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The isolation of apparently homoplastidic mutants induced by a nuclear recessive gene in *Arabidopsis thaliana*

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Summary. The nuclear recessive gene, *chm1*, of *Arabidopsis thaliana* is a mutator that induces a variety of plastid alterations giving rise to mixed cells and variegated leaves. The variegation is maternally transmitted but *chm1* is transmitted in a Mendelian fashion (Rédei 1973; Rédei and Plurad 1973). In order to characterize the different types of plastid alterations induced by *chm1*, isolating homoplastidic lines, each apparently containing one type of mutant plastid in its cells, was essential since such characterization cannot be carried out on mixed cells. We have used two genetic approaches to isolate several apparently homoplastidic mutant lines by the removal of the mutator from the genetic background, and the maternal transmission of the mutant plastids. The rapidity of obtaining homoplastidic lines in the absence of *chm1* indicated a non-stochastic sorting-out of plastids in mixed cells. That each of the *chm1*-free homoplastidic mutant lines was apparently homoplastidic for one type of mutant plastids was confirmed by electron microscopic observations. Here we report, for the first time, the production of different homoplastidic lines in the absence of the nuclear-mutator gene. Such genetically-stable homogeneous material should be a useful tool for studying the molecular mechanism(s) by which *chm1* induces a variety of heritable plastid alterations.

Key words: *Arabidopsis* – Mutator – Homoplastidic mutants – Chloroplast ultrastructure – Sorting-out

Introduction

Nuclear recessive genes that act as plastid mutators have been described in a number of genera of higher plants (Kirk and Tilney-Basset 1978; Gillham 1978; Grun 1976; Hagemann 1979). Some mutator genes induce one type of mutant plastids such as *iojap* and *cm* of maize (Shumway and Weier 1967; Coe et al. 1982; Thompson et al. 1983), *albostrains* (*as*), *Okina-mugi*, white-streak-3 (*wst3*) and *striata-4* of barley (Knoth and Hagemann 1977; Imai 1928; Ahokas 1976; Wettstein 1961), and *albomaculans* (*am*) of *Arabidopsis* (Röbbellen 1966). Other mutator genes induce many kinds of mutant plastids such as *chm1* (Rédei and Plurad 1973), mp_1 and mp_2 of *Epilobium hirsutum* (Michaelis 1968a, b), *pm* of *Oenothera hookeri* (Epp 1973; Sears 1983), *ā* of *Petunia hybrida*, and "*mutation allowing*" in *Nepeta cataria* (Potrykus 1970; Woods and DuBuy 1951). Physical and chemical agents have also been employed to induce chlorophyll-deficient plants. For example, N-nitroso-N-methylurea and N-nitroso-N-ethylurea have been used to induce plastid mutations in *Pelargonium zonale* and *Antirrhinum majus* (Hagemann 1976, 1979; Hagemann and Börner 1981). X-ray treatment was used to produce plastid mutations in *Arabidopsis* (Röbbelen 1962) and *Epilobium* (Michaelis 1958; 1967), while the radioactive isotopes ^{32}P and ^{35}S were used to produce both plastid mutations and variegated plants in *Epilobium* (Michaelis 1968a, b). Additionally, spontaneous mutations have contributed to the production of plastome mutants in *A. majus* (Hagemann 1971; Börner et al. 1980; Herrmann 1971a, b) and *P. zonale* (Börner et al. 1972, 1973; Börner 1980).

The recessive nuclear gene, *chm1*, *of Arabidopsis thaliana* increases the spontaneous rate of plastid mutation 10^6 fold (Rédei 1973). Homozygous *chm1* plants

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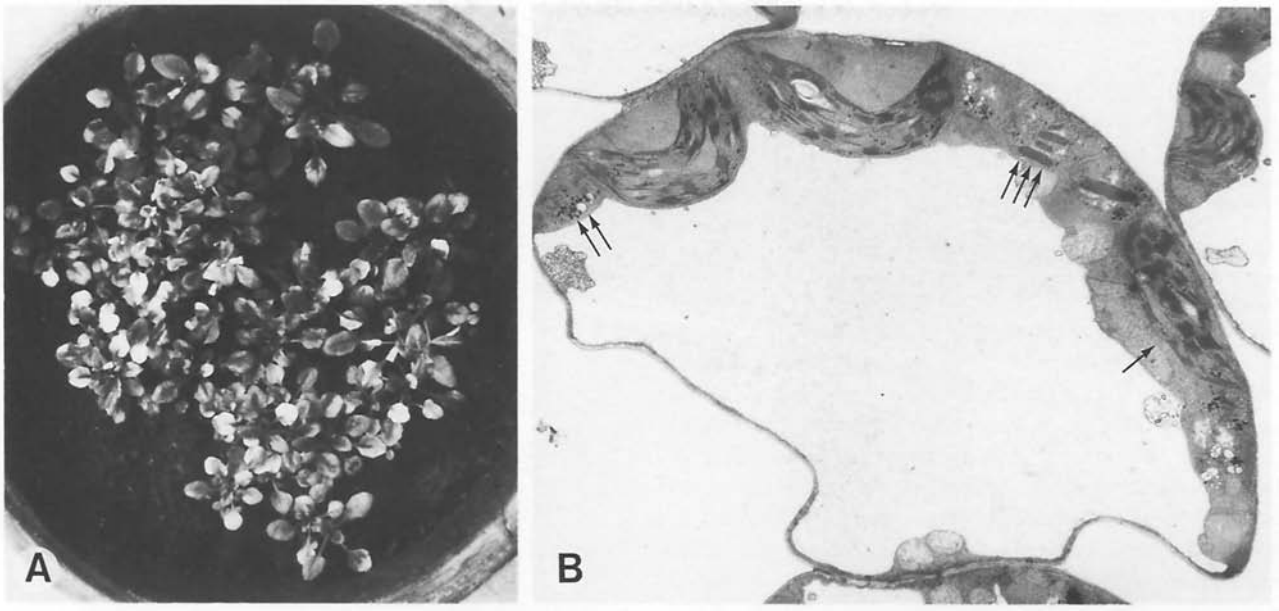

Fig. 1 A, B. The phenotype and ultrastructure of *chm1/chm1* plants. **A** The amount of color variegation in a *chm1/chm1* population of Arabidopsis. **B** A heteroplastidic cell from a *chm1/chm1* plant displaying → normal plastids, ⇉ a plastid lacking internal membrane structure and ⇉ a plastid with three giant grana and very few unorganized internal membranes. Enlarged 9,000 ×

show different color variegation, including white, yellow and pale-green as well as rough-leaf mutations (Rédei 1973). This variegation is maternally inherited but *chm1* itself is a recessive Mendelian gene. Long-term maintainance of stocks homozygous for *chm1* leads to the accumulation of a wide variety of mutant plastids per cell (Rédei and Plurad 1973). We have, therefore, decided to produce stable *chm1*-free homoplastidic lines, each containing only one type of mutant plastid in its cells. Here we report two genetic approaches to obtain such homoplastidic lines after the removal of the recessive mutator, *chm1*, from the nuclear background and the sorting out of the different mutant plastids from mixed cells either by maternal transmission through sexual reproduction or by tissue-culture propagation. This is the first report on the production of different stable homoplastidic mutant lines in a nearly isogenic background free of the nuclear-mutator inducer gene. Such material will be very useful in studying the molecular mechanisms by which nuclear genes control plastid function, development, and structure.

Materials and methods

Genetic stocks

All nuclear mutants used in this study were isolated from an *A. thaliana* (L.) Heynh., Columbia wild-type background, and were kindly provided by Dr. G. P. Rédei. The nuclear mutants used were: (1) *chm1* (for chloroplast mutator), a recessive nuclear gene on linkage group 3 induced by treating seeds with ethyl methane sulfonate (Rédei 1973), (2) the *gl1* (glabrous) gene, an exellent recessive marker approximately 15 map units from the *chm* locus, (3) the *as* (asymmetric leaf) gene, a recessive marker on linkage group 2, and (4) *y* (yellow), a semi-dominant mutation (not yet mapped).

Soil cultures

Seeds were planted in 12.5 cm clay pots filled with a mixture of soil and peat moss (approximately 5:1). The plants were grown under 8–10 h daily illumination in growth chambers. Between 400–500 foot candle illumination and 24 °C proved optimal for obtaining the best growth and highest yield of seeds.

Crosses of Arabidopsis

Arabidopsis, a member of the family Cruciferae, is a self-pollinating plant. Crosses were performed by removing the stamens from the flower bud while it was still closed to ensure that no selfing had occurred. Female parents were always marked with a recessive gene. The absence of the recessive phenotype in the F_1 progeny was an indicator that the cross was successful. The stigma was pollinated with pollen from at least two flowers.

Aseptic cultures

Plants were grown aseptically on a simple mineral medium, referred to as E-medium, according to Rédei (1965).

Organ and tissue culture

For tissue culture the Murashige-Skoog medium (MS) as described by Gamborg (1982) was used. The medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4D) and kinetin for callus cultures, or naphthalene acetic acid (NAA) and 6-benzyl-amino-purine (BAP) for regeneration cultures. The hormone concentrations used are given under Results.

Electron microscopy

Leaves were cut into 1 mm^2 pieces and fixed in 2.5% glutaraldehyde. 0.1 M PO_4 buffer pH 7.1 for 3 h at 4 °C. The leaf pieces were then post-fixed in 1% osmium tetroxide, 0.1 M PO_4 buffer

for 3 h at 4 °C, dehydrated in an acetone solution series (20%, 40%, 60%, 80% and 100%) and finally embedded in Epon resin. Thin sections of 700–900 A° were cut which were then stained with lead citrate and uranyl acetate. A JEOL 100 B transmission electron microscope was used for visualization.

Results

Isolation of homoplastidic lines

Maintaining stocks that are homozygous for the mutator, *chm1*, for a long period of time have led to the accumulation of several color variegations, including white, yellow and different shades of pale-green, in the plants (Fig. 1 A). As shown earlier by Rédei and Plurad (1973) several types of plastid alterations accumulate in the cells of such plants, giving rise to a heterogeneous population of mutant and normal plastids. We have confirmed the presence of mixed cells (heteroplastidic) carrying mutant and normal plastids in *chm1/chm1* variegated leaves (Fig. 1 B). Stable homoplastidic lines can be obtained only through the removal of the mutator and then allowing the sorting out of the mutant organelles from mixed cells to obtain plants with only one type of mutant plastid in their cells (homoplastidic lines). Two genetic approaches were applied to the isolation of homoplastidic lines.

In the first (Mourad and Rédei 1985), the pulse-and-chase method (Fig. 2), asymmetric leaf (*as*), wild-type females were crossed with variegated *chm1/chm1*, hairless (*gl1/gl1*) males. The F_1 was normal but heterozygous for *chm1* and no variegation was observed because no mutant plastids are transmitted through the male parent. In the F_2, 25% of the population displayed late sectoring since *chm1* is newly introduced (pulsed) and new plastid mutations require some time to sort out. F_2 plants which are *chm1/chm1* carry only a few types of mutant plastids. Mutant sectorial flowers were backcrossed as females to *CHM1GL1/CHM1GL1* males to block *chm1* (chase) but retain and propagate the mutant plastids in the cytoplasm of the progeny since they are transmitted through the egg. Among the progeny, mutant branches were backcrossed again as females to *CHM1GL1/CHM1GL1* males. Only variegated progeny were allowed to self and were maintained as separate lines.

In the second approach, hairless, variegated *chm1/chm1* females were crossed to wild-type males. In this method the mixture of mutant plastids was maternally transmitted and *chm1* was blocked immediately by the wild-type allele. Highly variegated F_1 plants were selected and allowed to self. In the F_2, only hairy variegated plants were advanced to an F_3. In the F_3, only families which were not segregating for hairless were maintained either aseptically on E-medium or in soil. Twenty three rough-leaf families and six hairy color-variegated families were isolated.

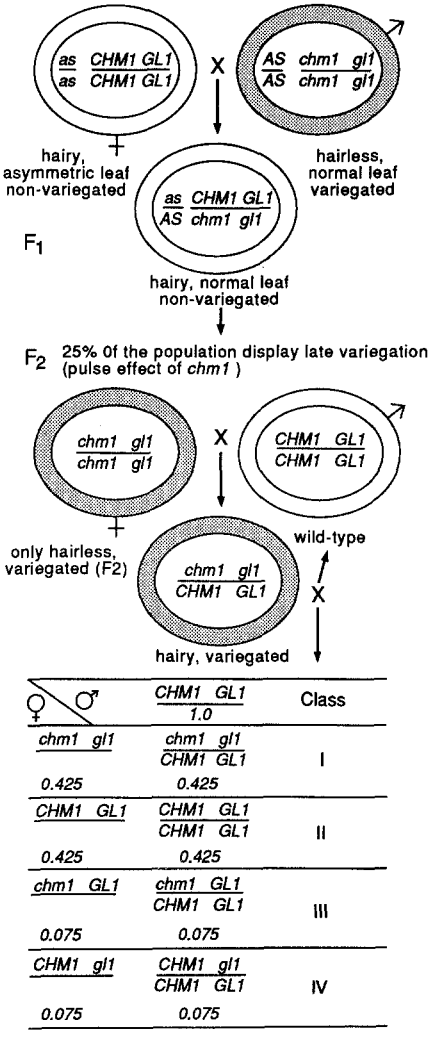

Fig. 2. Diagrammatic illustration of the pulse-and-chase method (designed from Mourad and Rédei 1985)

Verification of the absence of the mutator

Since the *chm1* locus is about 15 map units from *gl1* (Rédei 1973) the majority of the progenies having only hairy variegated individuals would be expected to be free of the nuclear mutator. Since some of the cytoplasmically mutant hairy plants may be recombinants, and hence may still carry one or two *chm1* alleles which because of homozygosity in the progeny will induce new forward as well as reverse mutation(s), their genetic constitution was verified by outcrossing them to *chm1* heterozygous testers. These testers were constructed by crossing asymmetric (*as/as*), non-variegated hairy (*CHM1GL1/CHM1GL1*) females (free of any cytoplasmic mutation) with *chm1gl1/chm1gl1* males. The F_1 tester will be non-asymmetric and cytoplasmically wild-type because *chm1* is recessive. Such a tester was used as the female parent and crossed to each of the 23 rough-leaf families and the six color-variegated families. The presence of *chm1* in the

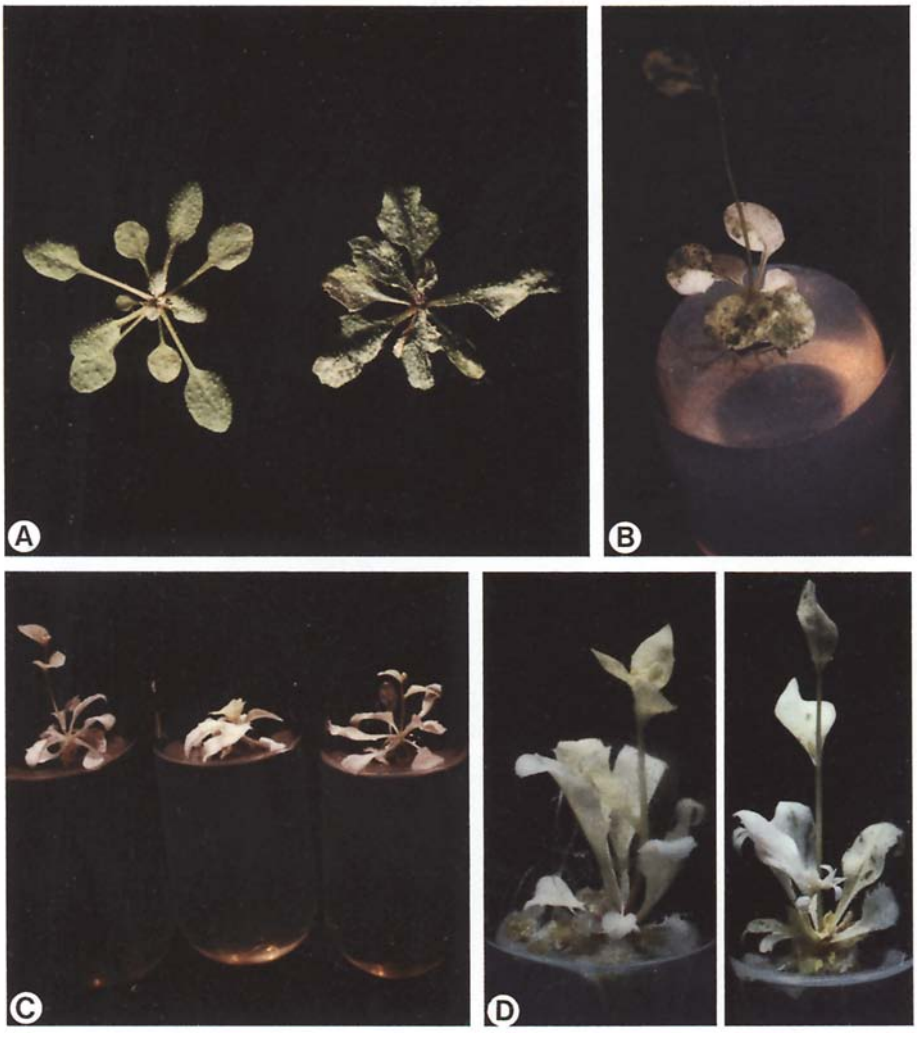

Fig. 3A–D. *chm1*-induced color-variegated and rough-leaf (RL) mutants. **A** Left, a Columbia wild-type plant and right, RL32, one of the eleven *chm1*-free rough-leaf mutants. **B** A highly variegated plant from the progeny of the *chm1*-free family 1j. **C** All pale apparent homoplastidic plants sorting-out from the *chm1*-free family 4g. **D** Left, a solid pale plant regenerated from a white leaf of a plant from the *chm1*-free family 1j and right, a pale plant regenerated from a solid white leaf of a plant from progeny of a family with the mutator present. The green spots covering the regenerated plant indicate a genotype *chm1*/*chm1* and the high activity of the mutator

chosen mutant family was revealed through the reappearance of variegation in 25% of their progeny if the chosen mutant was *CHM1*/*chm1*, and 50% if the chosen mutant was *chm1*/*chm1*. The absence of *chm1* in the chosen mutant was revealed by the lack of variegation in these progenies (100% non-variegated). Table 1 shows that 11 out of the 23 rough-leaf mutant families and three out of the six color-variegated mutant families did not segregate for variegation when crossed to the female tester indicating that they were *chm1*-free (Fig. 2, classes II and IV).

Description of the rough-leaf mutants

The rough-leaf mutants are green in color, have irregular leaf shape and rough-leaf surface (Fig. 3A). After the removal of the mutator gene from the nuclear background the rough-leaf lines maintained their phenotype upon selfing for several generations (Fig. 3A). To verify the cytoplasmic nature of the *chm1*-free rough-leaf mutants each rough-leaf family was reciprocally crossed with semi-dominant yellow (*y*) homozygotes and the recessive marker asymmetric leaf (*as*). The rough-leaf was used as a male when crossed with the *as* marker and as a female when crossed with *y*. The progeny from the two crosses were classified in both F_1 and F_2. The rough-leaf phenotype was transmitted maternally and was present in F_1 and F_2 only when rough-leaf females were crossed with *y*/*y* males. If the rough-leaf phenotype was contaminated with some recessive nuclear mutations they would have been identified in F_2 when the rough-leaf was crossed as a male parent.

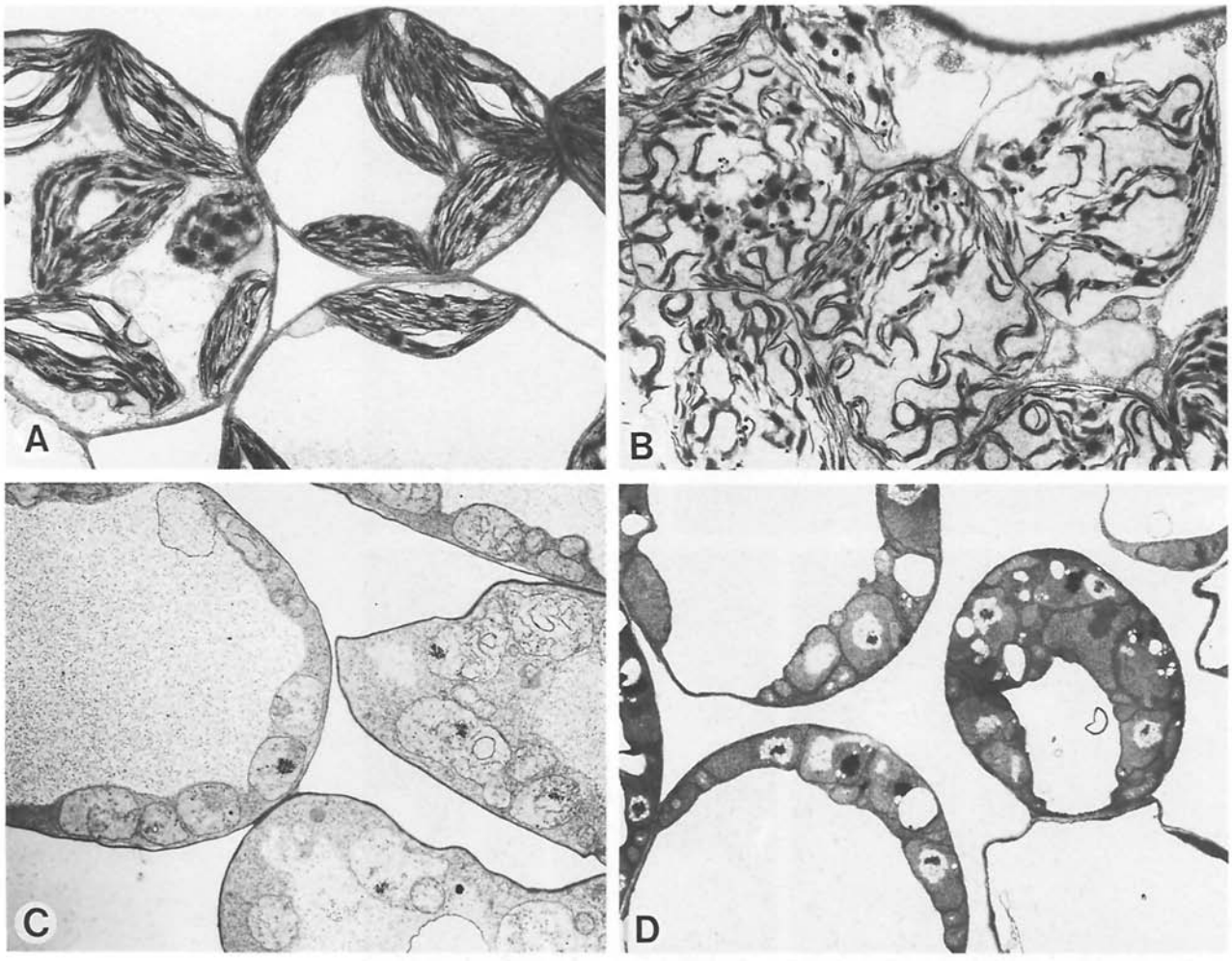

Fig. 4 A–D. Transmission electron microscopy of wild-type and *chm1*-free apparent homoplastidic mutants. **A** Normal wild-type plastids showing normal stacking of the lamellae into grana. Starch granules are accumulated. Enlarged 9,000 ×. **B** Apparent homoplastidic mutant RL32 showing identical mutant plastids with curved and even ring-shaped lamellae in the same cell. Enlarged 9,500 ×. Note that the size of the rough-leaf plastids are larger than the normal wild-type plastids. **C** Apparent homoplastidic yellow mutant showing cells carrying one type of plastid with very few lamellae scattered in the stroma in an unorganized fashion. Enlarged 5,400 ×. **D** Apparent homoplastidic white mutant showing cells carrying one type of plastid with no internal membrane structure. Enlarged 9,000 ×

The rough-leaf phenotype is not perfectly expressed at the cotyledonous stage but at later stages of development 100% of the population expressed it. All of the 11 *chm1*-free rough-leaf families displayed reduced fertility, expressed as low seed set per fruit, reduced total pollen per flower, and elevated levels of defective pollen (data not shown).

Description of the color-variegated mutants

Mutator-free famlies 1j, 4g and 5a were planted in E-medium in test tubes to select the most variegated plants. These were then used to initiate tissue culture for sorting out the mutant plastids and eventually obtaining plants homoplastidic, or apparently homoplastidic, for a single type of plastid. Progeny of the color-variegated *chm1*-free families, 1j, 4g and 5a, were grown on E-medium to ensure the survival of the very-pale mutants (Fig. 3B–D). From the mutator-free families all pale, apparently homoplastidic, plants were obtained (Fig. 3C). The solid pale-leaves were cloned in callus cultures. The medium used for callus culture was Murashige-Skoog supplemented with 0.00045 mM 2,4D and 0.001 mM kinetin, pH 5.8. From progeny of family 1j (mutator-free) a plant was regenerated from a solid pale-leaf giving rise to a solid-color plant (Fig. 3D). Plants regenerated from solid pale-color leaves obtained from progeny of families carrying the mutator had green spots indicating the presence and activity of *chm1* (Fig. 3D). For regeneration, young leaves with their petioles were excised from aseptically grown plants and cultured on Murashige-Skoog medium supplemented with 0.002 mM NAA and

Table 1. Verification of the absence of the mutator from the rough-leaf and highly color-variegated selected lines. Each line was crossed with a female tester (*as*/*AS*, *CHM1GL1*/*chm1gl1*). Only the progenies of crosses involving highly color-variegated families were planted on E-medium to assure the survival of all mutants

Line	S[a]	V[b]	NV[c]	NV:V
RL[d]				
24	79	19	60	3.2:1
27	72	16	56	3.5:1
30	80	24	56	2.3:1
32	98	0	98	4.0:0
36	77	22	55	2.5:1
42	68	0	68	4.0:0
45	85	31	54	1.7:1
48	85	0	85	4.0:0
49	81	20	61	3.1:1
50	76	18	58	3.2:1
51	86	0	86	4.0:0
54	88	20	68	3.4:1
55	82	0	82	4.0:0
57	70	19	51	2.7:1
58	76	0	76	4.0:0
60	116	24	92	3.8:1
63	82	0	82	4.0:0
66	91	22	69	3.1:0
68	71	0	71	4.0:0
69	74	0	74	4.0:0
70	86	0	86	4.0:0
71	77	0	77	4.0:0
89	75	16	59	3.7:1
CV[e]				
1j	64	0	64	4.0:0
4a	78	18	60	3.3:1
4g	72	0	72	4.0:0
5a	80	0	80	4.0:0
5m	76	21	55	2.6:1
10k	92	43	49	1.1:1

[a] Survivors
[b] Variegated
[c] Non-variegated
[d] Rough-leaf
[e] Color-variegated (sectorial)

0.005 mM BAP. Sub-culturing was done on the same medium every 4 weeks.

Ultrastructure of the homoplastidic mutants

Rédei and Plurad (1973) have described the different changes that occur in the defective plastid types induced by *chm1* in homozygous *chm1* plants. Here a transmission electron microscopy study was carried out on the mature leaves of the different *chm1*-free mutant families in order to strengthen the evidence that each mutant family is apparently homoplastidic for a single type of mutant plastid. RL32 became apparently homoplastidic for a rough-type plastid that has curved lamellae (Fig. 4B). RL63 looks similar but the plastids are more

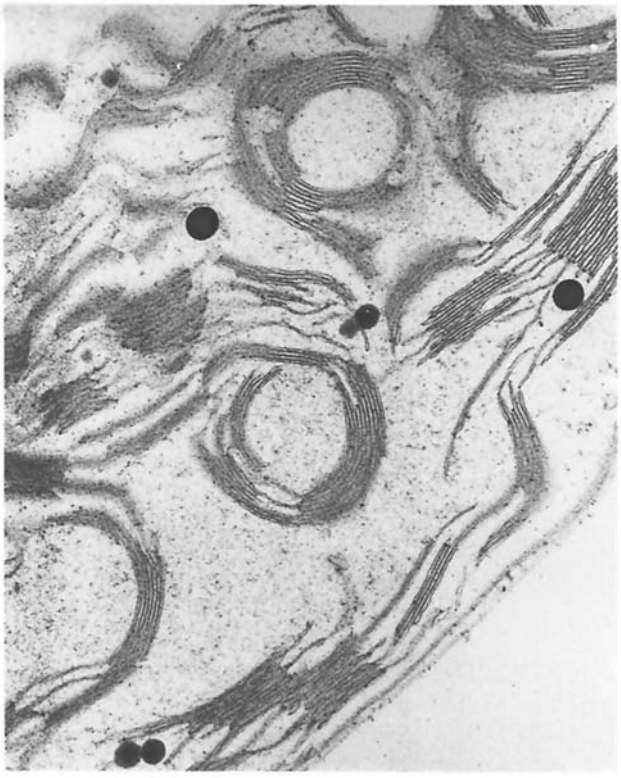

Fig. 5. The ultrastructure of RL63 plastid. Note the curvature of the lamellae, almost forming a circle or ring shape. Enlarged 54,000 ×

deformed than RL32. The curvature of the lamellae are very sharp and some of the thylakoids are circular in shape (Fig. 5). Some yellow plants were apparently homoplastidic for a plastid that carried very few lamellae scattered in the stroma (Fig. 4C). White plants became apparently homoplastidic for a mutant plastid lacking internal membrane structure (Fig. 4D).

Discussion

The nuclear gene *chm1* of *A. thaliana* is one of the most powerful plant organelle mutators. It enhances the spontaneous mutation rate of the plastid by about 10^6 fold (Rédei 1973). Its mutagenic effect on plastids appears to be stronger than the most effective physical and chemical mutagens known to induce plastome mutations (Kirk and Tilney-Bassett 1978). The penetrance and expression of *chm1* is 100%. The F_2 progeny of a cross *CHM1*/*CHM1* × *chm1*/*chm1* shows about 25% variegated plants, which represent the homozygous *chm1* individuals (Rédei 1973). Homozygous *chm1* plants show different color variegations, including white, yellow and pale-green (Fig. 1A), as well as rough-leaf mutations (Fig. 3A). Electron microscopic studies revealed different types of mutant plastids per cell in the *chm1*/*chm1*

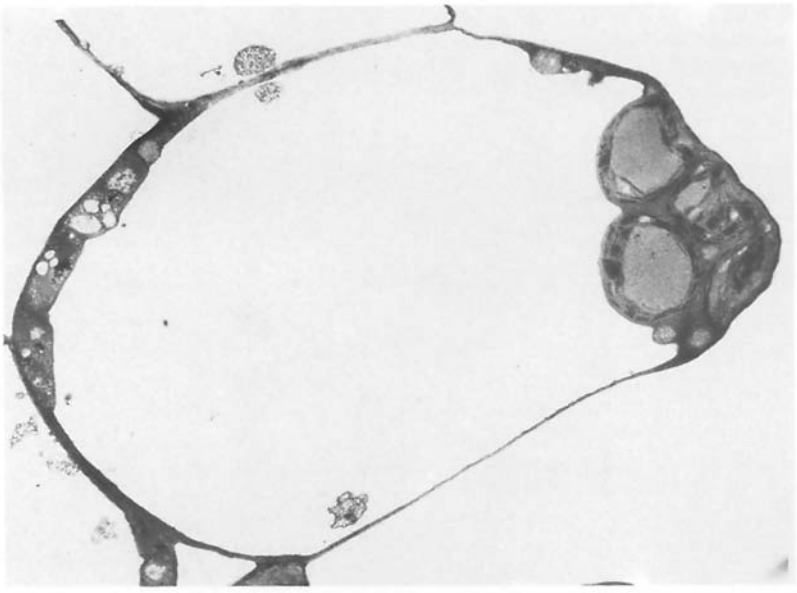

Fig. 6. A typical cell in the process of sorting-out displaying three mutant and four normal plastids at left and right, respectively. Enlarged 8,000 ×

plants (Fig. 1 B). Previous electron microscopic studies by Rédei and Plurad (1973) have shown that the mitochondria of *chm1/chm1* plants are normal in cells carrying aberrant plastids.

To study the mechanism by which the nuclear gene, *chm1*, causes plastid alterations, it is essential to obtain homoplastidic mutant lines with one type of mutant plastid in their cells. This is because molecular analysis cannot be carried out on a population that is constantly changing due to the high mutational effect of *chm1* on the plastids. We, therefore, decided to obtain homoplastidic mutant lines from *chm1/chm1* material using two genetic approaches. In the first approach, pulse-and-chase method (Fig. 2), the *chm1* gene was removed and then reintroduced again in a new genetic background to exert a pulse effect and induce a small number of plastid mutations. The mutant plastids were then chased by backcrossing twice to the wild-type. The second backcross eliminated *chm1* from 75% of the chromosomes (Fig. 2). In the second approach, the mixture of mutant plastids was maternally transmitted and *chm1* was blocked immediately by the wild-type allele. In the subsequent generations hairy variegated plants were selected. Because of the close linkage between *chm1* and the glabrous (*gl1*) gene [about 15 map units (Rédei 1973)] the majority of the hairy variegated plants were *chm1*-free (data not shown).

Color-variegated individuals and rough-leaf mutants were selected and the absence of *chm1* from their genetic background was confirmed by crossing them to a specially constructed female tester, *chm1/CHM1* (Table 1). That the *chm1*-free rough-leaf, yellow and white mutants isolated were apparently homoplastidic was confirmed by electron microscopic observations (Fig. 4 B–D). At the moment, these are the only nuclear-gene-induced homoplastidic lines (in the absence of the mutator) availabe.

The rapidity in obtaining homoplastidic leaf sectors of considerable size within a plant generation in the presence or absence of *chm1*, and the frequent microscopic observations showing mutant and wild-type plastids in groups at opposite sides of the cell (Fig. 6), led us to suggest a non-stochastic sorting-out of plastids in *chm1* mutants. We believe that sorting-out of mutant and normal plastids in *chm1*-free material is a non-random process and that the two daughter plastids remain in close vicinity within the viscous cytosol. Since electron microscopic observations frequently show plastids of one type grouped to one side of the cell (Fig. 6), the chances are greater that the two daughter plastids would go to one side of the cell wall rather than being separated during the process of cytokinesis. The multiple copies of the mitochondrial genes of yeast, and of the plastid genes of *Chlamydomonas*, showed similar tendencies of preferential sorting-out and genetic drift (Birky 1983; Birky et al. 1981). In *Medicago sativa*, two types of chloroplast DNA molecules, differing in an *Xba I* restriction site, were able to sort-out in one generation giving rise to plants with either one type or the other (Johnson and Palmer 1989; Rose et al. 1986). In a hybrid chimera of *Pelargonium*, the plastome cleavage pattern of one parent was found in the green tissue while the pattern of the other parent was found in the white tissue, indicating a quick non-random sorting-out of plastids within a plant generation (Metzlaff et al. 1982). Similar observations were made in somatic hybrids of *Datura* with seven Solanaceous species (Muller-Gensert and Schieder 1985), and of *Nicotiana* plastome-deficient mutants with wild-type (Gleba et al. 1985). Using our genetically-stable, *chm1*-free,

rough-leaf homoplastidic mutant lines we did not detect any differences between the banding pattern of their restricted purified chloroplast DNA and that of the wild-type (data not shown). Chui et al. (1990) found that the plastome of *Oenothera* plants which are homozygous for the mutator, *pm*, contained deletions at five discrete regions. Therefore, the mechanism of action by which the two mutators, *chm1* and *pm*, induce heritable plastid alterations is different. Actually any event(s) occurring in the chloroplast between replication, transcription and translation (or even at the level of assembly of some plastid polypeptides into protein complexes) may be the target of *chm1* action and could result in changes that are maternally inherited. In another study, mitochondrial, rather than chloroplast, DNA rearrangements appear to be associated with a color-variegated mutant of *Arabidopsis* that arose spontaneously and seems to be allelic to the *chm1* locus (J. M. Martínez-Zapater, personal communication). Whether the target of the nuclear-mutator gene, *chm1*, is the chloroplasts, the mitochondria, or both, we believe that our genetically-stable, mutator-free homoplastidic mutant lines, reported for the first time in this paper, are suitable genetic material to study the molecular mechanism(s) by which *chm1* induces heritable cytoplasmic changes.

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