

# The distinctive roles of five different *ARC* genes in the chloroplast division process in *Arabidopsis*

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## Summary

***ARC* (accumulation and replication of chloroplasts) genes control different aspects of the chloroplast division process in higher plants. In order to establish the hierarchy of the *ARC* genes in the chloroplast division process and to provide evidence for their specific roles, double mutants were constructed between *arc11*, *arc6*, *arc5*, *arc3* and *arc1* in all combinations and phenotypically analysed. *arc11* is a new nuclear recessive mutant with 29 chloroplasts compared with 120 in wild type. All the phenotypes of the double mutants are unambiguous. *ARC1* down-regulates proplastid division but is on a separate pathway from *ARC3*, *ARC5*, *ARC6* and *ARC11*. *ARC6* initiates both proplastid and chloroplast division. *ARC3* controls the rate of chloroplast expansion and *ARC11* the central positioning of the final division plane in chloroplast division. *ARC5* facilitates separation of the two daughter chloroplasts. *ARC5* maps to chromosome 3 and *ARC11* and *ARC6* map approximately 60 cM apart on chromosome 5.**

## Introduction

The division of young chloroplasts in expanding mesophyll cells ensures the presence of the full complement of chloroplasts in mature leaf cells. In the *Arabidopsis* leaf, cells mature from the base to the tip of the leaf (Pyke *et al.*, 1991). Earlier proplastid division in mitotic cells keeps pace with cell division but it is the subsequent post-mitotic divisions of young developing chloroplasts in young mesophyll cells which assure the size of the population of mature chloroplasts and photosynthetic competence.

The sequence of ultrastructural changes characteristic of chloroplast division in higher plants is well documented (Boffey, 1992; Leech and Pyke, 1988; Leech *et al.*, 1981; Orros and Possingham, 1989; Possingham *et al.*, 1988; Whatley, 1988). Chloroplast division always occurs when the young chloroplasts have grown to about 50% of their final volume and contain numerous thylakoid membranes and small granal stacks (Ellis and Leech, 1985). The chloroplasts become increasingly centrally constricted, twist around the isthmus and the two equally sized daughter chloroplasts finally separate. Following three rounds of chloroplast division in *Arabidopsis thaliana* ecotype Landsberg *erecta* (Ler) the mature mesophyll cells contain a mean of 120 chloroplasts (Pyke and Leech, 1992). The sequence of morphological changes leading to the development of the mature chloroplast complement based on our observations of developing mesophyll cells of *Arabidopsis* ecotypes is illustrated in Figure 1.

To identify the eukaryotic genes controlling the different phases of chloroplast division, we have used the strategy of isolating and analysing mutants of chloroplast division in *Arabidopsis*. In these *arc* (accumulation and replication of chloroplasts) mutants the mesophyll chloroplasts differ considerably from wild type in number, size and shape (for a review see Pyke, 1997). All the *arc* mutant phenotypes are stable and result from single nuclear recessive mutations, i.e. they follow normal Mendelian inheritance patterns in reciprocal backcrosses.

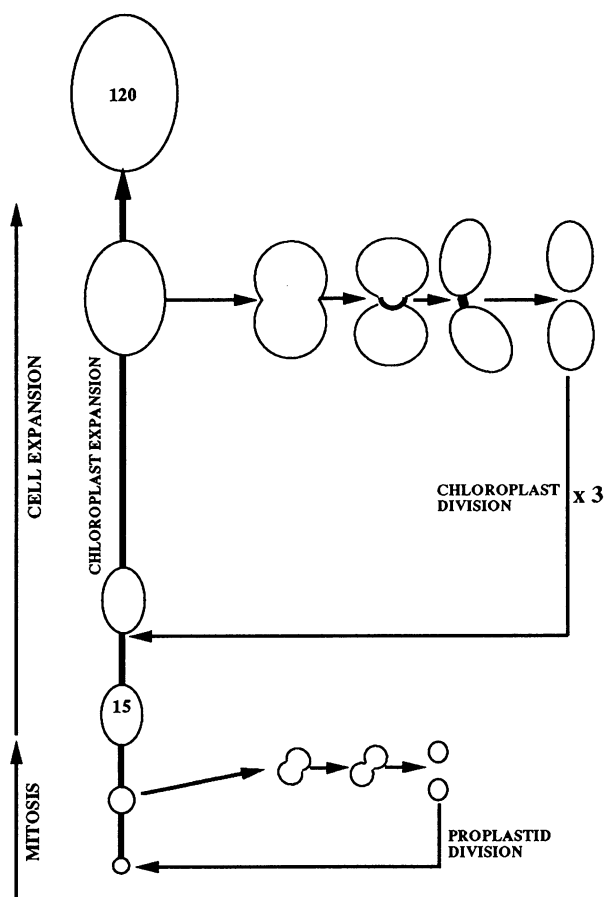
Eleven independent nuclear *ARC* genes involved in the control of chloroplast division have been identified so far. In view of their distinctive mutant phenotypes it seems likely that these *ARC* genes have several very different roles in the chloroplast division process. In order to investigate the genetic control of chloroplast division we used our *arc* mutants to examine the interactions and epistatic relationships of the *ARC* genes. Our approach was to construct and identify double *arc* mutants and analyse their chloroplast phenotypes. Five *arc* mutants with distinctive heritable phenotypes were chosen. *arc6* has the most dramatic alteration in chloroplast number with a mean of only two very large chloroplasts (Pyke *et al.*, 1994) forming large sheets covering the internal cell surface (Robertson *et al.*, 1995). *arc1* is unique because more chloroplasts are present per cell plan area (32/1000  $\mu\text{m}^2$ ) than in wild type (25/1000  $\mu\text{m}^2$ ) (Pyke and Leech, 1992, 1994). In *arc3* a few abnormally large chloroplasts are present (Pyke and Leech, 1992; Pyke and Leech, 1994) and in the *arc5* mutant chloroplast fission is arrested at the final stage and the 13 chloroplasts are large and dumb-bell shaped (Pyke and Leech, 1994;

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**Figure 1.** Diagram illustrating normal chloroplast development and chloroplast division in *Arabidopsis thaliana* (Ler) leaf development. The proplastids in meristematic mesophyll cells undergo a series of divisions ensuring that the young post-mitotic cells contain at least 15 proplastids. During cell expansion the young chloroplasts also expand and begin to form a central constriction which becomes increasingly narrow until separation of two equally sized daughter chloroplasts occurs. Chloroplasts undergo three rounds of division so that the mature mesophyll cell has a complement of 120 chloroplasts which are all of similar size and shape.

Robertson *et al.*, 1996). *arc11* is a recently isolated mutant, described here for the first time. In *arc11* there are two chloroplast populations, one equivalent in size range to wild-type chloroplasts and a second population of larger chloroplasts.

In this paper we analyse the 10 double mutant phenotypes constructed from *arc1*, *arc3-1*, *arc5*, *arc6-1* and *arc11* in all combinations and compare them with the parental single mutant phenotypes. The interactions of the different *ARC* genes are described and a model for the independence and order of *ARC* gene action proposed.

## Results

### The *arc11* mutant

The *arc11* phenotype is of great interest since all *arc11* mesophyll cells have a heterogeneous population of

chloroplasts as seen in Figure 2(b) compared with the homogeneous wild-type population (Figure 2a). Forty to 50% of the chloroplasts in *arc11* cells are within the wild-type size range whilst 50–60% are larger than wild type; some chloroplasts are 10 times larger than wild type (Figure 2c). The smallest mesophyll cells already show this heterogeneity of size among the population of young chloroplasts. The ultrastructural analysis of *arc11* chloroplasts confirms that they vary greatly in size and are frequently larger than wild type (Figure 2e,f). The arrangement of the appressed and non-appressed chloroplast thylakoid membranes and the density of the stroma in *arc11* resemble very closely those of normal wild-type chloroplasts (Figure 2e,f). The growth, vigour and fertility of the *arc11* plant is normal under optimal growth conditions and the whole plant phenotype is very similar to wild type, except that *arc11* begins flowering approximately 3 days earlier.

Chloroplast division is limited in *arc11* mesophyll cells, and apparently occurs only once since fully expanded *arc11* mesophyll cells have a mean of 29 chloroplasts (Figure 2d). In young ( $1000 \mu\text{m}^2$ ) post-mitotic cells of *arc11* the dividing chloroplasts are asymmetric giving the appearance of 'budding' (Figure 2b insert). In mature *arc11* cells it appears that separation after chloroplast division yields one large and one small daughter plastid.

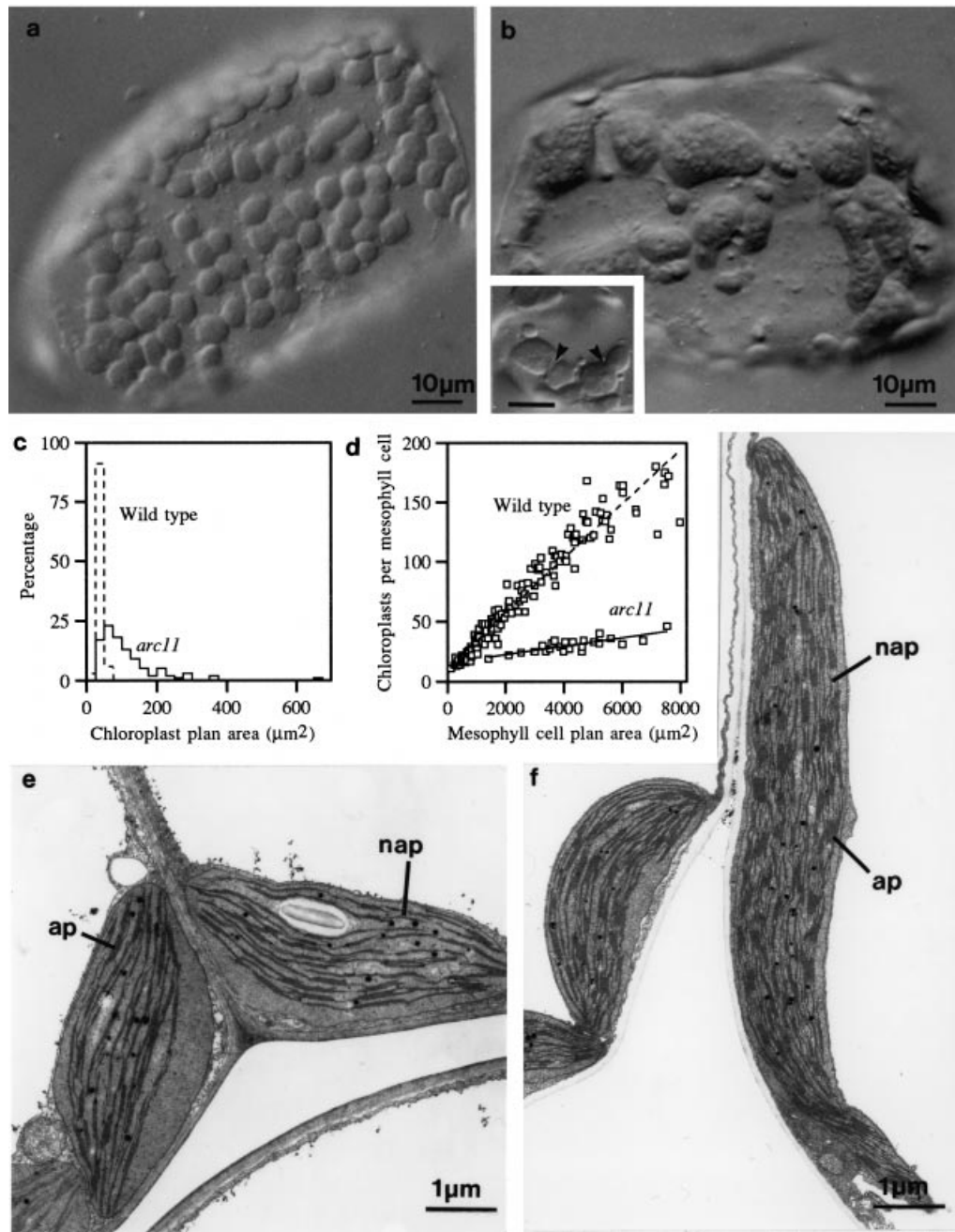
*arc11* is a mutation of a novel, independent *ARC* locus. The unique nature of the *ARC11* locus was revealed by F1 complementation analysis between *arc11* and the other 10 *arc* mutants.

The *arc11* mutant phenotype is stably inherited and in reciprocal backcrosses segregates as a monogenic nuclear recessive trait in a normal Mendelian manner. *ARC11* is 30 cM south of a transposed *Ac* on chromosome V (see Experimental procedures).

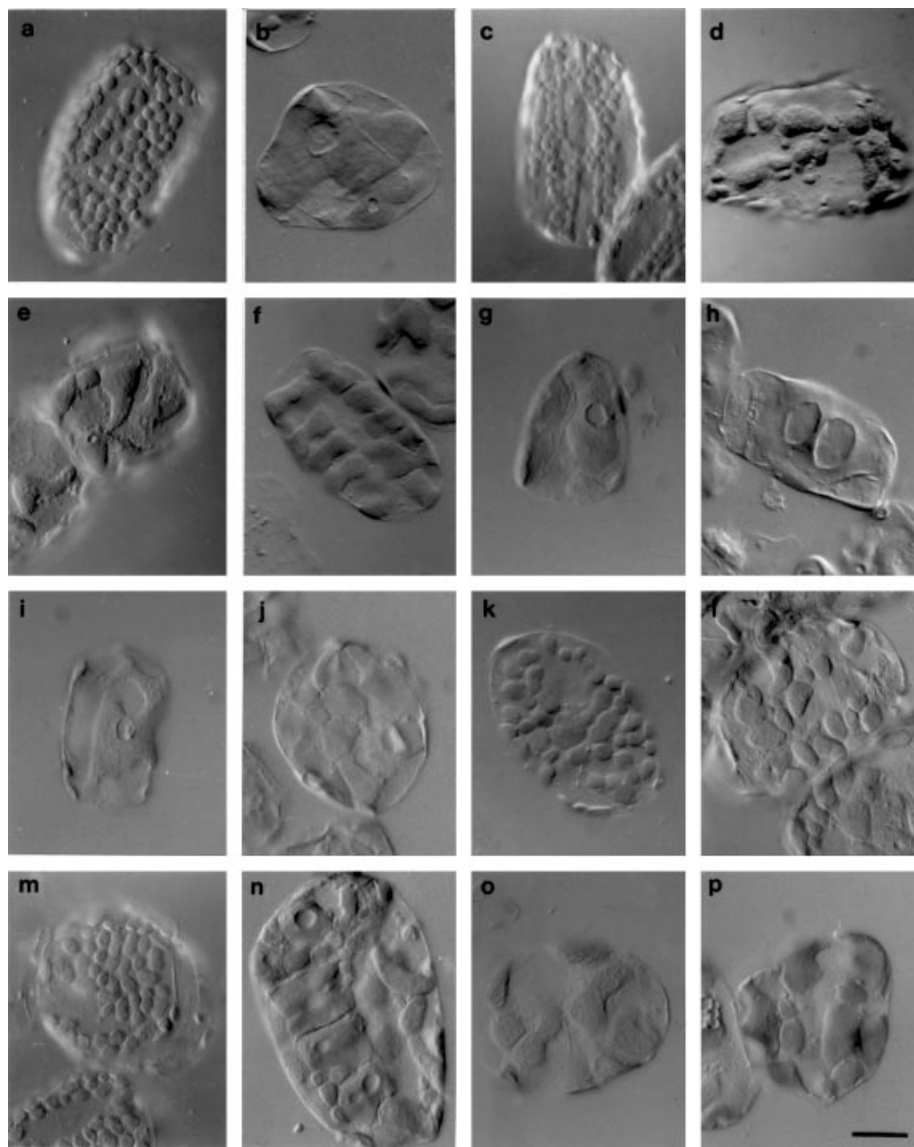
### Construction of double mutants

We constructed mutants homozygous recessive at two *arc* loci to study the extent of *ARC* gene interaction and to determine the hierarchy of *ARC* gene control in the chloroplast division process.

*Identification of double mutants in crosses giving no novel phenotypes in the F2 generation.* In crosses between *arc3* with *arc6*, *arc5* with *arc6*, *arc11* with *arc6*, *arc11* with *arc3*, *arc11* with *arc5* and *arc3* with *arc5*, the F2 plants segregated for wild type and parental types only but no novel F2 phenotypes were found. To identify the double mutant plants in these populations, F2 seedlings with each parental phenotype were allowed to self fertilise. The progeny from one of the parental F2s exhibited a 3:1 segregation pattern. The F3 segregation ratios were as follows:



**Figure 2.** Chloroplasts in the mesophyll cells of wild-type *Arabidopsis thaliana* (*Ler*) and in mesophyll cells of the *arc11* mutant. (a,b) Photomicrographs of isolated leaf mesophyll cells from wild-type (*Ler*) and *arc11*, respectively. The insert in (b) illustrates *arc11* chloroplasts in division. (c) Frequency distribution of chloroplast size, measured as plan area, for chloroplasts from fully expanded leaves of wild-type (*Ler*) and *arc11*. Percentages of chloroplasts of different sizes as measured from 143 wild-type chloroplasts (---) and from 100 *arc11* chloroplasts (—). The chloroplast plan area bin size (i.e. the interval of plan area size used in the histogram) is 25  $\mu\text{m}^2$ . (d) The relationship between chloroplast number per mesophyll cell and mesophyll cell plan area for wild-type (*Ler*) (---) and *arc11* (—). Each data point represents the measurements from one cell in which the cell area was measured and the chloroplast number counted. Regression values ( $r^2$ ) are 0.935 (wild type) and 0.663 (*arc11*). (e,f) Electron micrographs of wild-type (*Ler*) and *arc11* mesophyll cell chloroplasts, respectively. Note the extremes in chloroplast size in *arc11* mesophyll cells compared to the uniform size of chloroplasts in wild-type cells. *arc11* mesophyll chloroplast ultrastructure is very similar to wild-type with normal thylakoids and grana. ap, appressed membrane; nap, non-appressed membrane.



**Figure 3.** Isolated leaf mesophyll cells from fully expanded leaves of wild-type *Arabidopsis thaliana* (Ler) and *arc* mutants viewed with Nomarski differential interference contrast optics.

(a) Wild-type (Ler); (b) *arc6*; (c) *arc1*; (d) *arc11*; (e) *arc3*; (f) *arc5*; (g) *arc11 arc6*; (h) *arc3 arc6*; (i) *arc5 arc6*; (j) *arc1 arc6*; (k) *arc1 arc11*; (l) *arc1 arc3*; (m) *arc1 arc5*; (n) *arc11 arc3*; (o) *arc11 arc5*; and (p) *arc3 arc5*. The cells illustrated are representative of over 1000 cells viewed for each double mutant genotype. The wild-type, *arc1*, *arc3*, *arc1 arc3* and *arc1 arc5* images are taken from Pyke and Leech (1994). Bar = 25  $\mu$ m.

*arc3*  $\times$  *arc6*, 37 *arc3* : 11 *arc6* ( $\chi^2$  0.11,  $P > 0.7$ )

*arc5*  $\times$  *arc6*, 59 *arc5* : 18 *arc6* ( $\chi^2$  0.11,  $P > 0.7$ )

*arc11*  $\times$  *arc6*, 38 *arc11* : 10 *arc6* ( $\chi^2$  0.44,  $P > 0.5$ )

*arc11*  $\times$  *arc3*, 35 *arc3* : 13 *arc11* ( $\chi^2$  0.11,  $P > 0.7$ )

*arc11*  $\times$  *arc5*, 42 *arc11* : 13 *arc5* ( $\chi^2$  0.06,  $P > 0.8$ )

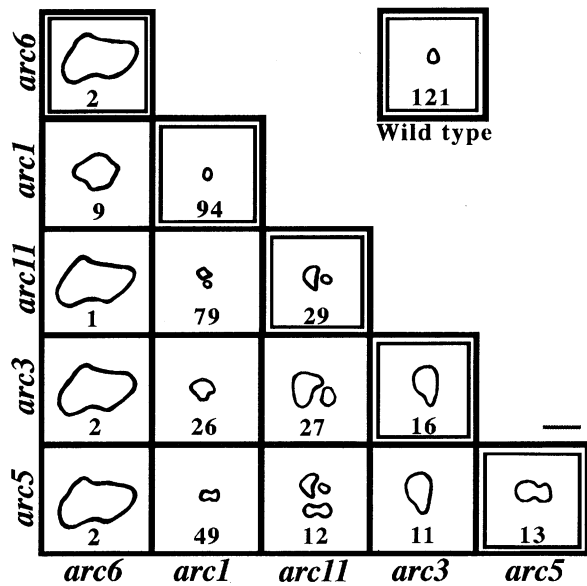
*arc3*  $\times$  *arc5*, 44 *arc5* : 11 *arc3* ( $\chi^2$  0.73,  $P > 0.3$ ).

Double mutants were confirmed by their lack of segregation in the subsequent F4 generation, and analysis of F1 progeny from crosses to parental lines confirmed that the double mutants were homozygous recessive at both mutant loci. F4 seedlings were used in the chloroplast and cell analysis of the double mutants.

*Identification of double mutants in crosses giving novel phenotypes in the F2 generation.* Among the F2 progeny of the crosses *arc1* with *arc6* and *arc1* with *arc11* four phenotypes were identified; wild type, maternal type, paternal type and a novel phenotype which had characteristics of both parents. F2 segregation data were consistent with a 9:3:3:1 ratio as follows:

*arc1*  $\times$  *arc6*, 43 wild type : 15 *arc1* : 18 *arc6* : 6 *arc1 arc6*  
( $\chi^2$  0.89,  $P > 0.8$ )

*arc1*  $\times$  *arc11*, 46 wild type : 13 *arc1* : 17 *arc11* : 4 *arc1 arc11*  
( $\chi^2$  0.756,  $P > 0.8$ ).



**Figure 4.** Diagrammatic representations of chloroplast shape and size for wild-type *Arabidopsis thaliana* (Ler), for five single *arc* mutants and for 10 double *arc* mutants.

Each square contains a line drawing representing the characteristics of the chloroplasts of a single genotype. The x and y axes give the genotypes of the parents. Single *arc* mutants, i.e. parental chloroplast phenotypes, are represented in squares bound by two lines. Double mutant phenotypes are represented in squares bound by a single line. The number in each square is the mean number of chloroplasts per mesophyll cell for that mutant. Bar = 25  $\mu\text{m}$ .

Selected putative double mutant plants were pollinated by relevant homozygous parental lines to confirm their genotypes. Lack of segregation of phenotypes in the F3 generation further confirmed that the putative double mutants were indeed homozygous recessive at both mutant loci. F3 seedlings were used in the chloroplast and cell analysis of the double mutants.

#### Analysis of double mutants

The photomicrographs in Figure 3(g–p) are representative of more than 1000 mesophyll cells from each double mutant. The characteristic shapes and relative sizes of the chloroplasts in the cells of each genotype are illustrated diagrammatically in Figure 4.

*arc11 arc6*, *arc3 arc6* and *arc5 arc6* double mutants. Each of these double mutants had only one or two chloroplasts per mesophyll cell (Table 1, Figure 3g–i) identical in size, distribution and appearance to *arc6* chloroplasts (Figure 3b). The double mutants segregated from the maternal phenotypes in F3 progeny. The relationship between the number of the chloroplasts per mesophyll cell and the size of the mesophyll cell across a range of cell sizes in all the double mutants is indistinguishable from that in the single *arc6* mutant, as shown in Figure 5(c–e).

The epistatic interaction between the mutant genes *arc11*, *arc3* and *arc5* with *arc6* can be interpreted as indicating that *ARC6* gene action is upstream of *ARC11* and *ARC3* and *ARC5* gene action.

*The arc1 arc6*, *arc1 arc11*, *arc1 arc3*, and *arc1 arc5* double mutants. *arc1 arc6* double mutants could be identified in F2 progeny by their novel chloroplast phenotype (Figure 3j). The mean chloroplast number (9) per cell in *arc1 arc6* is greater than the number in *arc6* (2) but less than in *arc1* (94) (Table 1, Figure 5f). Chloroplast size in *arc1 arc6* is also intermediate between the two parental lines: *arc1 arc6* chloroplasts are half the plan area of the *arc6* chloroplasts but more than 15 times larger than the *arc1* chloroplasts (Table 1, Figure 3c). Double mutant seedlings could be recognised by their pale and slightly twisted leaves indicative of the homozygous recessive *arc1* and *arc6* mutations, respectively.

*arc1 arc11* mutants (Figure 3k) were identified in the F2 generation by their pale leaf phenotype indicative of a homozygous *arc1* mutation, but also by the persistence of the variable chloroplast size per cell indicative of the homozygous *arc11* mutation. The double mutant cell has a chloroplast complement of 79, i.e. greater than *arc11* (29) but less than *arc1* (94) (Table 1). Mean chloroplast size in *arc1 arc11* is intermediate between the two parents as is the relationship between number of chloroplasts per mesophyll cell and mesophyll cell size (Figure 5g).

The *arc1 arc3* and *arc1 arc5* double mutants have novel phenotypes (Figure 3l,m) in which the characteristics of both parents are seen: the chloroplast number per mesophyll cell is greater than in either *arc3* or *arc5* single mutants (Figure 5h,i) but less than in the *arc1* parent. Chloroplast size in these double mutants is also intermediate between the two parental mutants (Table 1).

These results suggest that the *ARC1* gene acts independently of the *ARC6*, *ARC11*, *ARC3* and *ARC5* genes during the chloroplast division process.

*The arc11 arc3* double mutant. *arc11 arc3* double mutants (Figure 3n) segregated with the *arc11* chloroplast number amongst the *arc3* population in F3. *arc11 arc3* chloroplasts resemble *arc3* in the proportion of large chloroplasts with plan areas between 400 and 600  $\mu\text{m}^2$  but resemble *arc11* chloroplasts in number (27) and size range (25–675  $\mu\text{m}^2$ ) (Table 1). The relationship between the number of chloroplasts and the size of the mesophyll cells in *arc11 arc3* is also identical to *arc11* (Figure 5j).

The *arc11 arc3* chloroplast phenotype is therefore similar but not identical to *arc11*, suggesting that *ARC11* is partially epistatic to *ARC3*.

*The arc11 arc5* double mutant. The *arc11 arc5* double mutants (Figure 3o) segregated with the *arc5* chloroplast

**Table 1.** Chloroplast number, chloroplast plan area and mesophyll cell plan area for populations of mesophyll cells from fully expanded first leaves of wild-type and *arc* mutants of *Arabidopsis thaliana* cv. Landsberg *erecta*<sup>a</sup>

Genotype <sup>b</sup>	Chloroplasts	Chloroplast plan area <sup>d</sup>	Mesophyll cell plan area <sup>e</sup>	Chloroplasts
	(number per mesophyll cell) <sup>c</sup>	( $\mu\text{m}^2$ )	( $\mu\text{m}^2$ )	(number per 1000 $\mu\text{m}^2$ mesophyll cell plan area)
<i>L.er</i>	120	50 (0.5)	4800 (135)	25
<i>arc6</i>	2	1000 (34)	3700 (62)	0.5
<i>arc11 arc6</i>	1	1090 (37)	3100 (68)	0.3
<i>arc3 arc6</i>	2	1140 (28)	3500 (75)	0.6
<i>arc5 arc6</i>	2	1070 (35)	3700 (75)	0.5
<i>arc1</i>	94	30 (0.5)	2900 (68)	32
<i>arc1 arc6</i>	9	530 (23)	2900 (53)	3
<i>arc1 arc11</i>	79	50 (2.5)	2900 (90)	27
<i>arc1 arc3</i>	26	120 (5)	4200 (192)	6
<i>arc1 arc5</i>	49	80 (5)	4800 (145)	10
<i>arc11</i>	29	110 (9)	4200 (112)	7
<i>arc11 arc3</i>	27	190 (14)	3700 (91)	7
<i>arc11 arc5</i>	12	160 (9)	3500 (78)	3
<i>arc3</i>	16	290 (13)	3900 (80)	4
<i>arc5</i>	13	310 (11)	3500 (83)	4
<i>arc3 arc5</i>	11	260 (12)	3400 (58)	3

<sup>a</sup>Data for *arc1*, *arc3*, *arc5*, *arc1 arc3* and *arc1 arc5* are taken from Pyke and Leech (1994).

<sup>b</sup>The maternal parent of the double mutant is given first.

<sup>c</sup>Determined from a regression of chloroplast number per cell on mesophyll cell plan area using the value for mean mesophyll cell plan area (see e).

<sup>d</sup>Mean of at least 100 chloroplasts from at least 30 different mesophyll cells.

<sup>e</sup>Mean of at least 150 cells per genotype.

The mean plan area of cells in which chloroplasts were measured was not significantly different from the mean plan area of the 150 cells.

SEM values are shown in parentheses<sup>d, e</sup>.

number amongst the *arc11* population in F3. In addition, only a proportion of the chloroplasts in *arc11 arc5* had the characteristic 'arc5' dumb-bell shape indicative of arrest in the final stage of chloroplast division. *arc11 arc5* has 12 chloroplasts (Table 1) compared with 13 in *arc5* indicating that no chloroplast divisions have been completed. The relationship between the number of chloroplasts and the size of the mesophyll cell in *arc11 arc5* is also identical to *arc5* (Figure 5k). The range of chloroplast size in the *arc11 arc5* (20–450  $\mu\text{m}^2$ ) resembles neither the *arc5* (125–700  $\mu\text{m}^2$ ) nor the *arc11* (25–675  $\mu\text{m}^2$ ) distribution.

The appearance of the *arc11 arc5* double mutant is consistent with *ARC5* acting downstream of *ARC11* during chloroplast division.

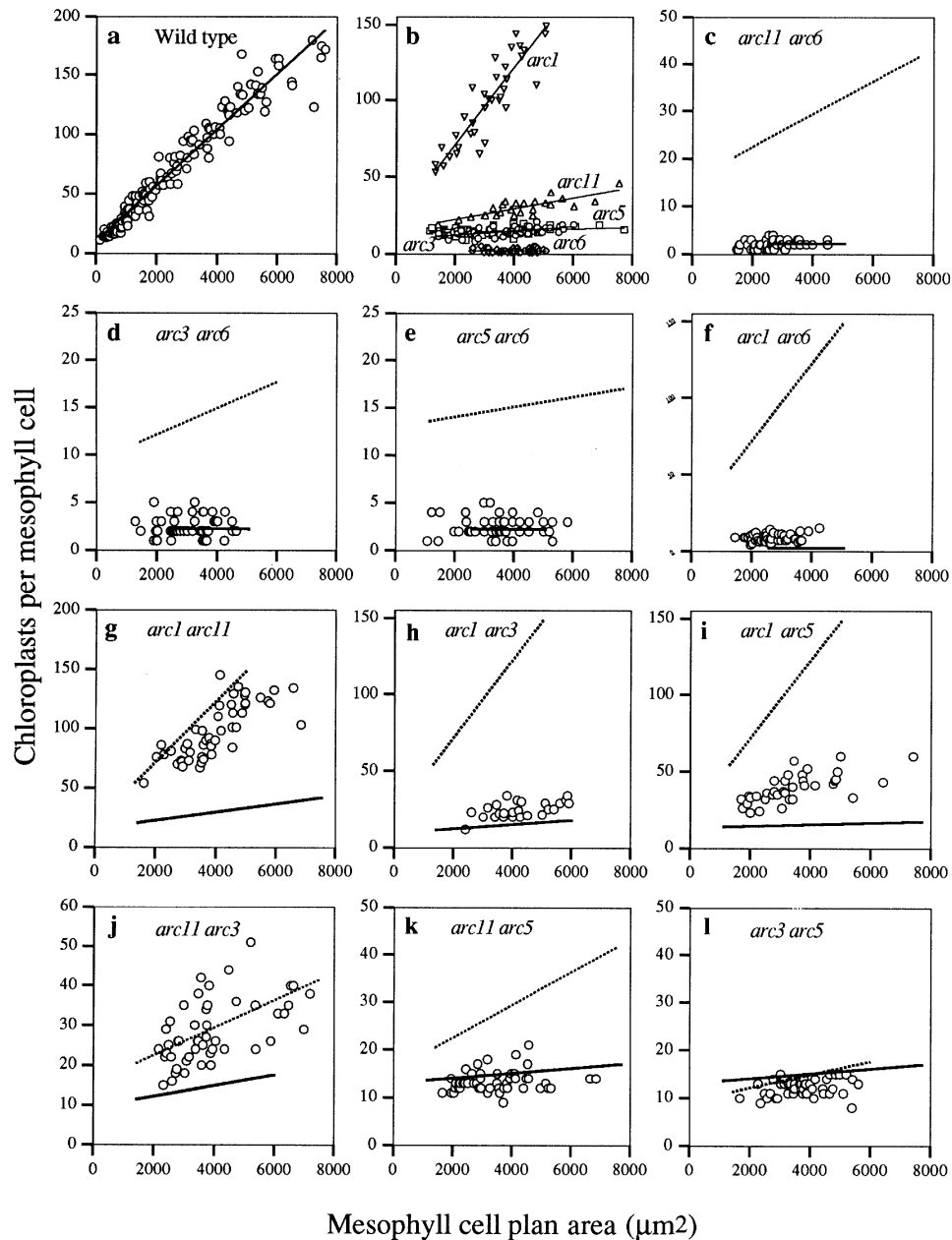
*The arc3 arc5 double mutant.* In fully expanded leaves, chloroplast numbers in *arc3* and *arc5* are very similar impeding the identification of double mutants (Table 1 and Figure 5l). However, in 16-day-old seedlings the appearance of *arc3* and *arc5* chloroplasts is very different and can be used to identify double mutants. At this stage *arc3*

chloroplasts are larger than wild type and have an amorphous shape (Figure 3e), but most *arc5* chloroplasts are arrested in chloroplast division and a central constriction can be clearly seen (Figure 3f). *arc3 arc5* mutants (Figure 3p) segregated with the *arc3* phenotype amongst the *arc5* population in F3 after 16 days of growth. The double mutant had no dumb-bell shaped chloroplasts characteristic of *arc5*.

The chloroplast phenotype of *arc3 arc5* suggests that *ARC3* and *ARC5* function in the same pathway and that *ARC3* acts upstream of *ARC5*.

#### Mapping of the *ARC* loci

We mapped *ARC5*, *ARC6* and *ARC11* using SSLP analysis on a small population of F2 mutant plants. *ARC5* maps to the top arm of chromosome 3 between nga162 (Bell and Ecker, 1994) and *AtDMC1* (Klimyuk and Jones, 1997). *ARC6* and *ARC11* both map to chromosome 5 but at distant locations. *ARC6* maps between ARMS marker m247 (Fabri and Schaffner, 1994) and CAPS marker DFR (Konieczny and Ausubel, 1993) on chromosome 5. *ARC11* maps close to the SLP microsatellite marker nga139 on chromosome

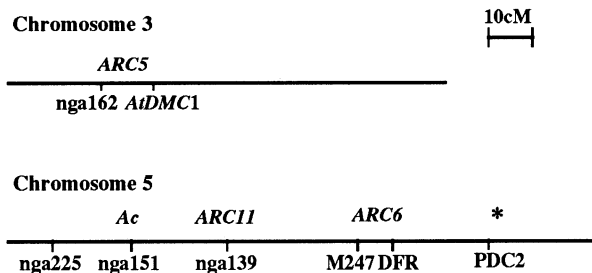


**Figure 5.** The relationship between chloroplast number per mesophyll cell and mesophyll cell plan area for wild-type *Arabidopsis thaliana* (Ler), five single *arc* mutants and 10 mutants homozygous recessive at two *arc* loci. (a) Wild-type (Ler); (b) *arc1* ( $\nabla$ ), *arc3* ( $\circ$ ), *arc5* ( $\square$ ), *arc6* ( $\blacklozenge$ ) and *arc11* ( $\triangle$ ) single mutants all in the Ler background. Before the construction of double mutants *arc6* was crossed into the Ler background, (c) *arc11 arc6*; (d) *arc3 arc6*; (e) *arc5 arc6*; (f) *arc1 arc6*; (g) *arc1 arc11*; (h) *arc1 arc3*; (i) *arc1 arc5*; (j) *arc11 arc3*; (k) *arc11 arc5*; and (l) *arc3 arc5*. Each graph in (c–l) shows the data points for the double mutant ( $\circ$ ) with the maternal (---) and paternal (—) regression lines. Each data point represents the measurement from one cell in which the cell area was measured and the chloroplast number counted. At least 50 cells were analysed for each double mutant except for *arc1 arc3* and *arc1 arc5*. Measurements for the *arc1*, *arc3*, *arc5* single mutants and *arc1 arc3* and *arc1 arc5* double mutants were taken from Pyke and Leech (1994). The values on the y axes differ to accommodate the chloroplast numbers per cell in the single and double mutants.

5. The *Ac* element maps close to *nga151* approximately 30 cm north of the *ARC11* locus leaving open the possibility that *Ac* is effecting the *arc11* mutation. These map positions are shown in Figure 6.

## Discussion

We were able to recover healthy double mutants from all the crosses and the double mutant combinations were not deleterious to plant growth. The Mendelian ratios obtained

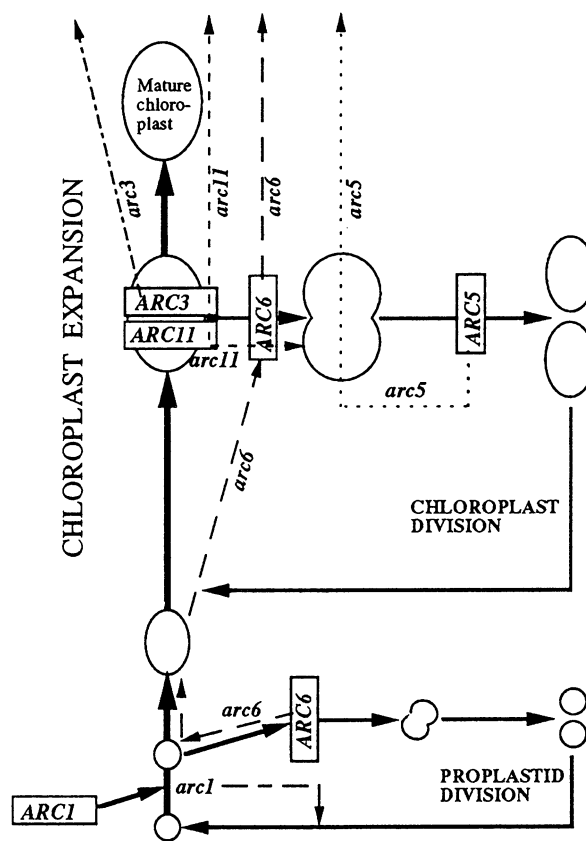


**Figure 6.** Map positions of *ARC5*, *ARC11* and *ARC6*. *Chromosome 3* shows the position of *ARC5* and chromosome 5 shows the positions of *ARC6*, *ARC11* and the *Ac* element. (Lister and Dean recombinant inbred map, updated November 1998). It is interesting to note that *AtFtsZ1;1\** (identical to part of clone MC015) maps to chromosome 5 distal to *PDC2* and *AtFtsZ2;1* (identical to part of the BAC clone F2H17) maps to chromosome 2 ([HTTP://genome-HTTP://www.stanford.edu/Arabidopsis/](http://genome-www.stanford.edu/Arabidopsis/)).

in the F<sub>2</sub> generation (*arc1* × *arc6*, *arc1* × *arc11*) and in the F<sub>3</sub> generation (*arc3* × *arc6*, *arc5* × *arc6*, *arc11* × *arc6*, *arc11* × *arc3*, *arc11* × *arc5* and *arc3* × *arc5*) confirm that the *arc* mutants are the result of mutations in independent nuclear recessive *ARC* genes. Based on our analysis of the double mutant chloroplast phenotypes and comparison with the single mutants we are able to determine the hierarchy of the five *ARC* genes in the control of the higher plant chloroplast division process. The *ARC* genes will be considered in the order in which they apparently operate in the proplastid and chloroplast division pathways as illustrated in Figure 7.

The *ARC6* gene acts upstream of *ARC11*, *ARC3* and *ARC5* since the *arc11 arc6*, *arc3 arc6* and *arc5 arc6* double mutants all have a chloroplast phenotype indistinguishable from *arc6*. Just as in the single *arc6* mutants both proplastid and chloroplast division are arrested in all the double mutants with *arc6*. Therefore *ARC6* acts pleiotropically during the initiation of both proplastid division and chloroplast division, possibly by encoding a promoter of chloroplast division.

In contrast, the chloroplast phenotypes of double mutants constructed with *arc1* are all novel and additive and appear to be intermediate between the two parental phenotypes, demonstrating that *ARC1* acts independently of the other four *ARC* genes. In the case of the *ARC1* locus it is not possible to conclude from the characteristics of the single *arc1* mutant alone whether the *arc1* lesion affects proplastid or chloroplast division or both processes. However, analysis of *arc1 arc6*, *arc1 arc11*, *arc1 arc3* and *arc1 arc5* double mutants provides clear evidence that *arc1* considerably accelerates proplastid division only and has no effect on chloroplast division. The chloroplast number in all the double mutants with *arc1* is enhanced above the paternal chloroplast number even for those mutants which contain a second mutant gene completely inhibiting the initiation or completion of chloroplast division. The difference between the chloroplast number in *arc3* (16) and *arc1 arc3* (26) is a measure of the acceleration of



**Figure 7.** A diagram illustrating the suggested hierarchy of *ARC* gene action in higher plant chloroplast division.

This model is based on Figure 1. The stages in the chloroplast division process affected by *ARC6*, *ARC1*, *ARC11*, *ARC3* and *ARC5* gene action were deduced from our analysis of the respective single mutants and double mutants. The effects of mutation at each *ARC* locus are indicated by broken lines. *ARC1* is involved in the down-regulation of proplastid division but is in a separate pathway. *arc1* leads to increased proplastid division. *ARC6* is involved in the initiation of both proplastid and chloroplast division. *arc6* completely suppresses proplastid and chloroplast division but allows extended expansion until the chloroplasts are 20 times larger than wild type. *ARC11* is involved in the central positioning of the division constriction. In *arc11* the constriction is asymmetric. *ARC3* controls chloroplast expansion. The abnormally rapid expansion of *arc3* chloroplasts prevents chloroplast division. *ARC5* facilitates the separation of the two daughter plastids and in *arc5* the chloroplasts remain dumb-bell shaped and continue to expand.

proplastid division which occurs in the presence of *arc1*. In addition, the effect of *arc3* can be seen by the increase in the size of the chloroplasts in *arc1 arc3* beyond the sizes of *arc1* chloroplasts. It is particularly interesting that in the *arc1 arc5* mutant the presence of *arc5* seems to act pleiotropically and to have affected the expression of *arc1* and to have further enhanced proplastid division so that about twice as many young plastids are present in *arc1 arc5* mesophyll cells as in *arc1 arc3*.

*ARC3* has an important role in the initiation of chloroplast division since in *arc3* the chloroplast number (16) is the same as the final proplastid number, i.e. no chloroplast division occurs. Further understanding of the role of *ARC3*



comes from the analysis of the *arc3 arc5* double mutant whose chloroplast number and phenotype is identical to that of *arc3*. The lesion in *ARC3* completely prevents the initiation of all chloroplast division: there are no dumb-bell shaped profiles in *arc3 arc5* as would be seen if division had proceeded and then been stopped, as in *arc5*. In *arc3 arc5* the expansion of the chloroplasts continues unchecked until the proportion of the cell surface covered by chloroplasts becomes the same as in *arc3*. Ellis and Leech (1985) provided evidence that chloroplast division only occurs in young chloroplasts when they are less than a certain size. We suggest that *ARC3* ensures that the young chloroplasts expand to this optimal size to coincide with the initiation of chloroplast division. As a result of the *arc3* mutation, expansion of the young chloroplasts is unchecked and the chloroplasts are too large to be able to divide at the time chloroplast division would normally be initiated.

The mutations which give rise to *arc3* and *arc11* have in common that they disrupt the normal relationship between chloroplast division and chloroplast expansion although *ARC3* and *ARC11* have different roles in this coordination. In *arc11*, the young chloroplasts divide asymmetrically (Figure 2b and insert) and apparently only once giving rise to daughter chloroplasts of different sizes. The appearance of the *arc11 arc3* double mutant provides considerable further support for our interpretation of the role of *ARC11*. The *arc11 arc3* double mutant has the *arc11* chloroplast heterogeneity and number per cell (29) but the mean sizes of both the small and large chloroplasts are larger than in *arc11*. Thus, in *arc11 arc3* a lesion in the *ARC3* gene facilitates chloroplast expansion resulting in larger chloroplasts as in *arc3* and the lesion in the *ARC11* gene allows only one round of asymmetric chloroplast division resulting in the typical *arc11* chloroplast number. Further evidence supporting our interpretation of the role of *ARC11* comes from the examination of the *arc11 arc5* double mutant. In *arc11 arc5*, chloroplasts capable of division in *arc11* cannot complete this division because of the presence of *arc5* and are seen as larger than wild-type dumb-bells typical of *arc5*. The presence of *arc5* has prevented the final separation of the two daughter plastids. In *arc11 arc5* there is definite interaction in the expression of the two mutant genes since the dumb-bell shaped chloroplasts appear symmetrical in the mature cells. We do not know the exact time during chloroplast development when *ARC11* operates; *ARC11* could function just before chloroplast division is initiated (as illustrated in Figure 7) or at any time earlier during the chloroplast expansion phase.

The analyses of the double mutants *arc1 arc5* and *arc3 arc5* confirm that the *ARC5* gene plays a very late role in the final stages of the chloroplast division process and effects the separation of the daughter

plastids. Similar to *arc5*, in *arc1 arc5* all the chloroplasts are arrested in the dumb-bell configuration and the daughter plastids never separate. In *arc1 arc5* the chloroplast number is intermediate between the two parents confirming that *ARC1* and *ARC5* are on different pathways. The appearance of the *arc3 arc5* double mutant confirms that *ARC5* acts downstream of *ARC3* on the same pathway.

On the basis of the analysis of single and double *arc* mutants it is clear that *ARC6* is involved in the initiation of a series of divisions in the wild-type *proplastids* therefore the post-mitotic cells contain 15 young chloroplasts. The chloroplasts then expand and reach an optimal size for chloroplast division. The co-ordination of chloroplast division with chloroplast expansion is mediated by *ARC3* with *ARC11* also being involved. *ARC6* is pleiotropic and is also involved in the initiation of the *chloroplast* division process. During division, chloroplasts become centrally constricted and the position of the narrow isthmus involves *ARC11*. The final separation into two equally sized daughter chloroplasts involves *ARC5*. The chloroplasts continue to expand and further rounds of division occur. The full wild-type chloroplast complement of 120 chloroplasts in the Landsberg *erecta* ecotype of *Arabidopsis* is attained after three complete rounds of chloroplast division. *ARC1* down-regulates proplastid division in an independent pathway to *ARC6*, *ARC11*, *ARC3* and *ARC5*.

We used only one of four allelic *arc6* mutants and only one of two allelic *arc3* mutants in this study. The four *arc6* mutants are equally extreme and their chloroplast phenotypes are apparently identical: the two *arc3* mutants are also similar. We isolated only one mutant each of *arc5*, *arc1* and *arc11* despite screening more than 20 000 individuals. *arc5-1* is a strong allele since every single chloroplast is halted at the very last stage of chloroplast division. *arc1-1* and *arc11-1* also have chloroplast phenotypes greatly altered from wild type but are still stable. Even more severe alleles of *arc1*, *arc3*, *arc5*, *arc6* and *arc11* than the ones we have isolated may well be lethal. It is particularly interesting that no genetic mutants have been found for the stages between the initial (*arc3*) and final (*arc5*) stages of chloroplast division. It is theoretically possible that these intervening stages occur completely spontaneously without genetic control because of the physical characteristics of the chloroplast envelope (Leech *et al.*, 1981).

Recent work has identified plant nuclear homologues of the prokaryotic cell division gene *FtsZ* in *Arabidopsis* (Osteryoung and Vierling, 1995; Osteryoung *et al.*, 1998) and in the moss *Physcomitrella* (Strepp *et al.*, 1998). *FtsZ*, a cytoskeletal protein related to tubulin, is a vital component of the prokaryotic cell division machinery. In

*Escherichia coli*, FtsZ is recruited to the equator of a dividing cell to form a ring around the division site. All FtsZ genes currently identified in plant species show sequence homology to one of two distinct groups; those which contain a chloroplast transit peptide sequence (FtsZ1) and those which do not (FtsZ2). The *Arabidopsis* FtsZ protein AtFtsZ1-1 has been shown to be imported into chloroplasts (Osteryoung and Vierling, 1995). Antisense repression of *Arabidopsis* FtsZ genes from either of these two families decreases chloroplast numbers in the mesophyll cells (Osteryoung *et al.*, 1998). At present there is no evidence to suggest that either of the AtFtsZ genes are homologues of any known ARC loci.

Our analysis of double *arc* mutants has shown that normal chloroplast division in *Arabidopsis* is a complex process and involves the integrated action of several independent unlinked nuclear genes. The rapidly increasing availability of polymorphic markers on the *Arabidopsis* chromosomes will facilitate the fine mapping of the ARC loci and the isolation of the ARC genes.

## Experimental procedures

### Plant material

Wild-type *Arabidopsis thaliana* plants Landsberg *erecta* (Ler) and *arc* mutant plants were grown in controlled conditions as described previously (Pyke and Leech, 1991). The *arc1*, *arc3* and *arc5* mutants were isolated from an EMS mutagenised population of *Arabidopsis thaliana* (Ler) (Lehle seeds, Tucson, AZ, USA) (Pyke and Leech, 1992; Pyke and Leech, 1994; Robertson *et al.*, 1996). *arc6* was not tagged but was isolated from a T-DNA mutagenised population of *Arabidopsis thaliana* WS (Feldmann and Marks, 1987; Feldmann, 1991; Pyke *et al.*, 1994; Robertson *et al.*, 1995) obtained from the Nottingham *Arabidopsis* Stock Centre (University of Nottingham, Nottingham, UK; stock no. N3115). The *arc11* mutant was isolated from a population of *Arabidopsis thaliana* (Ler) mutagenised with the *Nael* deleted *Ac* element (Dean *et al.*, 1992; Lawson *et al.*, 1994). Twelve progeny from one full green seedling for each of 350 lines were analysed for mutant *arc* phenotypes. Two of the 12 seedlings analysed from line 122 (transformant 02213-3) displayed the *arc11* phenotype. Seeds of all the genotypes used in this paper can be obtained from the Nottingham *Arabidopsis* Stock Centre ([HTTP://nasc.nott.ac.uk](http://nasc.nott.ac.uk)).

### Double mutant construction

Double mutants were constructed by crossing homozygous *arc1*, *arc3*, *arc5*, *arc6* and *arc11* plants in all combinations. All F1 progeny were phenotypically wild type and allowed to self fertilise. F2 and F3 seeds were sown in 12 × 8 gridded arrays. Tissue was sampled from the apical half of fully expanded first leaves of each seedling and placed into 3.5% (v/v) glutaraldehyde in 12 × 8 arrays of a corresponding microtiter plate (Pyke and Leech, 1991) and chloroplast number per cell, chloroplast size and mesophyll cell size determined. Putative double mutants were allowed to grow, self fertilise and set seed.

### Analysis of chloroplast number, chloroplast size and mesophyll cell size

Chloroplasts in individual isolated fixed mesophyll cells were counted using Nomarski differential interference contrast optics (Nikon Optiphot, Nikon, UK). Mesophyll cell plan areas (Pyke and Leech, 1991) and individual chloroplast plan areas (Pyke and Leech, 1992) were measured directly from the microscope using an image analysis system (Seescan Imaging Ltd, Cambridge, UK). Fully expanded first leaves were always used.

### Ultrastructural analysis

For ultrastructural analysis, first leaves from *Ler* wild type and *arc11* were harvested 16 days after sowing and examined after fixation and embedding in Spurr's resin as described previously for *Arabidopsis* (Pyke *et al.*, 1994).

### Mapping of the ARC loci

To map the position of *ARC5*, an F2 population originating from a cross between *arc5* (*Ler*) and Columbia was used. Genomic DNA for PCR was isolated from leaves of 300 F2 mutant seedlings (Dellaporta *et al.*, 1983). SSLPs (Bell and Ecker, 1994) and the CAPS marker *AtDMC1* (Klimyuk and Jones, 1997) and the recombinant inbred map (Dean and Lister, 1998) were used.

*ARC6* was mapped using the *Arabidopsis* RFLP Mapping Set, ARMS (Fabri and Schäffner, 1994), on a population of F2 mutant progeny from a cross between *arc6* (WS) and *Ler*. Genomic DNA was isolated from 22 F2 mutant progeny by CTAB extraction (Dean *et al.*, 1992), digested with *EcoR1* and Southern blotted. Nineteen of the 25 ARMS markers showed polymorphisms between WS and *Ler* (Rutherford, 1996). Eight of these markers were used as probes in the preliminary mapping of *ARC6*. The CAPS marker DFR (Konieczny and Ausubel, 1993) was used to verify the map position of *ARC6*.

The identification of the *arc11* mutant in a population of *Arabidopsis thaliana* carrying a transposed *Ac* element potentially offered a good opportunity to isolate the first ARC gene. To establish the tagged status of *arc11* we analysed the co-segregation of the *arc11* mutant phenotype with the *Ac* element in mutant siblings from the F2 of a backcross between *arc11* (*Ler*) and Columbia. Genomic DNA was digested with *SspI*, separated, Southern blotted and probed with the 904 bp *HindIII-EcoRI* fragment of *Ac* (Dean *et al.*, 1992) labelled with 50 µCi <sup>32</sup>P-dCTP. *Ac* was found in 59 of the 66 individuals tested. The segregation of the *Ac* element away from the *ARC11* locus can be explained by excision of *Ac* leaving a mutagenic footprint in the *ARC11* gene (Bancroft *et al.*, 1993). Plant DNA sequences flanking the *Ac* were isolated by IPCR. Genomic DNA from *arc11* was double digested using *BstY1* and *BclI* and self-ligated. After an initial 5 min at 94°C the conditions for the amplification were as follows: 30 sec at 94°C; 30 sec at 55°C and 3 min at 72°C. The cycle was repeated 34 times. The primer pairs used for the amplification of the plant DNA flanking the 5' and 3' regions of the *Ac* were B34 and D74 (Briza *et al.*, 1995) and DL6 (Long *et al.*, 1993) and D71 (Briza *et al.*, 1995), respectively. Primers designed from the 150 bp 5' and 1.1 kb 3' IPCR products were used to amplify over the region of *Ac* excision in the *arc11* individuals lacking the *Ac* element. Sequence analysis of the resulting PCR fragments showed that the *arc11* plants lacking *Ac* had the wild-type sequence, so the *Ac* element is not located within the *ARC11* gene and cannot be used to isolate the gene.

To map the position of *ARC11*, genomic DNA from F2 mutant seedlings originating from a backcross between *arc11* (*Ler*) and Columbia were used with SSLP markers (Bell and Ecker, 1994). To map the position of the *Ac* element CIC (Creusot *et al.*, 1995) and Ecker (Ecker, 1990) YAC libraries were probed with the 1.1 kb 3' JPCR fragment. Hybridisation to the YAC filters was carried out as described by Schmidt *et al.* (1992) except that the washing conditions were  $3 \times$  SSC, 0.1% SDS for 10 min followed by  $0.1 \times$  SSC, 0.1% SDS for 10 min. Three YAC clones anchored to nga151 on chromosome 5 were identified.

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