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Multiple gene control of plastome-genome incompatibility and plastid DNA inheritance in interspecific hybrids of *Zantedeschia*

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Abstract From interspecific hybridisation in the genus *Zantedeschia*, we have previously produced albino, variegated and virescent hybrids. The inhibition of chloroplast development in these hybrids is due to plastome-genome incompatibility. The albino hybrids did not develop prolamellar bodies in their etioplasts in darkness and formed only distended membranes, but no grana in light. This indicates that the block to chloroplast development occurs before or during the development of etioplasts. In albino leaf sectors of variegated hybrids, chlorophyll and carotenoid contents were only 2–4% of those in green plants. The mRNA levels for five plastid-development-related genes were also severely reduced, but they were still readily detectable using Northern hybridisation techniques. In the green sectors of variegated hybrids, chlorophyll content and mRNA levels of the fives genes were slightly reduced. We have earlier shown that F_1 hybrids between *Z. aethiopica* and *Z. odorata* were albino when *Z. odorata* plastids were present but virescent when *Z. aethiopica* plastids were present. When the F_1 hybrids with *Z. aethiopica* plastids were backcrossed to *Z. odorata*, the progeny were albino, virescent or green. *Z. odorata* plastids were inherited only from maternal parents in the F_1 progenies but inherited from either maternal or paternal parents in the backcross. The increased compatibility with, and inheritance of *Z. odorata* plastids in the backcross suggests that multiple genes play a major role in the plastome-genome incompatibility. An increase in the number of genes from the parent contributing the plastids improves chloroplast development in the hybrids of the genus *Zantedeschia*.

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Introduction

Incompatibility between the plastome (plastid genome) and nuclear genome inhibits chloroplast development and chlorophyll formation. Such incompatibility (hybrid variegation) was first described in interspecific hybridisation in the genus *Oenothera* (Renner 1936; Stubbe 1960; Kirk and Tilney-Bassett 1978), then in *Trifolium* (Pandey et al. 1987; Przywara et al. 1989), *Pelargonium* (Metzlaff et al. 1982, Pohlheim 1986), *Impatiens* (Arisumi 1985) and *Zantedeschia* (Yao et al 1994b, 1995). When two species are hybridised, the incompatibility may be only in a one-cross direction (Pandey et al. 1987) or in both directions (Przywara et al. 1989; Yao et al. 1995). In the genus *Oenothera*, five types of plastomes and six types of genomes have been classified (Kirk and Tilney-Bassett 1978). Hybrids with different combinations of plastome and genome showed different leaf colours. In compatible plastome-genome combinations the leaves were green, whereas with incompatible combinations the leaves could be albino, pale-green or virescent (yellow young leaves becoming green with ageing). Plastome-genome incompatibility has also been observed in somatic hybrids between distantly related species. In these cases the incompatibility has been shown to result from a biased inheritance of plastid DNA (ptDNA) (Donaldson et al. 1993).

The control mechanisms for plastome-genome incompatibility are poorly understood, although this phenomenon is now more widely observed. In the albino sectors of *Trifolium repens* × *T. hybridum* hybrids, the internal membranes of chloroplast were absent (Pryzwara et al. 1989). A study with *Oenothera* found that genetically programmed chloroplast dedifferentiation occurred in an incompatible plastome-genome combination (Glick and Sears 1994); there was no report of plastid-development related gene expression in the albino hybrids. Studies us-

ing chlorophyll-deficient mutants demonstrated that the expression of nuclear and plastid genes encoding chloroplast proteins are co-ordinately regulated in plant cells. A mutation in a plastid gene or a nuclear gene encoding a plastid protein can inhibit the expression of many genes in the plastome and nuclear genome encoding chloroplast proteins (Leon and Arroyo 1998). Nuclear genes playing significant roles in chloroplast development have been cloned using T-DNA- (Reiter et al. 1994; Mandel et al. 1996) and transposon (Carol et al. 1999) tagged mutants. However, their controlling role in plastome-genome interaction has not been determined.

The genus *Zantedeschia* contains one evergreen species, *Z. aethiopica* (AE) and five winter-dormant species (Letty 1973). A summer-dormant species, *Z. odorata* (OD) falls in between AE and the winter-dormant species according to morphological analysis (Perry 1989) but is more closely related to *Z. aethiopica* based on cytogenetic data (Yao et al. 1994a). Interspecific hybridisations between species within the winter-dormant section are generally compatible with the production of mostly green hybrids, although some albino and virescent hybrids have been produced (New and Paris 1967). Hybridisation between OD and AE has produced only virescent and albino hybrids. Analysis of plastid ptDNA has demonstrated that the virescent hybrid plants contain the plastome of AE, whereas the albino hybrids have the plastome of OD. Because of biparental (pt)DNA inheritance, some hybrids containing plastids from both parents were variegated with green and white leaf sectors (Yao et al. 1994b, 1995). All hybrids produced from crosses between either OD or AE and winter-dormant species were albino. These albino plants only survived in tissue culture (Yao et al. 1995). Plastome-genome incompatibility in this genus is fatal between distantly related species.

The small number of species, biparental ptDNA inheritance and different types of incompatible plastome-genome interactions make *Zantedeschia* a useful genus for studying plastome-genome incompatibility. To understand the consequences of plastome-genome incompatibility, we investigated plastid ultrastructure, pigment composition and the expression of plastid-development-related genes in albino and variegated hybrids. We also have analysed F_2 and backcross progenies of AE × OD hybrids. This analysis was used to estimate the number of genes involved in the control of plastome-genome incompatibility in *Zantedeschia*.

Materials and methods

Plastid ultrastructure

In vitro-grown plants were used for plastid structure observation, as albino plants can not grow in the greenhouse. Variegated plants can grow in the greenhouse, but identification of green sectors and white sectors is difficult following dark treatment. *In vitro*-grown shoots of albino hybrids of AE × *Z. elliottiana* (Yao et al. 1995) and AE were subcultured on medium with 1.0 mg/l BA (N^6-benzyladenine) in the dark or light for 5 weeks. Leaf tissue was then collected from these cultures and cut into approximately 0.2-cm squares and fixed in 3% glutaraldehyde, 2% formaldehyde in 0.1*M* phosphate buffer, pH 7.2. The subsequent electron microscopy procedures were as described by Przywara *et al.* (1989).

Pigment determination

Plants of AE, OD and variegated F_1 hybrids of OD × AE were grown in the greenhouse. Three samples of 1 g of leaf tissue were taken from each parent species and from green sectors and albino sectors of the variegated hybrids. The leaf pigments were extracted with 80% acetone. Total chlorophyll was determined as described by Arnon (1949), and pigments were separated by HPLC (high-performance liquid chromatography) using the procedures of Gilmore and Yamamoto (1991) and the relative concentrations determined.

Interspecific hybridisation

The F_1 hybrids used in this study were produced from the AE × OD cross of our previous study (Yao et al. 1995). F_2 plants were produced by cross-pollinating F_1 siblings because in any particular inflorescence stigmas will have lost their receptivity by the time pollen is released. For backcrosses to AE or OD, inflorescences were emasculated by removal of the male zone of the spadix. For an individual inflorescence, hand-pollinations were repeated twice at 2-day intervals. Pollinated inflorescences were covered with paper bags tied with a fine wire. At 8–10 weeks after pollination, embryos were dissected. This showed that their development was retarded compared with embryos in the parental species. Embryo culture techniques (Yao et al. 1995) were used to recover F_2 and backcross plants. Albino plants were maintained in tissue culture, and virescent and green plants were transferred to a greenhouse approximately 4 weeks after germination. Plants were transferred to a field plot after 1 year.

Southern and Northern blot analysis

Total DNA was isolated from leaf tissue according to Rogers and Bendich (1988). Southern blots were prepared by digesting *Zantedeschia* DNA (5 µg per lane) with *Eco*RI, separating the DNA fragments on 0.7% agarose gels and transferring to Hybond-N^+ nylon membranes (Amersham, UK). For use as a probe, the 2.5-kb *Hind*III fragment from the ptDNA clone pZAC1 of AE (Yao et al. 1994) was purified from an agarose gel and labelled with [^{32}P]-dCPT using the Megaprimer DNA Labelling Kit (Amersham). The membranes were prehybridsed and hybridised in 0.5 *M* $NaPO_4$ buffer (pH 7.2) with 1 m*M* EDTA and 7% SDS at 65°C and washed using 0.4 × SSC and 0.2% SDS at 65 °C. Fuji X-ray film was used for autoradiography.

Total RNA was prepared from leaf tissue following the method of Cathala et al. (1983). Samples were collected from green leaves of AE and OD, virescent leaves of AE × OD F_1 hybrids, green sectors and white sectors of variegated hybrids of OD × AE and yellow and green leaves of backcross plants from OD × (AE × OD). All plants were grown in a greenhouse. Approximately 10 µg of each RNA sample was fractionated on formaldehyde-agarose gel and subsequently blotted on Hybond-N^+ membrane following the manufacturer's recommendation. The blots were sequentially hybridised in 0.43 *M* $NaPO_4$ buffer (pH 7.2) with 20 m*M* EDTA and 7% SDS at 60°C. Probes were made from plasmid inserts containing a specific gene and were labelled with [^{32}P]-dCTP using the Megaprimer DNA Labelling Kit (Amersham). After hybridisation, the blot was washed in 2 × SSC/0.2% SDS at 60°C. After each probing, the membranes were stripped in 0.1% SDS at 100°C for 1 h.

The nuclear DNA clones used for probes were the chlorophyll a/b binding protein gene (cab) of peach (Bassett et al. 1998) and the ribulose bisphosphate carboxylase small subunit (rbcS) of apple (Beuning 1994). Chloroplast DNA clones used for probes were

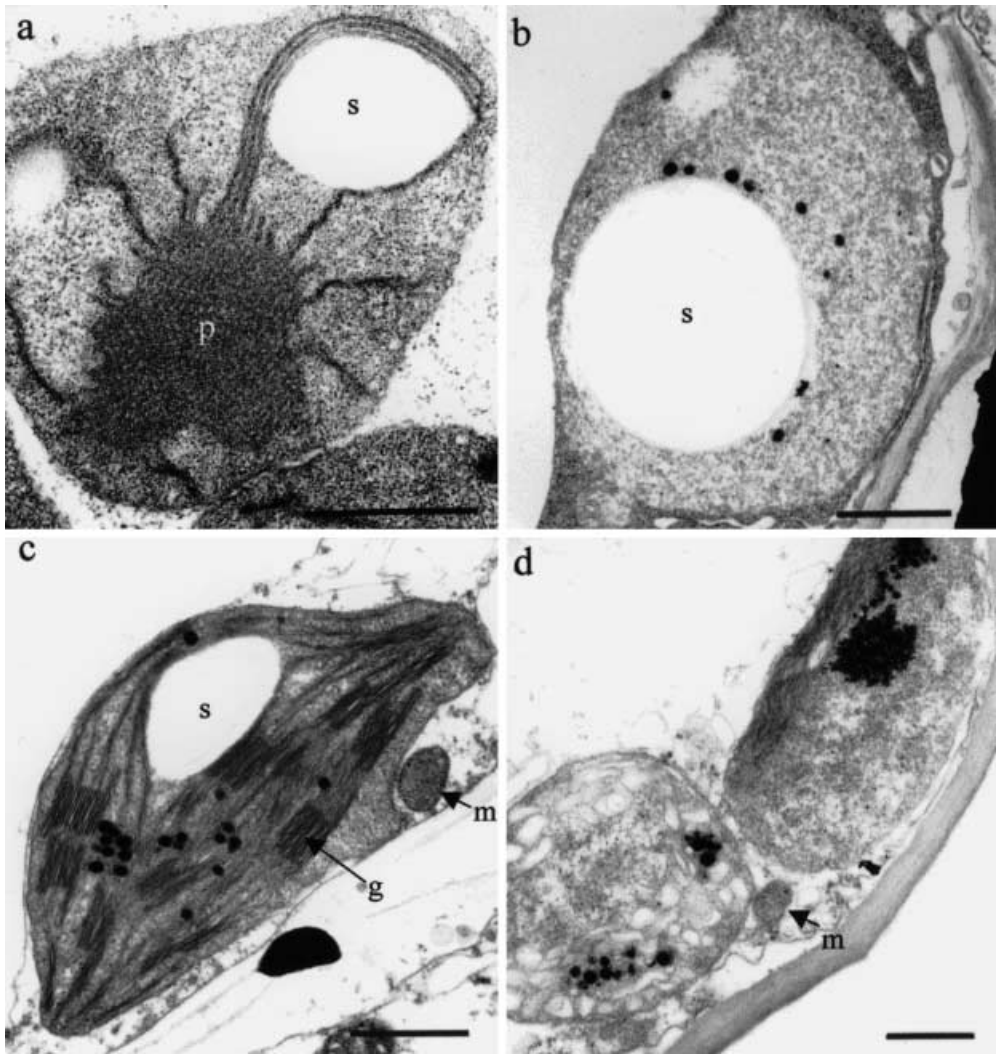

Fig. 1a–d Ultrastructure of plastids of *Z. aethiopica* (AE) and an albino hybrid grown under light and dark conditions. **a** AE grown in the dark, **b** an albino hybrid grown in the dark, **c** AE grown in the light, **d** an albino hybrid grown in the light. *g* Grana, *m* mitochondria, *p* prolamellar body, *s* starch grain, *Bar*: 1 µm

the ribulose bisphosphate carboxylase large subunit (rbcL) (Zurawski et al. 1981), the psbA gene encoding a membrane protein in PSII from spinach (Zurawski et al. 1982) and the 23s rRNA gene from mung bean (Palmer and Thompson 1981). The nuclear 18s rRNA gene of apple (Simon and Weeden 1992) was used as a probe to check the even loadings of RNA samples.

Results

Ultrastructure of plastid in albino hybrids grown under light and dark conditions

In leaves that developed in the dark, typical prolamellar bodies were observed in plastids of the parent AE (Fig. 1a) but not in plastids of albino hybrids (Fig. 1b). Large starch grains were present in the plastids of both green genotypes and albino hybrids. For AE leaves grown in the light, chloroplasts showed thylakoid membranes, grana and large starch grains (Fig. 1c), whereas the plastids in albino hybrids showed only abnormal membranes, no grana and small starch grains (Fig. 1d). The ultrastructure of the plastids was similar in all green samples examined and similar in all albino samples examined. The differences in plastid structure between albino hybrids and AE indicated that plastid development in leaves of the albino hybrids was abnormal under both light and dark conditions.

Table 1 Chlorophyll contents of parents and variegated hybrids

Plant material	Chlorophyll (µg)/g fresh weight
Z. odorata (OD)	1126 ± 25.7[a]
Z. aethiopica (AE)	1155 ± 45.6
OD × AE, green sector	1088 ± 51.9
OD × AE, albino sector	17 ± 1.2

[a] Mean ± SE (n = 3).

Green and yellow pigment contents in leaf tissues of F_1 hybrids

The green leaves of OD and AE, and the green leaf sectors of variegated hybrids contained approximately 1100 µg chlorophyll per gram of fresh weight (Table 1). However,

Table 2 F_2 and backcross plants produced using *Z. aethiopica* (AE) and *Z. odorata* (OD) as parents

Cross	Plants in tissue culture			Plants established in greenhouse		
	Numbers of embryos cultured	Numbers of embryos germinated	Numbers of albino plants	Total	Green	Virescent
(AE × OD) × (AE × OD)	568	58	18	28	2	26
(AE × OD) × OD	182	25	5	9	3	6
OD × (AE × OD)	162	98	53	5	2	3
AE × (AE × OD)	191	96	0	43	9	34
AE × OD	30	30	0	30	0	30

the albino sector of the variegated hybrids contained only 17 µg chlorophyll per gram of fresh weight, approximately 1.5% of that present in green leaf tissue.

The pigments in green leaf tissue of AE and albino leaf tissue of the variegated hybrids were separated using HPLC. Five major peaks were detected from the green leaf tissue. Pigment identification for peaks was achieved by comparison with published analyses made using HPLC. (Gilmore and Yamamoto 1991). These peaks were identified as violaxanthin, lutein, chlorophyll a, chlorophyll b and β-carotene. Corresponding peaks for all pigments were detected in the albino leaf sectors of the variegated hybrids, but the levels were reduced to between 1% and 6% of those in the green leaf tissue (data not shown).

Production of F_2 and backcross hybrids between *Z. aethiopica* and *Z. odorata*

The genetic control of plastome-genome incompatibility and ptDNA inheritance was analysed by producing F_2 and backcross progeny plants from F_1 hybrids of AE × OD (Table 2). Although embryo culture techniques were used to recover plants, a large number of embryos did not germinate, and many of those that germinated were albino. In tissue culture, it was difficult to distinguish virescent plants from green plants. These were identified only after transfer to the greenhouse.

We have previously described two types of virescence, young yellow leaves greening evenly or the yellow leaves with pale green veins (Yao et al. 1995). In this second type of virescence, greening of the leaf spreads from the veins through the leaf with time. The type of virescence was dependent on the genotype of AE used in the cross with OD. In this study, additional types of virescence were identified in both the F_2 plants and the backcrosses. In some, such as the backcross to AE, virescence was very mild. Some of the seedlings from the backcross to OD and some F_2 seedlings showed a more severe virescence, with young leaves almost white and very slow in greening.

Plastid DNA inheritance in F_2 and backcross hybrids

In the F_2 population of AE × OD, green, virescent and albino plants were produced, even though only virescent

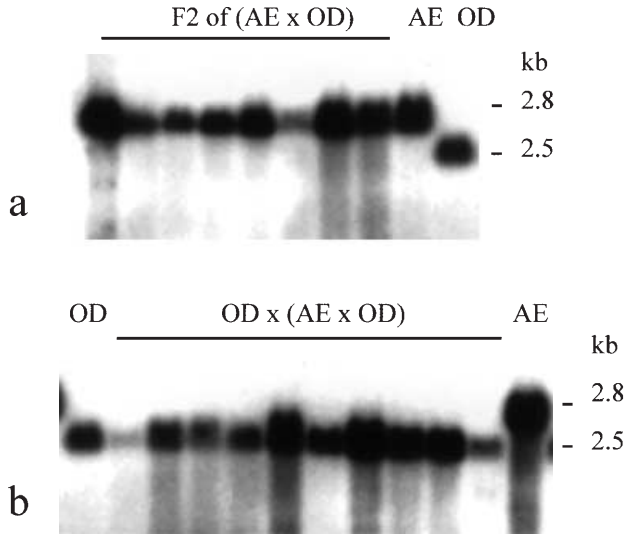

Fig. 2a, b Detection of *Z. aethiopica* (AE) ptDNA in the F_2 progeny plants of AE x *Z. odorata* (OD) (**a**) and of OD ptDNA in the backcross progeny plants of OD × (AE × OD) (**b**). *Eco*RI digested DNA was separated on a 0.7% agarose gel and transferred to a nylon membrane. The membrane was probed with ptDNA clone pZAC1

plants had been produced in the original F_1 population. The plastid type was determined in 10 of the F_2 progeny (4 albino, 2 green and 4 virescent) using Southern hybridisation with the ptDNA clone pZAC1 as a probe. This probe detects a 2.8-kb *Eco*RI fragment for AE and a 2.5-kb *Eco*RI fragment for OD. All F_2 plants regardless of their leaf colour showed the 2.8-kb *Eco*RI fragment only (Fig. 2a), indicating inheritance of AE ptDNA in the F_2 progeny plants. This result is consistent with our previous observation that only AE ptDNA was present in the F_1 hybrids of AE × OD (Yao et al. 1994). The variation in leaf colour was therefore due to differences in the number and/or dose of nuclear genes involved in plastome/genome incompatibility. It is likely that, as a result of nuclear gene segregation, albino plants contain more incompatible genes from OD while the green plants contain more compatible genes from AE.

The ptDNA type in backcross progenies to both parents was also investigated. Only OD ptDNA was detected in the plants from the backcross OD x (AE × OD) (Fig. 2b). In the backcross using OD as the paternal parent [(AE x OD) × OD], 8 of the 9 plants (green and vi-

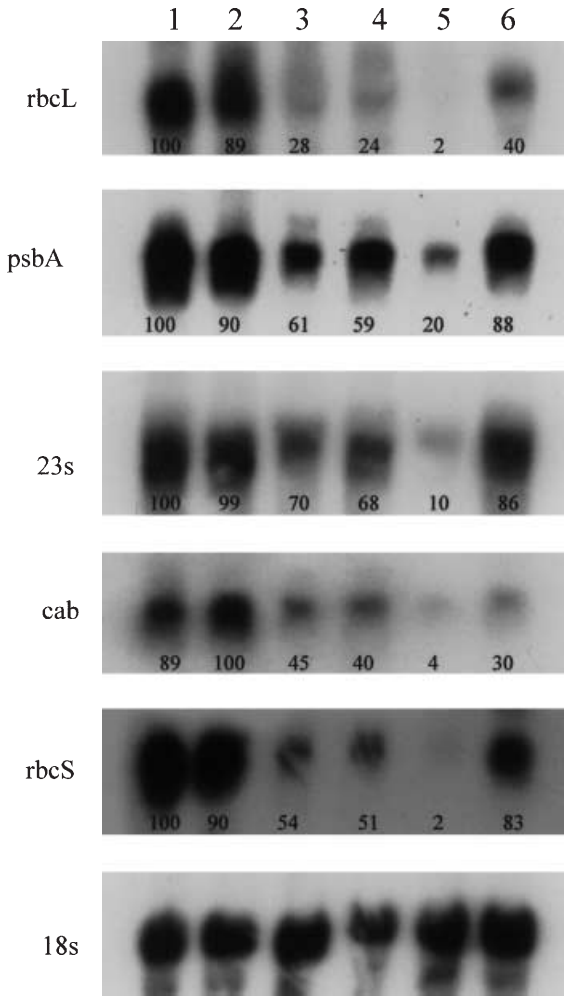

Fig. 3 Detection of mRNA for genes relating to chloroplast development in F_1 hybrids using Northern analysis. RNA samples were prepared from green leaves of *Z. aethiopica* (*AE*) (*1*), green leaves of *Z. odorata* (*OD*) (*2*), pale-green virescent leaves (*3*) and yellow virescent leaves with green veins (*4*) of AE × OD hybrids, albino leaf sectors (*5*) and green sectors (*6*) of variegated OD x AE hybrids. These samples were separated on a formaldehyde-agarose gel and transferred to a nylon membrane that was sequentially probed with six probes as indicated on the *left side* of the figure. The *numbers* in each panel show the relative level of the hybridisation signal as a percentage of the maximum, corrected to the 18s rRNA signal level

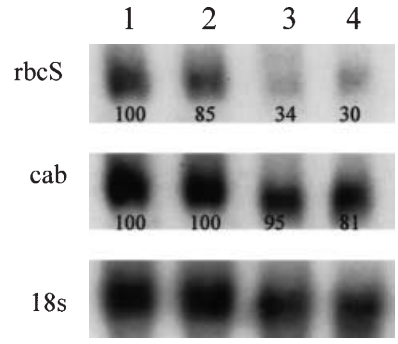

Fig. 4 Detection of mRNA for genes relating to chloroplast development in backcross hybrids using northern analysis. RNA samples were prepared from green leaves of *Z. aethiopica* (AE) (*1*), green leaves of *Z. odorata* (OD) (*2*), yellow virescent leaves (*3*) and green leaves developed from virescent tissue (*4*) of OD × (AE × OD) hybrids. These RNA samples were separated on formaldehyde-agarose gel and transferred to a nylon membrane that was sequentially probed with three probes as indicated on the *left side* of the figure. The *numbers* in each panel show the relative level of hybridisation signal as a percentage of the maximum, corrected to the 18s rRNA signal level

rescent) tested contained OD ptDNA and 1 plant with pale yellow leaves contained maternal AE ptDNA (data not shown). This result indicates a strong selection for OD plastids as the proportion of OD in the nuclear genome increased. For the backcross AE × (AE × OD), only AE ptDNA was detected in the 4 plants tested.

Expression of plastid-development-related genes in F_1 and backcross hybrids

Steady-state levels of RNA were examined for three plastid genes (rbcL, psbA and 23s rRNA) and two nuclear genes (cab and rbcS) using heterologous probes in Northern analyses. In general, RNA levels for all these five genes were reduced in the F_1 hybrids of AE and OD (Fig. 3). Although the albino leaf sector of variegated hybrids showed the most severe reduction in RNA level for all five genes, these tissues still contained a detectable RNA level for each gene. The green leaf sections of variegated hybrids were not visibly different from normal green leaves in colour, but the RNA levels for plastid-development-related genes were still significantly reduced. There was a greater reduction of RNA for rbcL and cab than for psbA. Two types of virescent leaves were examined, pale-green and yellow leaves with green veins. No differences in RNA levels were detected between these leaves although they both had reduced levels compared to that found in normal green leaves.

In the backcross OD × (AE × OD), young leaves of some plants showed a more severe white/yellow virescence than was found in the F_1 parent. However, mature leaves of these plants were green. Northern hybridisation showed that both the virescent and mature leaves of the backcross contained reduced amounts of RNA for rbcS and cab (Fig. 4). This result is similar to that observed for the F_1 hybrid plants.

Discussion

In darkness, proplastids normally develop into etioplasts, which are characterised by the presence of one or several prolamellar bodies (Ryberg et al. 1993). Following the transfer of dark-grown leaves into light, the regular structure of the prolamellar bodies gradually disappear, and the protothylakoids of the etioplastids are transformed into thylakoids and the membranes start to overlap, eventually forming typical grana. Plastids in *Zantedeschia* albino hy-

brids had no prolamellar body, no lamellar membranes in the dark nor grana in the light (Fig. 1). Pryzwara et al. (1989) noted the absence of internal chloroplast membranes in the albino hybrids of *Trifolium repens* × *T. hybridum*. A number of studies have reported on the ultrastructure of plastids in chlorophyll-deficient mutants. For example, prolamellar bodies were never observed in etioplasts of several maize mutants (Mascia and Robertson 1978) but were observed in etioplast-like plastids of the L1B1 mutant of mangrove tree (*Rhizophora mangle*) grown in the light (Corredor et al. 1995). Our study reports the ultrastructure of etioplasts in albino interspecific hybrids and shows the absence of prolamellar bodies in these hybrids. This result indicates for the first time that plastid development is blocked at an early stage. Large starch grains have been found in the etioplasts of albino hybrids grown on tissue culture medium containing sucrose (Fig. 1). The formation of starch in plastids of albino hybrids indicates that at least the enzymes for starch synthesis are active in the albino plastids.

Expression of plastid genes and nuclear genes encoding chloroplast proteins has been studied in albino mutants (Reiter et al; 1994, Mandel et al. 1996) and photobleaching plants (Mayfield and Taylor 1987). These studies concluded that the development of chloroplasts requires the co-ordinated expression of plastid genes and nuclear genes encoding chloroplast proteins (Leon and Arroyo 1998). We have found in the present study that the expression of plastid and nuclear genes encoding chloroplast proteins was reduced in virescent and albino hybrids (Figs. 3, 4). This result also indicates a similar type of co-ordinated gene expression.

It is worthwhile to note mRNA levels and chlorophyll contents in albino and virescent leaf tissues. First, although the mature green leaves in virescent hybrids appear to be as green as the mature leaves of the parents, they have reduced RNA levels for genes required for chloroplast development. This suggests that the incompatible interaction persists in mature leaves. Second, albino leaves still contain detectable levels of RNA for these genes. Although pigment levels in albino leaves were low, the chlorophyll contents were higher than in some albino mutants previously described (Mandel et al 1996). These data, together with the range of chlorophyll-deficiency phenotypes, suggest progressive plastome-genome compatibility in different *Zantedeschia* hybrids.

Leaf colour and ptDNA inheritance patterns in F_1, F_2 and backcross hybrids of AE and OD are summarised in Fig. 5. This scheme is based on the results presented in the present study and on previously published data (Yao et al. 1994). In the backcross OD × (AE × OD), virescent, green and albino plants have been produced and they all contain OD plastids. If only one gene were involved in controlling plastome-genome incompatibility, there should be only two nuclear genotypes in the backcross progeny, one the same as the parent OD and the another the same as the F_1 hybrids. Plants with the OD genotype should be green, but plants with the F_1 genotype and OD plastids should be albino (Yao et al. 1994).

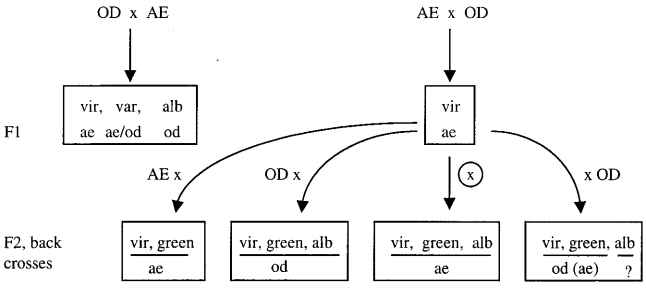

Fig. 5 Summary of leaf colour and ptDNA inheritance patterns in F_1, F_2 and backcross hybrids of *Z. aethiopica* (*AE*) and *Z. odorata* (*OD*) (based on the results presented in the present study and from Yao et al. 1994). *vir* Virescent, *var* variegated, *alb* albino, *ae* AE plastids, *od* OD plastids, *ae/od* green sector containing AE plastids and white sector containing OD plastids, *od (ae)* most plants containing OD plastids and only one plant found with AE plastids, *?* plastid type not tested

The production of virescent plants with OD plastids indicates that more than one gene is involved in controlling plastome-genome incompatibility.

The number of genes involved in the control of plastome-genome incompatibility has not yet been determined for genera such as *Pelargonium* (Pohlheim 1986) and *Oenothera* (Glick and Sears 1994). In our work, although embryo abortion and failure to germinate in vitro may lead to biased segregation data for genetic analysis, some conclusions can still be drawn. In the F_2 population of AE × OD, 2 green plants were identified among the 28 seedlings that were grown in a greenhouse (Table 2). The expected ratio among the F_2 progeny would be 1 green : 8 virescent : 7 albino for two genes and 1 green : 26 virescent : 37 albino for three genes. The result suggests that two or three genes were involved in regulating plastome-genome incompatibility in this cross. For the backcross AE × (AE × OD), segregation of 2 genes would account for the observed ratio of green to virescent progeny.

F_1 hybrids of AE × OD have only AE plastids and, for the reciprocal cross OD × AE, a small proportion of hybrids have OD plastids. F_1 hybrids with OD plastids were albino. The backcrosses OD × (AE × OD) and (AE × OD) × OD produced green, virescent and albino plants, and all but one contained OD ptDNA (Fig. 5). The production of virescent plants with OD plastids indicates that an increase in OD gene improves the development and inheritance of OD plastids in the backcrosses

In conclusion, our data suggest that a small number of genes play a major role in the plastome-genome incompatibility and, further, that an increase in the number of genes from the parent contributing the plastids improves chloroplast development in the hybrids between *Z. aethiopica* and *Z. odorata*.

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