cells statoliths and other plastids also show grossly abnormal morphology and the statoliths are greatly increased in size. During *arc*6 stomatal guard cell development the perturbation in proplastid population dynamics affects plastid segregation and 30% of stomata lack plastids in one or both guard cells. Our evidence would suggest that *ARC*6 is expressed throughout the vegetative cells of the *Arabidopsis* seedling with major effects on both the proplastid phenotype and the proplastid population. *ARC*6 is the first gene to be identified in *Arabidopsis* which has a global effect on plastid development in cells arising from both the shoot and root meristems, and is of major importance in the nuclear control of plastid differentiation in higher plants.

Key words: *Arabidopsis*, chloroplast division, proplastid division, meristem, guard cell, *arc*6

INTRODUCTION

Several nuclear *ARC* loci which control chloroplast development in leaf mesophyll cells have been identified by mutant analysis in *Arabidopsis thaliana* (Pyke and Leech, 1992, 1994; Pyke et al., 1994). These mutants were identified by their reduction in or increase in chloroplast number per leaf mesophyll cell compared to wild type. The most extreme of these mutants, *arc*6 (Pyke et al., 1994), has only two enormously enlarged chloroplasts per mesophyll cell, which do not increase in number during mesophyll cell expansion, inferring that chloroplast division has been severely perturbed in *arc*6 mesophyll cells. Consequently *arc*6 is the most radically altered chloroplast number mutant in higher plants. Despite the abnormal chloroplast morphology, *arc*6 plants grow normally with no dramatic change in whole plant phenotype and are fully fertile (Pyke et al., 1994). The mutant phenotype of *arc*6 mesophyll cells is highly stable and segregates as a single nuclear locus.

Since the number of chloroplasts in *arc*6 cells is considerably lower than the estimated proplastid number per mitotic cell in *Arabidopsis*, of 14 (Pyke and Leech, 1992, 1994), it is possible that proplastids in young *arc*6 seedlings may also be perturbed in a radical way (Pyke et al., 1994). Proplastids are the progenitor organelles from which specific types of plastid can differentiate and are characteristic of meristematic cells in shoot and root apices (Kirk and Tilney-Bassett, 1978; Thomson and Whatley, 1980). Chloroplasts in mature leaf cells develop from proplastids in younger cells (Possingham, 1980), whereas in roots plastid differentiation leads to the formation of amyloplasts, in particular statoliths in the columella cells and other small root plastids (Whatley, 1983). Although the shoot and root meristems of *Arabidopsis* have been relatively well characterised morphologically (Medford et al., 1992; Dolan et al., 1993), proplastid development in *Arabidopsis* meristems has not been examined. Reports of the few studies that have been made of other higher plants indicate that meristematic cells at the shoot apex contain between 10 and 20 proplastids (Cran and Possingham, 1972; Lyndon and Robertson, 1976; Fujie et al., 1994). In order to maintain the continuity of plastids through cell lineages in the plant, proplastids must divide during mitosis and segregate into daughter cells at cell division in an ordered manner (Birky, 1982; Possingham and Lawrence, 1983) and it is generally assumed that cells lacking plastids as a consequence of abnormal proplastid segregation would not be viable. The rate of proplastid division in relation to cell division is also critical to competent cell development, for the number of proplastids in the young post-mitotic cell will influence the subsequent size of the plastid cell population arising by differentiation and plastid division.

The availability of the *Arabidopsis arc*6 mutant with it's normal fertile plant phenotype gives us a unique opportunity

to examine the control of plastid development in the tissues of a higher plant. To gain an insight into the role of *ARC*6 in the control of early plastid development we have examined cells from the shoot meristem, root meristem and stomatal guard cells of the wild-type (*ARC*6) and the *arc*6 mutant of *Arabidopsis* to determine how early in plastid development the *arc*6 mutant phenotype can be recognised.

MATERIALS AND METHODS

Plant growth

Wild-type *Arabidopsis* plants of the ecotype Wassilewskija (WS) and the *arc*6 mutant were grown in controlled conditions as described previously (Pyke and Leech, 1991). The *arc*6 mutant was isolated from a T-DNA mutagenised *Arabidopsis* population in the WS background (Pyke et al., 1994).

Ultrastructural analysis

For ultrastructural analysis, shoot and root apices were harvested from 5-day-old seedlings. The shoot apex and the first leaf primordium of young *Arabidopsis* seedlings were examined after fixation and embedding in Spurr's resin, as previously described for *Arabidopsis* leaf tissue (Pyke et al., 1994). To examine the shoot apical meristem, whole seedlings were embedded intact and sectioned longitudinally. Root tips were dissected from 5-day-old seedlings and fixed and embedded using the same protocols. Measurements of plastid profiles from electron micrographs were made using a Seescan image analysis system (Cambridge, UK) (Pyke and Leech, 1991). Sectioning of young *Arabidopsis* seedlings embedded in polyethylene glycol (PEG) and staining with 4′,6′-diamidino-2-phenylindole (DAPI) was carried out using methods described previously (Marrison and Leech, 1992).

Characterisation of guard cell chloroplasts

Epidermal strips for visualising stomatal guard cells were peeled from the lower surface of first leaves between 20 and 25 days after sowing. Chloroplasts in stomatal guard cells were visualised by mounting epidermal strips in 1%(w/v) $AgNO_3$. Sizes of guard cell chloroplasts visualised by $AgNO_3$ staining were determined directly from the microscope by image analysis. Images of guard cell chloroplast autofluorescence in epidermal strips were obtained using a Nikon FXA fluorescence microscope with a filter combination of dichroic mirror 510, excitation filter 450-490 nm and barrier filter 515F.

RESULTS

Proplastids in the shoot apical meristems of *arc*6 and wild-type *Arabidopsis*

The shoot apex of the *arc*6 mutant of *Arabidopsis* consists of a central meristematic region surrounded by leaf primordia which develop on the flanks of the apical dome (Fig. 1). The morphology of the *arc*6 shoot apex (Fig. 1) does not differ from that of the wild-type shoot apex, which has been well characterised (Medford et al., 1992; Fujie et al., 1994). The apical dome itself consists of three cell layers L1, L2 and L3 (Fig. 1) and it's ultrastructure can be clearly observed by electron microscopy (Fig. 2A,B). Transects through the three layers of the wild-type apical meristem cells reveal populations of proplastids recognisable by their dense staining, two envelope membranes and limited internal membranes occasionally connected with the internal envelope membrane or appressed (Fig. 2A,B). The profile shape of wild-type proplastids is very uniform (Fig. 2B,D), typically ellipsoidal with average length/breadth dimensions of 1.2 μm×0.7 μm and a cross-sectional area which is very uniform throughout the proplastid population (Table 1). Serial sectioning in the wild-type apical meristem confirmed that each proplastid profile in a cell transect represents an individual proplastid. Proplastid profiles in wild-type cell transects average four per cell (s.e.=0.2, n=61), e.g. see Fig. 2D. Proplastids in both the wild-type and *arc*6 apical meristem are normally evenly distributed through-

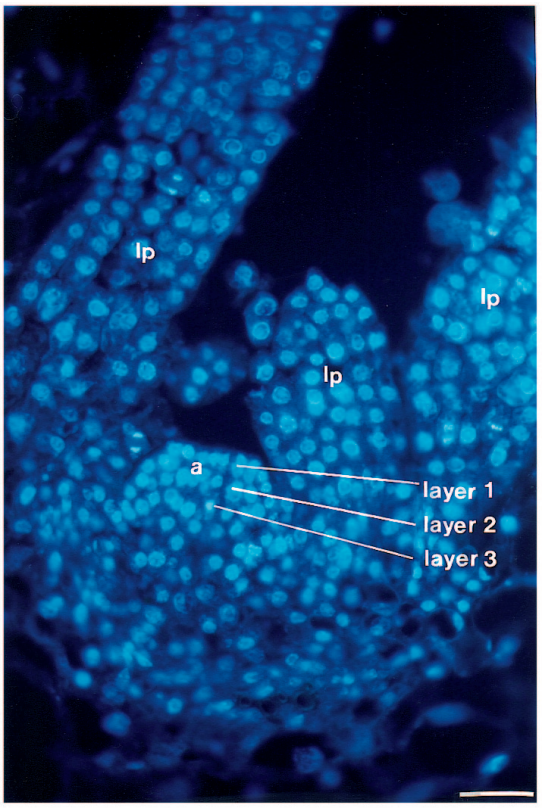

Fig. 1. Longitudinal PEG-embedded section (5 μm thick) through the shoot apex of an 8-day-old seedling of the *arc*6 mutant of *Arabidopsis*, stained with the DNA-binding fluorochrome, 4′,6′-diamidino-2-phenylindole (DAPI). There are three cell layers: layer 1, layer 2 and layer 3. a, apical meristem; lp, leaf primordium. Bar, 25 μm.

Table 1. Mean plastid transect area measured from electron micrographs of apical meristem cells and leaf primordia cells of wild type and the *arc*6 mutant of *Arabidopsis*

	Mean plastid transect area (μm²)	
	Wild type	*arc*6
Apical meristem	0.54 (0.03)	1.24 (0.25)
Leaf primordia	1.38 (0.07)	2.39 (0.15)

A total of 100 plastid transects from apical meristem cells and 150 plastid transects from leaf primordia cells were measured from at least three seedlings of wild type and *arc*6. Standard errors are shown.

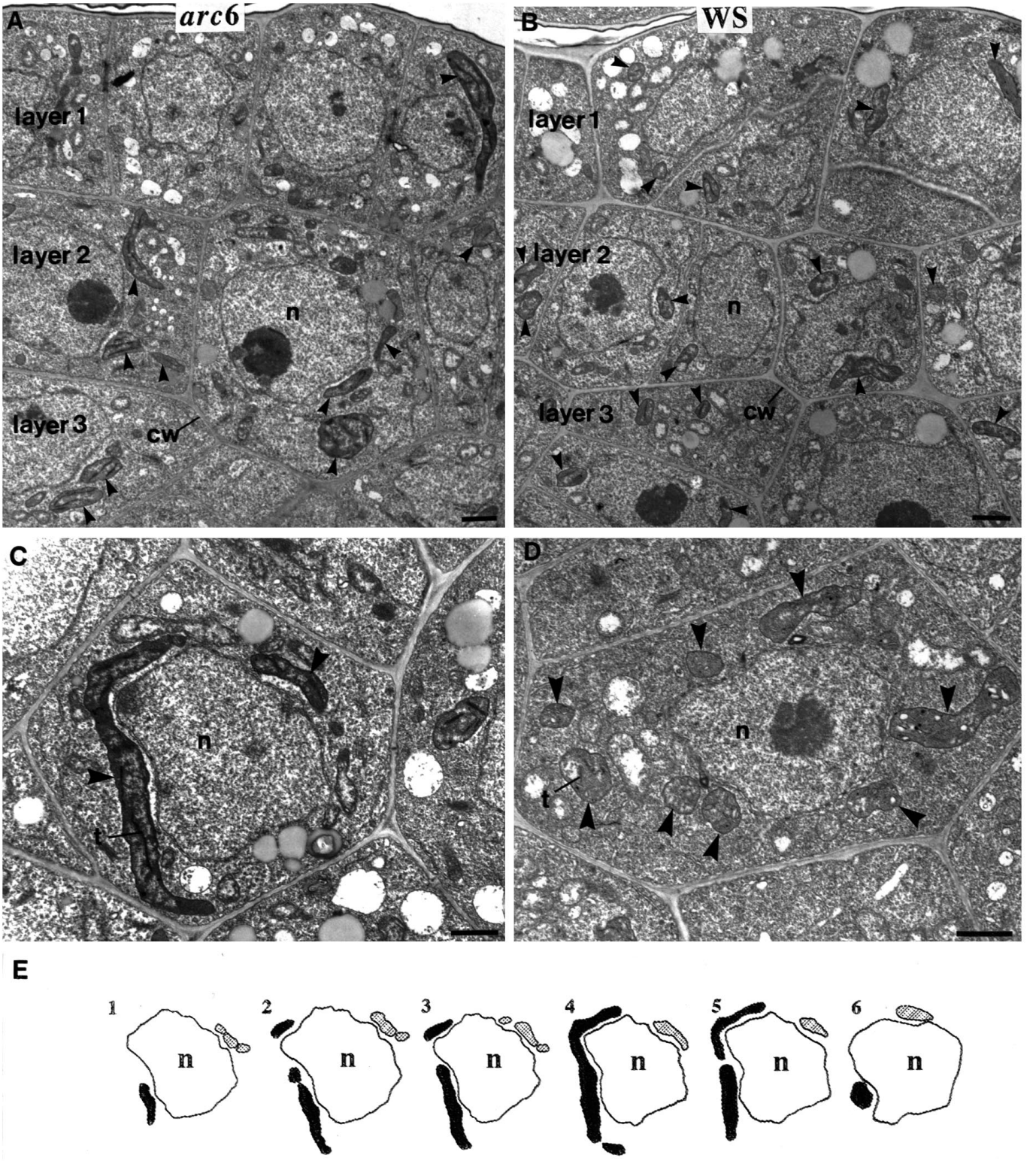

Fig. 2. Electron micrographs of longitudinal sections (90 nm thick) through the *Arabidopsis* shoot apical meristem of: (A and C) *arc*6; (B and D) wild type (WS). All proplastids are identified by arrowheads. Three cell layers, layer 1, layer 2 and layer 3, are evident in the apex in both *arc*6 (A) and wild type (B). Note the large increase in size and the variable morphology of *arc*6 proplastids compared to wild-type plastids. A close association between proplastids and the nucleus is clearly seen in both *arc*6 (C) and wild-type (D) cells. The section shown in C was the fourth in a set of sequential sections through this *arc*6 cell. In E tracings of these sequential sections showing profiles of the two *arc*6 proplastids (shaded and hatched profiles) and the nucleus are shown. The sections in the series represent a total depth of 1.5 μm through the *arc*6 cell. The tracings clearly show how several individual *arc*6 proplastid profiles in one section are actually part of the same large *arc*6 proplastid in the cell. cw, cell wall; n, nucleus; t, thylakoid. Bar, 1 μm.

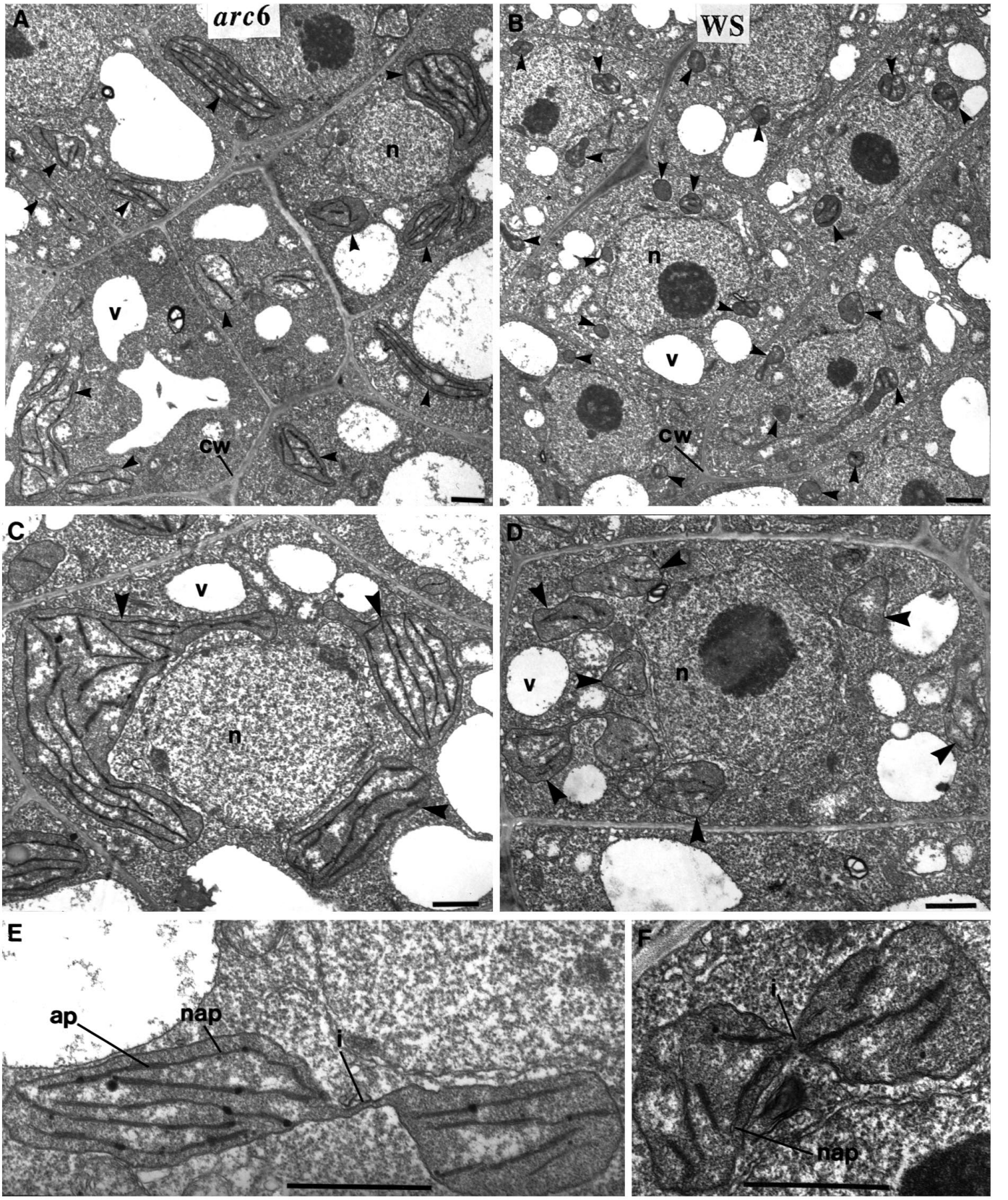

Fig. 3. Electron micrographs of longitudinal sections through leaf primordia of *Arabidopsis*. (A and C) *arc*6; (B and D) wild type (WS). Note the plastids in *arc*6 cells appear more highly developed. Putative division profiles for E, *arc*6 and F, wild-type plastids show a centrally constricted isthmus joining two equal sized daughter plastids. ap, appressed thylakoid membrane; nap, non-appressed thylakoid membrane; i, isthmus; cw, cell wall; n, nucleus; v, vacuole;. Bar, 1 μm.

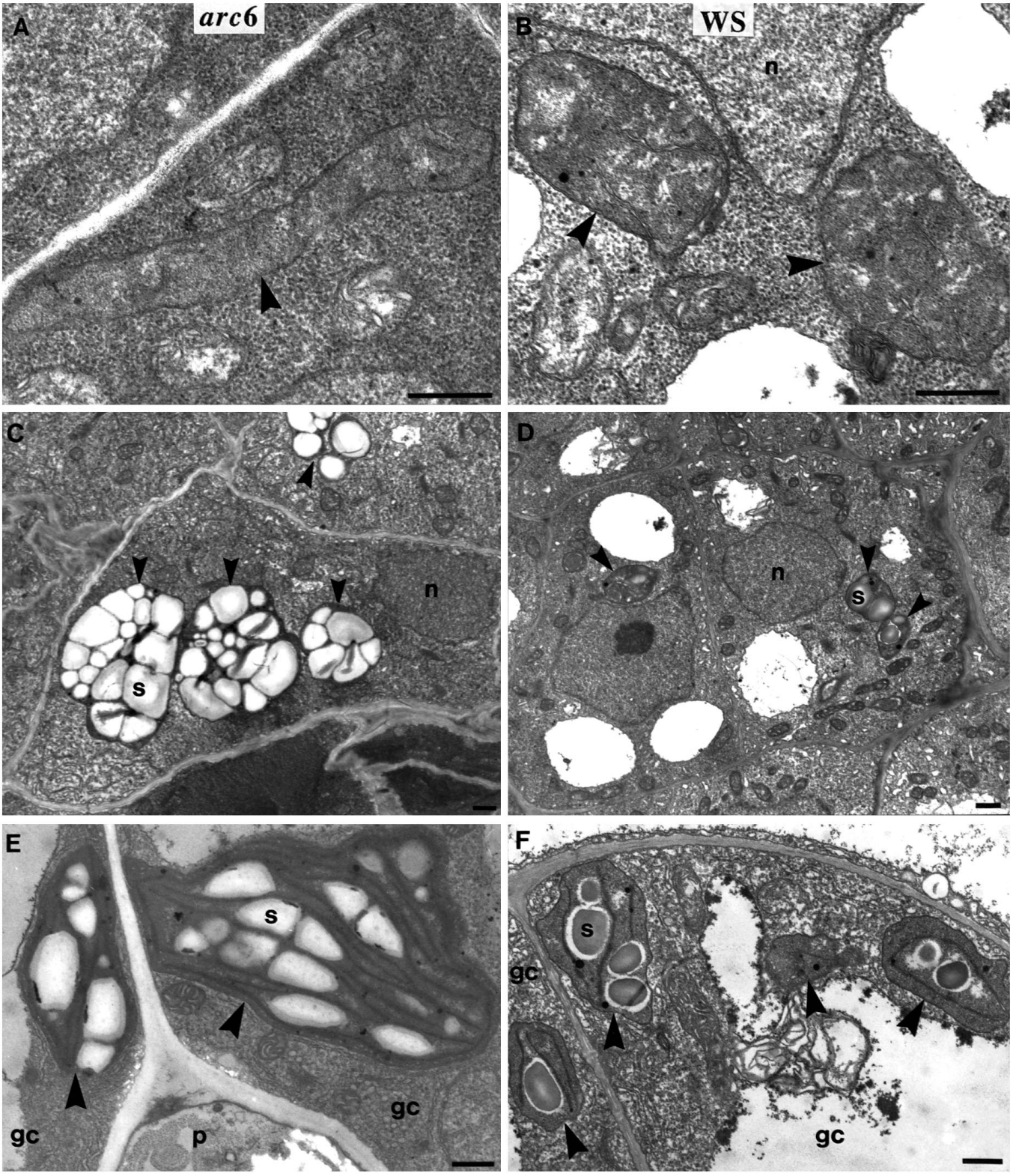

Fig. 4. Electron micrographs of longitudinal sections through *Arabidopsis* root tips of (A and C) *arc*6; (B and D) wild type (WS). All plastids are identified by arrowheads. The *arc*6 plastids in the root meristem (A) are elongated and have abnormal morphology compared to the ellipsoid, rounded root plastids in wild type (B). In the collumella cells in the root cap, the statoliths are packed with starch and are greatly increased in size in *arc*6 cells (C) compared to wild type (D). Electron micrographs of chloroplasts from an *arc*6 guard cell (E) and a wild-type guard cell (F). gc, guard cell; n, nucleus; p, stomatal pore; s, starch grain. Bar, 500 nm.

out the cytoplasm and in wild-type cells often form a distinct ring around the nucleus (Fig. 2D).

In *arc*6, the extremely large proplastids appear to be wrapped around the nucleus (Fig. 2C). In very marked contrast to the ellipsoidal wild-type proplastids the profiles of *arc*6 proplastids in the three layers of the apical meristem are highly variable in size and shape (Fig. 2A,C) and the electron micrographs reveal that the *arc*6 mutation has had a radical effect on proplastid morphology in cells throughout the apical meristem. Transects through *arc*6 apical meristem cells contain on average only two (s.e.=0.16, n=52) proplastid profiles per cell, and these profiles are highly variable in size with an average twofold increase in area compared to wild type (Table 1). Although the *arc*6 proplastids are larger and more irregular in shape, their internal structure is similar to wild type and a small amount of internal membrane is present. Because of their enlarged size, a complete three-dimensional reconstruction of an *arc*6 proplastid requires a series of sequential sections. In Fig. 2E sequential sections are shown through a representative cell from the *arc*6 apical meristem (Fig. 2C) and clearly show how small individual *arc*6 proplastid profiles contribute to a single large proplastid with a complex three-dimensional morphology. *arc*6 proplastids have many surface protuberances producing an amoeba-like structure, similar to but smaller than the mature *arc*6 chloroplasts in mesophyll cells (Pyke et al., 1994). Because of these differences in size and shape, comparison of numbers of proplastid profiles per cell transect will underestimate the reduction in proplastid number in *arc*6 cells compared to wild type. We can conclude from the electron micrographs that there is a very significant reduction in proplastid number per cell in the *arc*6 apical meristem associated with an average doubling in proplastid size and an increase in the complexity of plastid morphology.

Plastids in leaf primordia of *arc*6 and wild-type *Arabidopsis*

In order to ensure that we compared exactly similar regions of the mutant and wild-type meristem, cell samples were always taken from the central region of the first leaf primordia. Dramatic differences in proplastid morphology and number in the apical meristem of wild-type and *arc*6 *Arabidopsis* are maintained and enhanced in the young leaf primordia (Fig. 3). Wild-type leaf primordial cells contain between four and eight plastids undergoing differentiation from proplastids to chloroplasts with small amounts of internal thylakoid membrane and a simple ellipsoid profile (Fig. 3B,D). In *arc*6 leaf primordial cells (Fig. 3A,C) the plastid profiles are significantly enlarged compared to wild type (Table 1), often with a more complex profile shape (Fig. 3C). In *arc*6 plastids the internal membrane system is well developed, with organised thylakoid membranes orientated parallel with the long axis of the plastid. Chloroplast profiles contain an average of six to ten stacks of three or four appressed membranes. This is in marked contrast to wild-type plastids in which the thylakoid membranes are much less well developed with little obvious organisation (Fig. 3B,D). The increased size and more complex internal membranes in the *arc*6 leaf primordial plastids suggest that these plastids are at a more advanced stage of development than in wild-type tissue of the same age.

In some wild-type leaf primordial cells, plastids undergoing division can be identified by a central constriction separating two approximately equal sized daughter plastids (Fig. 3F). *arc*6 plastids must also be capable of division within the meristematic tissues because segregation into both daughter cells at cell division is clearly ensured, since all the cells in the *arc*6 apical meristems, in leaf primordia and in the mature leaf contain plastids. Occasionally we have observed *arc*6 proplastid profiles reminiscent of wild-type plastid division with a thin isthmus forming two equal sized daughter plastids (Fig. 3E).

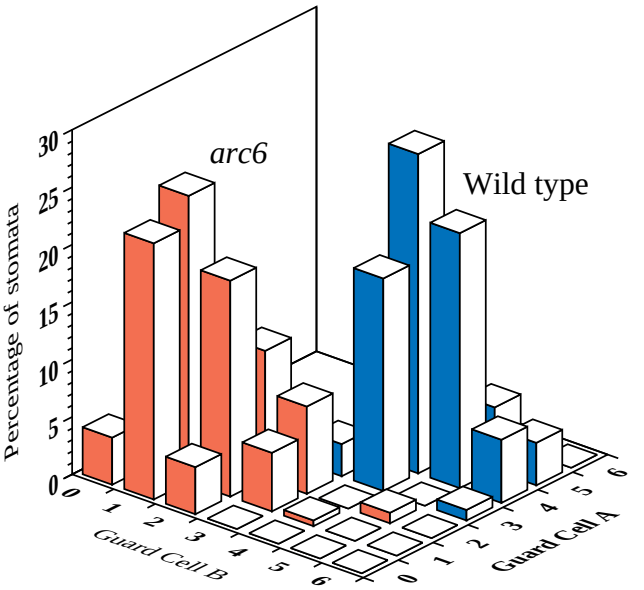

Fig. 5. Analysis of the segregation of plastids in the pair of guard cells forming stomata in wild-type and *arc*6 leaves: 200 stomata were examined in wild-type and in mutant leaves. Column height represents the % of the stomata with the stated number of plastids in the cells of the guard cell pair, cell A and cell B.

Plastids in root tip cells of *arc*6 and wild-type *Arabidopsis*

In order to determine whether the effect of the *arc*6 mutation is restricted to the shoot, we analysed plastids in the root tip of wild-type and of *arc*6 *Arabidopsis* seedlings. In cells of the wild-type *Arabidopsis* root meristem, the plastids are an ellipsoid with few internal membranes (Fig. 4B) and similar in structure to plastids from the wild-type apical meristem. In contrast *arc*6 root plastids have a more elongated morphology (Fig. 4A), which in general is more pleiomorphic than the ellipsoidal wild-type plastids, but wild-type and *arc*6 root plastids are similar in size. In contrast, statoliths, the specialised root plastids found in the columella cells are greatly enlarged (Fig. 4C) compared to wild-type plants (Fig. 4D), and contain more starch. These observations show that the *arc*6 mutation also significantly perturbs root plastid development in addition to shoot and leaf plastid development and that *ARC*6 functions in cells throughout the *Arabidopsis* seedling.

Plastids in the stomatal guard cells of *arc*6 and wild-type *Arabidopsis*

The stomatal guard cells in the leaf epidermis provide an ideal tissue in which to follow changes in plastid population size and segregation during development, since all the plastids of the cell can be viewed simultaneously by light microscopy within whole

unsectioned guard cells in epidermal strips. The L1 layer of cells in the shoot apex gives rise to the epidermis from which stomata differentiate. Plastids in the *arc*6 apical L1 cells are abnormal compared to wild type and there are very few of them (Fig. 2A).

If the rate of proplastid division relative to cell division is radically affected by *arc*6 the number of proplastids in the guard mother cell will be significantly reduced and so will the number in the guard cells. *arc*6 guard cells consistently contain fewer plastids per guard cell than wild type, so clearly there is a large difference between the plastid segregation pattern in *arc*6 and wild-type stomatal guard cells (Fig. 5). In both wild-type and *arc*6 stomata there is a predominance of guard cell pairs with equal plastid numbers, in 20% of wild-type stomata each guard cell has four chloroplasts (Fig. 6A,B) and in 20% of *arc*6 stomata each guard cell has only one chloroplast. We made the very important observation that a significant proportion of guard cells in *arc*6 stomata contain no plastids: a characteristic which is never observed in wild-type stomata. Indeed 4% of *arc*6 stomata lack plastids in both guard cells (Fig. 6G,H). In the *arc*6 cell population sampled, 21% of stomata show 1/0 plastid segregation in the guard cell pair (Fig. 6E,F) and 4% show a 2/0 plastid segregation. In those *arc*6 guard cells containing only a single chloroplast, the chloroplast is enlarged (Fig. 6C,E) compared to chloroplasts in wild-type guard cells containing four or more chloroplasts (Fig. 6A,B). Examination of the ultrastructure of guard cell chloroplasts (Fig. 4E,F) confirmed that *arc*6 guard cell chloroplasts are four to five times larger than wild type but are similar in form and internal structure.

DISCUSSION

We have clearly demonstrated the importance of the *ARC*6 gene for normal proplastid development in *Arabidopsis*. *ARC*6 is the first gene to be identified in *Arabidopsis* which has a global effect on plastid development in cells arising from both the shoot and root meristems. Although other genes have been identified which affect chloroplasts in *Arabidopsis*, i.e. pale cress (Reiter et al., 1994), proplastids in the meristems of this mutant have normal morphology and the mutant gene specifically affects the chloroplast differentiation process. Our results show that the *ARC*6 gene functions very early in proplastid development prior to chloroplast differentiation, since proplastids in both shoot and root meristems are already grossly affected by the *arc*6 mutation. In addition our studies have shown that *arc*6 does not appear to have a significant effect on the ability of plastids in *arc*6 plants to function normally, since the growth of the *arc*6 plant is not severely limited by the *arc*6 mutation (Pyke et al., 1994). We have also established that *arc*6 causes severe disruption to both proplastid and chloroplast division. It remains to be determined whether perturbation of chloroplast division in *arc*6 leaves is a direct consequence of the *arc*6 mutation or a perturbation of proplastid development in the shoot meristem. Many morphological similarities have been shown between the proplastid and chloroplast division processes (Chaly and Possingham, 1981; Whatley, 1988) so it is possible that the *ARC*6 gene is involved in a process common to both proplastid and chloroplast division. There is considerable potential to use other *arc* mutants, which specifically affect different stages of chloroplast division (Pyke and Leech, 1992, 1994) to establish how different *ARC* genes interact in the control of proplastid and chloroplast division.

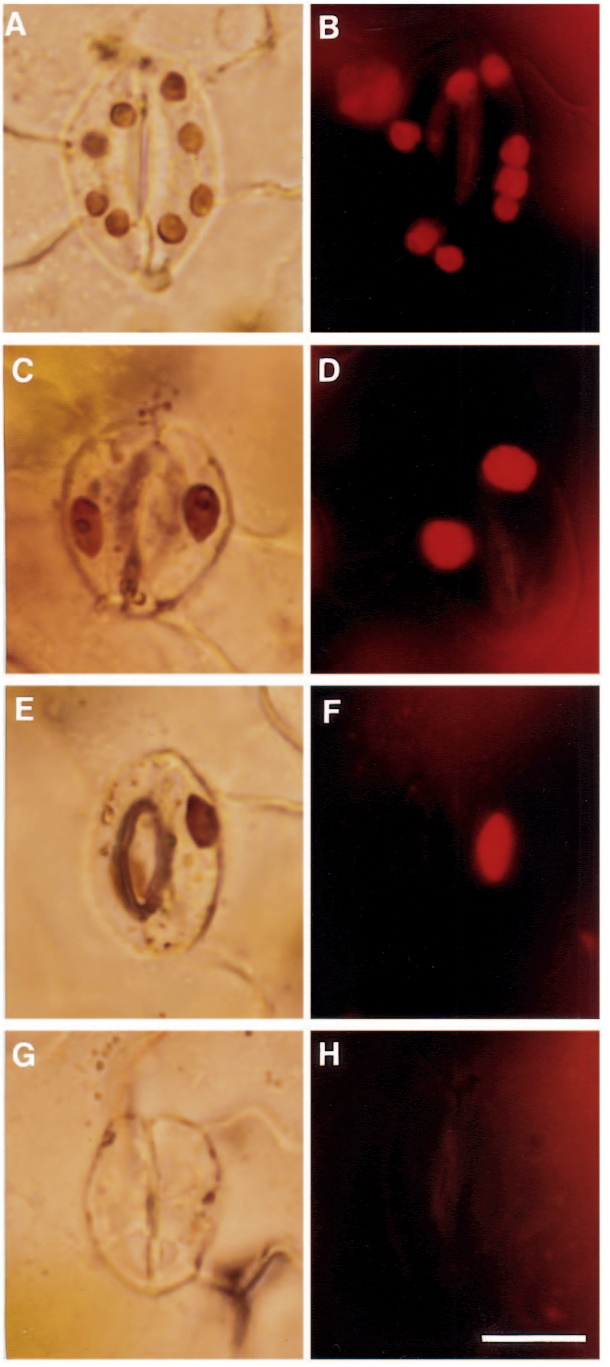

Fig. 6. Guard cell plastids in stomata of (A,B) wild-type and (C-H) *arc*6 *Arabidopsis* visualised by (left-hand side) silver nitrate staining and (right-hand side) chlorophyll fluorescence. Plastid numbers in cell A and cell B of each guard cell pair are shown as the number in A/number in B. (A,B) Wild-type, plastids segregate 4/4; (C,D) *arc*6, plastids segregate 1/1; (E,F) *arc*6, plastids segregate 0/1; (G) *arc*6, plastids absent; (H) *arc*6, plastids absent.

Since apoplastidy in higher plant cells is extremely rare (Sears, 1980) a control must exist in normal meristematic cells which prevents the formation of apoplastid daughter cells. In

*arc*6 cells, with only two chloroplasts, the mechanism controlling segregation must be especially stringent. Characterisation of the *arc*6 mesophyll cell phenotype suggests that *arc*6 chloroplasts in expanding mesophyll cells do not divide (Pyke et al., 1994), but *arc*6 proplastids in meristem cells are apparently capable of limited division; no cells in *arc*6 meristems or leaf mesophyll cells lack plastids. The putative division profiles of enlarged *arc*6 proplastids in leaf primordia cells have been observed, so division of *arc*6 plastids in dividing cells may be a rare but consistent event. The close association of proplastids with the nucleus in meristematic cells may be important in determining their segregation into daughter cells at division by virtue of their spatial arrangement. This type of plastid distribution has been reported previously in dividing cells of higher plants (Whatley, 1986; Baluska et al., 1993) and could ensure partitioning of some plastids into both daughter cells at division (Birky, 1982). *arc*6 chloroplasts appear to wrap around the nucleus and this may facilitate the approximately equal partitioning of plastids into the two daughter cells. Compared to the apical meristem, the control of plastid segregation during stomatal formation appears to be less stringent, since guard cells lacking chloroplasts do exist in *arc*6. Stomatal guard cells lacking plastids have only been reported once previously (Nelson and Mayo, 1975) in an orchid species, and the absence of plastids in the guard cell of the *arc*6 mutant of *Arabidopsis* could prove very useful in further examination of the role of guard cell plastids in stomatal function.

The increase in size and internal membrane organisation of *arc*6 plastids reflects an advanced state of development of *arc*6 plastids. It is possible that mutation in the *ARC*6 gene allows the premature differentiation of proplastids into either chloroplasts or statoliths, resulting in prematurely large organelles and a disruption of the normal balance between plastid differentiation and cell development.

In this study we have shown the *ARC*6 gene of *Arabidopsis*, identified originally by its abnormal chloroplast number in the *arc*6 mutant, to be crucial to the early development of plastids in both shoot and root cells. The global role of this gene in *Arabidopsis* suggests that it is of major importance in the nuclear control of plastid differentiation in higher plants.

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