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Olive: a key gene required for chlorophyll biosynthesis in *Antirrhinum majus*

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***Olive* (*oli*) is a recessive nuclear mutation of *Antirrhinum majus* which reduces the level of chlorophyll pigmentation and affects the ultrastructure of chloroplasts. The *oli*-605 allele carries a Tam3 transposon insertion which has allowed the locus to be isolated. The *oli* gene encodes a large putative protein of 153 kDa which shows homology to the products of two bacterial genes necessary for tetrapyrrole-metal chelation during the synthesis of bacteriochlorophyll or cobyrinic acid. We therefore propose that the product of the *oli* gene is necessary for a key step of chlorophyll synthesis: the chelation of magnesium by protoporphyrin IX. Somatic reversion of the *oli*-605 allele produces chimeric plants which indicate that the *oli* gene functions cell-autonomously. Expression of *oli* is restricted to photosynthetic cells and repressed by light, suggesting that it may be involved in regulating the rate of chlorophyll synthesis in green tissues.**

Key words: Antirrhinum majus/chlorophyll biosynthesis/protoporphyrin IX Mg-chelatase-methyltransferase/transposon-tagging

Introduction

The synthesis of chlorophyll pigments is essential for the light reactions of photosynthesis, yet little is known about its genetic control. Production of chlorophylls from their tetrapyrrole precursor, protoporphyrin IX (Proto IX) occurs in the chloroplast, and identification of intermediate metabolites has suggested a pathway by which Proto IX is converted into the functional pigments, chlorophyll *a* and *b* (reviewed by Rüdiger and Schoch, 1988; Beal and Weinstein, 1990). Although the activities of many enzymes involved in the pathway have been demonstrated in crude tissue extracts or intact organelles, very few have been purified and characterized in isolation. Therefore the enzymology of certain steps of the pathway, and the mechanisms which control chlorophyll synthesis as a whole, remain unclear. One key reaction, the chelation of magnesium into Proto IX, is of particular interest because it is the first step unique to chlorophyll production. There is indirect evidence to suggest that Mg-chelation limits chlorophyll synthesis (Griffiths, 1975; Fuesler *et al.*, 1981), but attempts to purify the chelatase enzyme have been unsuccessful (Walker and Weinstein, 1991a,b).

Significant progress has been made in the molecular genetic analysis of bacteriochlorophyll biosynthesis in two closely related species of purple bacteria, *Rhodobacter*, which are particularly amenable to genetic analysis and are capable of growth without photosynthesis (Kiley and Kaplan, 1987; Scolnik and Marrs, 1987). Chlorophyll-deficient mutants are therefore viable. This has made it possible to identify, by mutation, genes necessary for chlorophyll synthesis and many have been isolated and characterized (Yen and Marrs, 1976; Biel and Marrs, 1983; Taylor *et al.*, 1983; Zebo and Hearst, 1984; Young *et al.*, 1989; Coomber *et al.*, 1990; Yang and Bauer, 1990). Biochemical comparisons between mutant and wild-type strains have also allowed the metabolic lesions caused by different mutations to be identified, and as a result, some gene products have been tentatively assigned enzymatic functions (Biel and Marrs, 1983; Yang and Bauer, 1990). Although the bacteriochlorophylls of purple photosynthetic bacteria differ slightly from the chlorophylls found in higher plants, they are produced from the same precursors by a pathway which is conserved until its later stages (reviewed in Rüdiger and Schoch, 1988). Therefore *Rhodobacter* has the potential to provide a useful genetic model for plant chlorophyll synthesis.

Mutations which lead to a reduction of chlorophyll are also common in higher plants. Barley, for example, has at least 86 different nuclear genes which can be mutated to give chlorophyll-deficient phenotypes (von Wettstein *et al.*, 1971). Their use in dissection of the chlorophyll biosynthetic pathway has, however, been limited because they have pleiotropic effects on chloroplast structure and composition. These effects may have two possible explanations. First, mutations which disrupt any of the metabolic activities of the organelle can potentially lead to a loss of chlorophyll, along with other plastid components, as a result of photo-oxidative damage (reviewed by Somerville, 1986). Secondly, synthesis of chlorophyll and development of the organelle appear to be interdependent (von Wettstein *et al.*, 1971), and the accumulation of chlorophyll precursors which has been found in some mutants may therefore be the result of defects in plastid development (von Wettstein *et al.*, 1971; Gough, 1972; Newell and Rienits, 1975; Mascia, 1978). Because of these pleiotropic effects, it has not proved possible to distinguish clearly mutants with blocks in chlorophyll biosynthesis from those defective in other chloroplast activities, or to identify the majority of gene products by comparing mutant and wild-type plants biochemically.

An alternative approach, which can allow identification of gene products and functions, is the use of mutations as the basis for isolation and characterization of the genes themselves. A good illustration is the successful analysis of anthocyanin pigment synthesis in maize and *Antirrhinum*. The main advantage of these two species is that they contain mutagenic transposable elements (reviewed by Nevers *et al.*, 1986). Not only have transposon-induced mutations defined genes necessary for anthocyanin synthesis, but they have also

Fig. 1. A leaf of the *oli*-605 mutant showing clones of green revertant cells against a mutant yellow background. Darker green clones include palisade cells; those which appear lighter green are confined to the underlying spongy mesophyll.

allowed their isolation using the technique of transposon tagging (Fedoroff *et al.*, 1984; Martin *et al.*, 1985).

Here we describe the application of this strategy to the isolation and analysis of the *olive* (*oli*) gene which is necessary for chlorophyll pigmentation in *Antirrhinum majus*. The predicted product of *oli* shows homology to the products of two bacterial genes necessary for tetrapyrrole-metal chelation, suggesting that it is associated with activity of protoporphyrin IX Mg-chelatase, a key enzyme of chlorophyll biosynthesis. The level of *oli* mRNA is reduced by light, and we propose that the level of *oli* expression may influence the rate of chlorophyll synthesis.

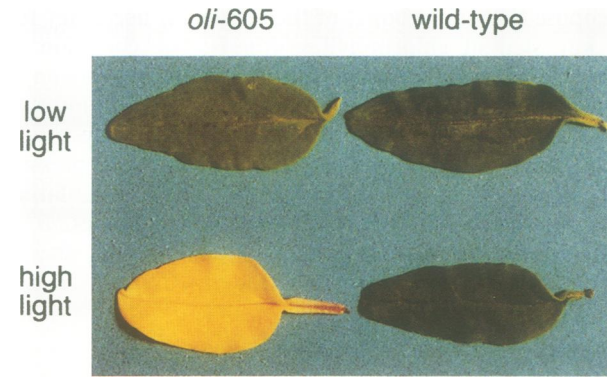

Fig. 2. The effect of light intensity on the *oli*-605 mutant phenotype. Leaves from the *oli*-605 mutant and its wild-type progenitor grown under low light (10.0 μmol/m^2/s) or higher light (50.0 μmol/m^2/s), at 25°C to prevent somatic reversion of the mutant allele.

Results

Description of the oli-605 mutant phenotype

Mutants homozygous for the *oli*-605 (*oli*-605) allele were obtained in the M_2 generation of a transposon mutagenesis programme (Luo *et al.*, 1991). They produce yellow leaves and stems with a high frequency of dark green spots and sectors (Figure 1). The frequency of the dark green spots is dependent upon temperature: plants grown at 25°C produced many fewer than those grown at 15°C. The colour of mutant tissue suggests that expression of *oli* is necessary for chlorophyll pigmentation, but not for the production of yellow carotenoid pigments.

Although mutant tissue appeared yellow when plants were grown in fields outside, the corresponding tissue of plants raised in the glasshouse in winter was light green. One explanation for this difference was that the phenotype of mutant tissue is sensitive to light intensity. To investigate this possibility, plants were grown at 25°C, to suppress green spotting, under five different intensities of illumination. Mutant plants grown under low light were found to contain more chlorophyll than those grown at higher light intensity (Figure 2). Under low light ($\leq$21.0 μmol/m^2/s) mutant tissue was able to accumulate about half as much chlorophyll as was present in wild-type leaves under the same conditions (Figure 3A). The chlorophyll *a*/*b* ratio in the mutant leaves was not significantly different from that in wild-type, indicating that the *oli*-605 mutation affects accumulation of both the *a* and *b* pigments to a comparable degree (Figure 3B).

The effects of the *oli*-605 mutation on the distribution and ultrastructure of chloroplasts was also examined. No difference was observed between wild-type leaf tissue (at either low or higher light intensity) and green mutant tissue grown under low light. All contained numerous chloroplasts in photosynthetic cells (Figure 4A), and these showed a well-developed system of thylakoids and granal stacks (Figure 4C). In contrast, the photosynthetic cells of yellow mutant leaves grown under more intense illumination contained fewer chloroplasts (Figure 4B). These chloroplasts were larger and less regular in shape. They also had fewer thylakoid membranes, which were occasionally paired but not stacked into grana, and they contained larger and more

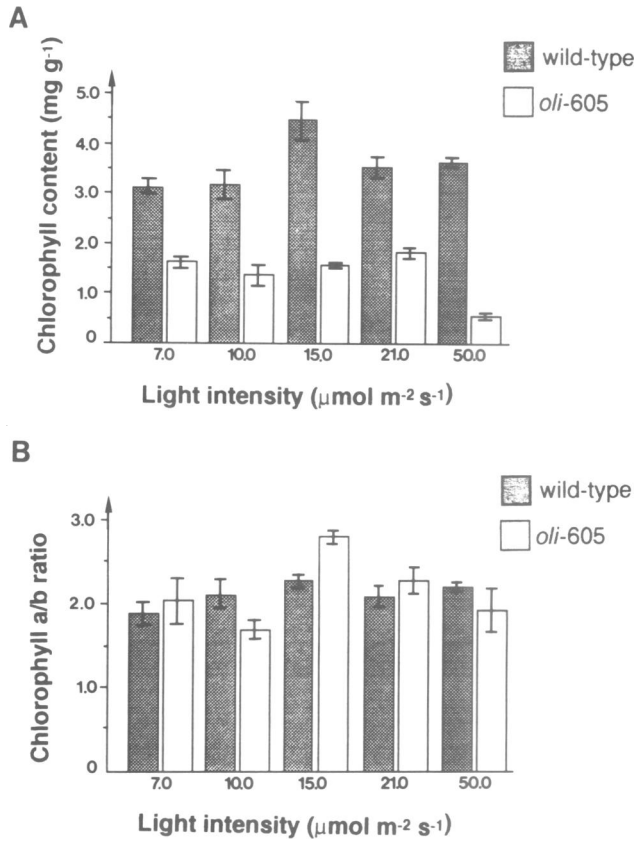

Fig. 3. The effect of light intensity on (**A**) chlorophyll content and (**B**) chlorophyll *a*/*b* ratio in wild-type and *oli*-605 mutant plants. Each value represents the mean of measurements made on at least 10 mature, fresh leaves from plants grown at 25°C. Bars indicate standard error values.

numerous lipid bodies (Figure 4D). The *oli*-605 mutation therefore appears to have effects on chloroplast number and ultrastructure, in addition to chlorophyll pigmentation.

Cloning the oli locus by transposon-tagging

One explanation for the appearance of dark green spots on a mutant background was that the *oli*-605 mutation was caused by a transposon insertion. Excision of the transposon during development of the plant could restore *oli* expression in clones of cells, accounting for the variegated phenotype. The sharpness of the boundary between mutant and wild-type clones also suggested that mutant cells could not be complemented by their wild-type neighbours.

Three further observations suggested that the *oli*-605 allele carried a transposon. First, the mutation was germinally unstable: about 19% of the progeny produced by self-pollination of *oli*-605/*oli*-605 homozygotes had a wild-type phenotype (Luo *et al.*, 1991). Secondly, the frequency of dark green clones produced by somatic excision was reduced ~1000-fold when mutant plants were grown at 25°C rather than at 15°C. Thirdly, a similar reduction in the frequency of dark green spots occurred in a genetic background homozygous for *Stabiliser*, a genetic inhibitor of transposition. This response to temperature and *Stabiliser* is characteristic of mutant alleles carrying the transposon Tam3 (Carpenter *et al.*, 1987). The involvement of Tam3 was therefore tested further by Southern hybridization. DNA from five *oli*-605/*oli*-605 homozygotes recovered in the M_2 generation of the transposon mutagenesis experiment, and from their wild-type progenitor, was digested with *Eco*RI, and probed with an internal fragment from a cloned copy of Tam3. Several hybridizing bands, corresponding to different copies of Tam3, were seen in DNA from all six

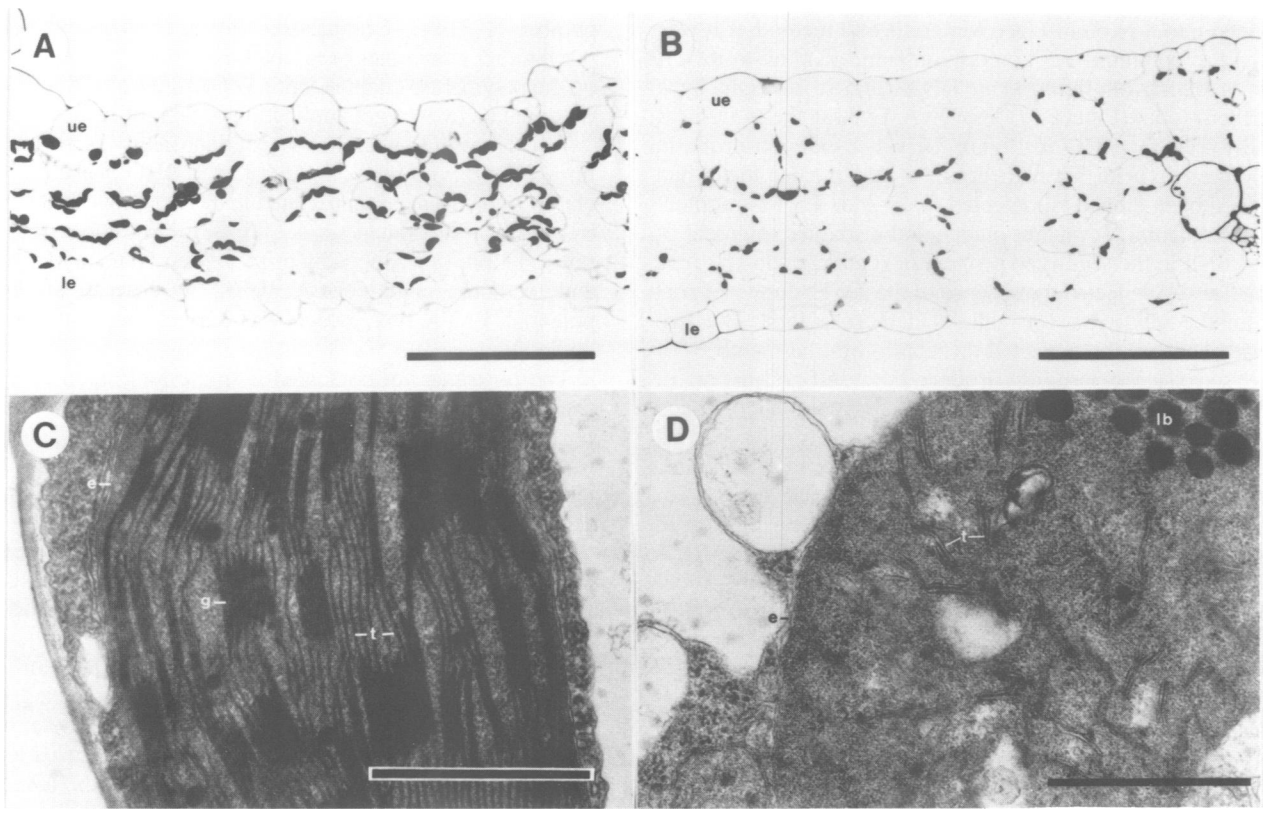

Fig. 4. The effect of the *oli*-605 mutation on chloroplast structure. The panels show transverse sections of (**A** and **C**) mature wild-type leaves and (**B** and **D**) yellow leaves from the *oli*-605 mutant grown in higher light. Scale bars represent 1 mm in the light micrographs, A and B, and 10 μm in the subsequent electron micrographs. ue, upper epidermis; le, lower epidermis; t, thylakoid; g, granum; e, chloroplast envelope; lb, lipid body.

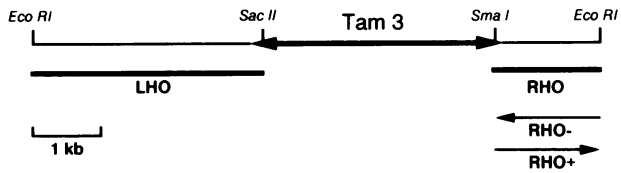

Fig. 5. Restriction map of the 8.2 kb *Eco*RI fragment from the *oli*-605 allele, and the probes derived from it. The broad arrow indicates Tam3; broad lines, double-stranded probes, and narrow arrows, single-stranded probes produced by primer-extension in the direction of the arrows. LHO is an *Eco*RI−*Sac*II fragment containing the sequence flanking the left-hand end of the Tam3 insertion; RHO is a *Sma*I−*Eco*RI fragment which includes the right-hand flanking sequence.

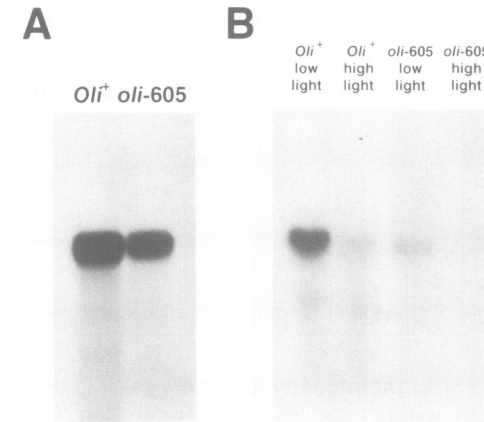

Fig. 6. Northern blots showing the effects of (**A**) the Tam3 insertion and (**B**) light intensity on *oli* expression. Total RNA from the leaves of *oli*-605 mutants and their wild-type progenitor was probed with a fragment of the *oli* cDNA. Plants for A were grown under glasshouse conditions, those for B, under either low or higher light (50.0 or 10.0 μmol/m^2/s).

plants, and some of these were present in the five mutants but absent from their wild-type progenitor (data not shown). If one of these bands was responsible for the *oli*-605 mutation, it should not only segregate with the mutant *oli*-605 allele, but its loss should also accompany reversion of *oli*-605 to wild-type. This was tested using four segregating families produced by self-pollination of four independent *Oli*$^+$/*oli*-605 revertants. Two DNA samples were prepared from each family: one from a pool of mutant plants, the second from a pool of their wild-type siblings. These were digested with *Eco*RI and probed with Tam3. A copy of Tam3 responsible for the *oli*-605 mutation was expected to produce a strongly hybridizing fragment in DNA from mutant plants, all of which were *oli*-605/*oli*-605 homozygotes. In contrast, the wild-type siblings were expected to yield a much weaker band of around one-third the intensity because these plants should have carried at least one revertant *Oli*$^+$ allele which would have lost the copy of Tam3. One band, with a size around 8.2 kb showed the expected distribution and was cloned into λ NM1149 (see Materials and methods). It was found to contain a 3.6 kb copy of Tam3 flanked to its left by ~3.2 kb of *Antirrhinum* DNA, and to its right by a further 1.4 kb (Figure 5). The DNA flanking the right-hand end of Tam3 (RHO in Figure 5), was used as a probe in Southern hybridization to confirm that the *oli* locus had indeed been cloned. It detected the 8.2 kb *Eco*RI fragment in DNA from M_2 mutant plants, and a smaller fragment of ~4.6 kb in the wild-type progenitor (data not shown). The smaller size reflected the absence of the 3.6 kb copy of Tam3 from the wild-type *Oli*$^+$ allele in these plants. The two bands showed the expected distribution in the members of the segregating families produced by self-pollination of *Oli*$^+$/*oli*-605 revertants: mutants had only the 8.2 kb band which contained Tam3, while their wild-type siblings carried both the 8.2 and 4.6 kb bands, the smaller of which was, as expected, about twice as intense as the larger.

In order to determine where in the *oli* gene Tam3 had inserted, Northern hybridization was carried out with RNA from mutant and wild-type plants. A single-stranded probe, produced from the right flanking sequence by primer-extension towards Tam3 (RHO−, in Figure 5), detected a large transcript of ~4.5 kb in RNA from wild-type leaves (Figure 6A). A transcript of the same size was also seen, at a lower abundance, in *oli*-605 mutants. Because RNA had been extracted from plants that had been grown at 25°C to prevent somatic reversion of the mutant allele, the observation of a transcript in the mutant suggested that the presence of Tam3 reduced, but did not abolish, expression from the *oli*-605 allele. Probes consisting of either the complementary strand of the right-hand flanking sequence (RHO+, in Figure 5), or of both strands of the left-hand flank (LHO in Figure 5), failed to detect a transcript in either mutant or wild-type RNA. These results indicated that Tam3 had inserted near the upstream end of the *oli* coding sequence.

Analysis of the structure of the oli gene

The sequence of the DNA flanking Tam3 in the *oli*-605 allele was determined. [The sequence data reported here have been deposited in the EMBL Data Library under the accession number X73144]. Comparison with that of its wild-type progenitor, which had been obtained from DNA amplified by the polymerase chain reaction (PCR), revealed that Tam3 was flanked by a target duplication of 8 bp of *oli* sequence.

Two large cDNA clones were identified by screening a library from wild-type leaves with the RHO probe, and 3′ cDNA sequences not represented in the clones were obtained by RACE (Frohman *et al.*, 1988). The start of *oli* transcription was mapped by primer extension (see Materials and methods), and located 30−32 bp downstream of a potential TATA box, which was part of the Tam3 target duplication.

The *oli* transcript contained a single long open reading frame, which began with a sequence fitting the consensus for translation start sites (Kozak, 1981) and had the potential to encode a large protein, OLI, of 1379 amino acids (Figure 7). The 26 N-terminal amino acids of OLI were relatively rich in Ser (27%, compared with 7% in the remainder of the protein), and relatively poor in Asp, Glu and Tyr, containing only one of these amino acids, Glu (at position 17). Such a composition is characteristic of chloroplast target peptides (von Heijne *et al.*, 1989), and suggested that OLI might have a chloroplast location.

Database comparisons revealed two bacterial proteins with significant homology to OLI. The more similar of these, with 39% identical amino acids, is the product of the *bchH* locus of the purple photosynthetic bacterium *Rhodobacter capsulatus* (Figure 7) (Bollivar and Bauer, 1992). *bchH* encodes an enzyme necessary for one of the first steps unique

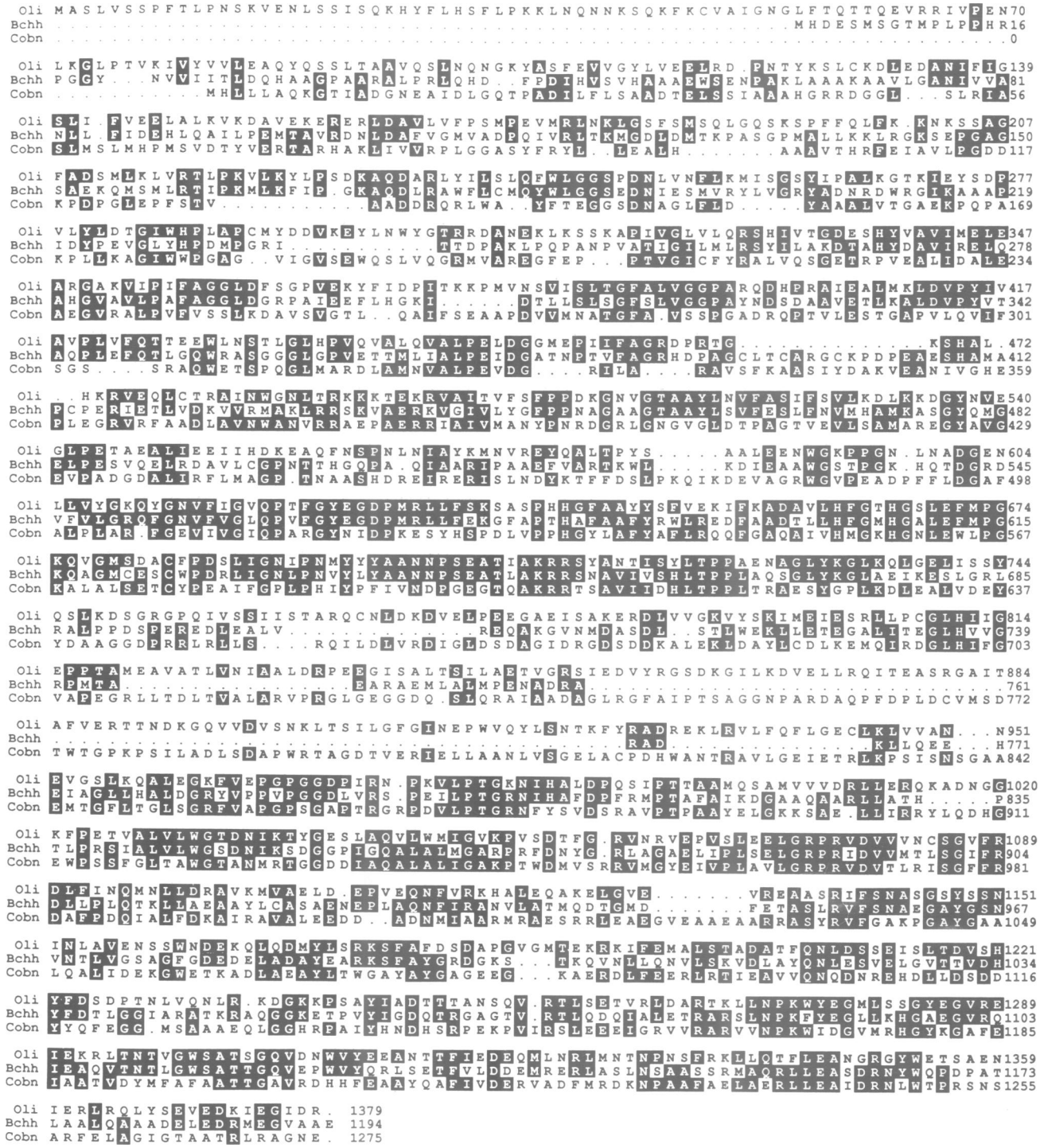

Fig. 7. Homology between OLI and the products of *bchH* and *cob*N. Amino acids are represented by the standard one-letter code. Identical amino acids are indicated by black boxes.

to the synthesis of bacteriochlorophyll. The second prokaryotic protein with homology to OLI was encoded by the *Pseudomonas denitrificans cobN* gene, which is necessary for synthesis of cobyrinic acid, a cyclic tetrapyrrole (containing cobalt) which is a precursor of cofactor B_{12} (Crouzet *et al.*, 1991). Although the similarity between OLI and the product of *cobN* was lower than that for *bchH* (29% identity), those regions of OLI which showed the greatest similarity to *cobN* were also the most similar to the *bchH* product (Figure 7).

Analysis of oli expression

Northern hybridization had revealed *oli* transcripts in wild-type leaves. The expression of *oli* in photosynthetic organs was further investigated by *in situ* hybridization. A fragment from the *oli* cDNA was subcloned, in both orientations, into a pBluescript vector. Complementary RNA probes labelled with digoxigenin were synthesized from these subclones using T7 RNA polymerase and hybridized with sections of wild-type and mutant leaves. In transverse sections of wild-type leaves, the antisense probe produced a strong signal

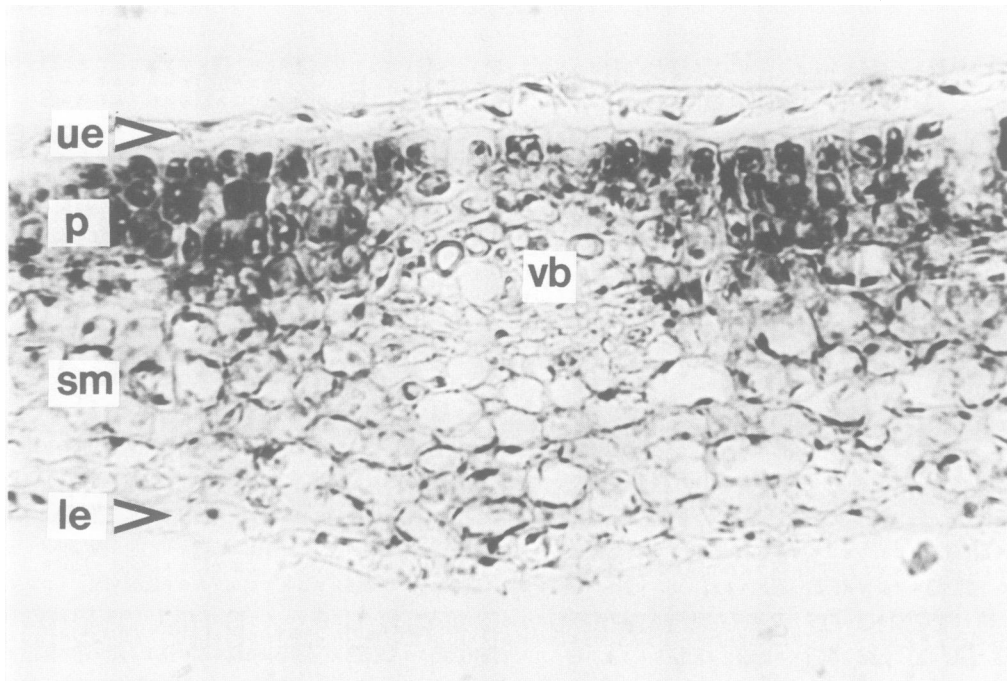

Fig. 8. Detection of *oli* RNA in wild-type leaf tissue. *oli* cDNA was hybridized to transverse sections of mature leaves and detected in a reaction producing a dark purple precipitate. ue and le denote upper and lower epidermis respectively; p, palisade mesophyll; sm, spongy mesophyll and vb, vascular bundle.

in the photosynthetic cells of the palisade and spongy mesophyll, but not in the cells of the epidermis and vascular tissue which are not photosynthetic (Figure 8). A similar pattern of hybridization was observed in mutant leaves, although the intensity of the signal was lower, as expected (data not shown). Control hybridization with the complementary, sense probe produced no significant signal (data not shown).

The phenotypic effects of the *oli*-605 mutation had been found to be less severe in plants which had been grown under low intensity light. Because the mutant *oli*-605 allele produces a transcript indistinguishable in size from that of the wild-type, one explanation for this response was that expression of the mutant allele was higher at lower light intensity. To investigate this possibility, RNA was extracted from the leaves of wild-type and mutant plants that had been grown at 25°C to suppress somatic reversion, under either low or higher light (15 and 50 μmol/m^2/s, respectively). This was used in Northern hybridization with a fragment of the *oli* cDNA as probe. Mutant tissue grown in low light contained significantly more *oli* transcript than that grown in higher light (Figure 6B). This indicated that expression of the *oli*-605 allele was inhibited by light. The same appeared true of the wild-type *Oli*$^+$ allele, because wild-type plants contained higher levels of transcript when grown in low light. The presence of Tam3 in the *oli*-605 allele therefore appears to reduce the level of *oli* transcript, but not to alter the response to light shown by the wild-type gene.

The speed of the response of *oli* expression to light was investigated using wild-type plants growing in a 12 h day/12 h night cycle. RNA was extracted from young leaves (harvested over the course of 24 h) and probed with *oli* cDNA. The abundance of *oli* transcript declined steadily during the light period; increased rapidly to a high level within 1 h of the return to darkness and remained at this high level throughout the night (Figure 9). In order to confirm that the response of *oli* expression was attributable to differences in illumination, the same Northern blot was probed with a cDNA clone of the light-induced *petH* gene from pea, which encodes ferredoxin NADP$^+$-reductase (FNR), a component of the chloroplast electron-transport chain (Newman and Gray, 1988). As expected, the level of FNR transcript showed the opposite response to light. It increased during the day and declined in darkness, although its response was slower than that of *oli*.

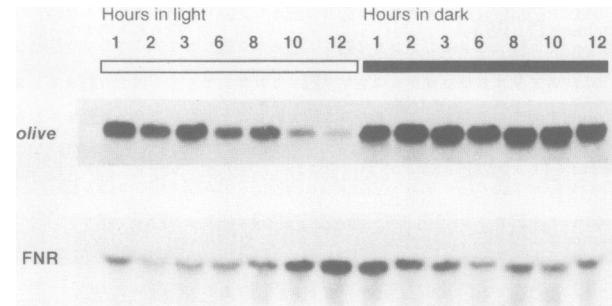

Fig. 9. Northern hybridization showing the response of *oli* and FNR gene expression to light. RNA was extracted from green wild-type tissue harvested at different times in a 12 h light/12 h dark cycle, and hybridized with probes from either the *oli* cDNA or the pea FNR cDNA.

Discussion

Biochemical role of OLI

We have shown that the *oli*-605 allele, which conditions a chlorophyll-deficient phenotype, carries the transposon Tam3, and have isolated the *oli* locus using Tam3 as a probe. The locus has the potential to encode a large protein, OLI,

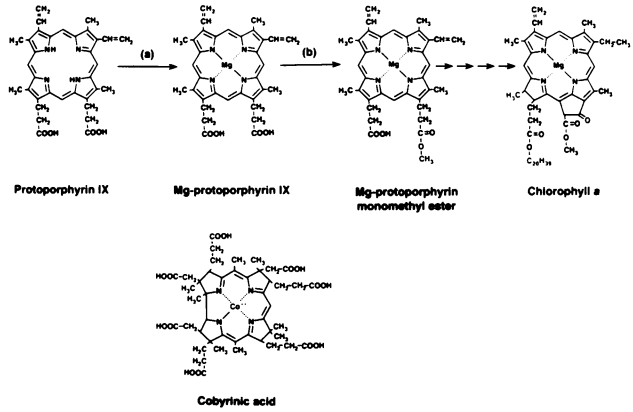

Fig. 10. Early steps in the chlorophyll biosynthetic pathway and the structure of cobyrinic acid. Only the Mg chelation (a) and methylation (b) reactions are shown in detail. Activity of Proto IX Mg-chelatase, responsible for reaction a has been detected in intact chloroplasts and *Rhodobacter* cells (Walker and Weinstein, 1991a; Gorchein, 1972). The methyltransferase, catalysing reaction b, has been partially purified from higher plants and *Rhodobacter* and found to use Mg-Proto IX, rather than the unchelated Proto IX, as substrate (Hinchigeri *et al.*, 1981, 1984). Therefore, although reactions a and b have not been resolved genetically, they can be distinguished biochemically (see discussion in text).

of 1379 amino acids. OLI shows extensive sequence homology to the product of the *bchH* gene of the purple photosynthetic bacterium, *Rhodobacter capsulatus* (Bollivar and Bauer, 1992), necessary for the production of bacteriochlorophyll (Biel and Marrs, 1983). The homology between the products of *oli* and *bchH*, together with the similarity of the mutant phenotypes, strongly suggest that the two genes have the same role in chlorophyll biosynthesis. Mutations in the *bchH* genes of both *R.capsulatus* and *R.sphaeroides* appear to block conversion of protoporphyrin IX (Proto IX) to Mg-Proto IX monomethyl ester (MPME) at the start of the bacteriochlorophyll biosynthetic pathway (Figure 10) (Biel and Marrs, 1983; Taylor *et al.*, 1983). This conversion can be resolved into two steps: chelation of Mg to give Mg-Proto IX (reaction 'a' in Figure 10), followed by methylation (reaction 'b' in Figure 10). Although these steps can be distinguished biochemically, they have not been separated genetically. In addition to *bchH*, mutations in three other genes, *bchD*, *I* and *K*, block the conversion of Proto IX to MPME (Biel and Marrs, 1983; Taylor *et al.*, 1983; Zebo and Hearst, 1984; Young *et al.*, 1989; Coomber *et al.*, 1990; Yang and Bauer, 1990). All these mutations cause the accumulation of Proto IX, suggesting that they all affect the first step (a) in the pathway. However, some may affect step b, if the Mg-Proto IX intermediate is unstable and breaks down to form Proto IX. A third possibility is that the two steps are catalysed by a single enzyme complex, with subunits encoded by the four genes, *bchD*, *H*, *I* and *K*.

We have also observed significant homology between the products of *bchH*, *oli* and the *cobN* gene of *Pseudomonas denitrificans* which provides further evidence for the function of *bchH* and *oli*. *cobN* is necessary for the insertion of cobalt (Co) into hydrogenobyrinic acid diamide to form cobyrinic acid, a precursor of cofactor B_{12} (Crouzet *et al.*, 1991). Cobyrinic acid shows obvious structural similarities to the porphyrin precursors of chlorophyll, being a cyclic tetrapyrrole containing a central metal atom, in this case Co (Figure 10). Unlike the insertion of Mg into Proto IX, there is no methylation step associated with Co chelation, suggesting that *cobN* encodes the Co-chelatase responsible for this step. In view of the homology with the product of *cobN*, it seems likely that the proteins encoded by *bchH* and *oli* are associated with the Mg chelation step of bacteriochlorophyll or chlorophyll synthesis.

The homology between the products of *oli*, *bchH* and *cobN* also implies that the three genes share a common ancestor. Many diverse bacterial taxa produce cofactor B_{12} and can therefore chelate Co, but relatively few are capable of chlorophyll synthesis. This suggests that the ancestral gene may have encoded Co-chelatase in a non-photosynthetic bacterium, and that this was the origin of the Mg-chelatase gene which made chlorophyll production, and thus photosynthesis, possible.

The activity of Proto IX Mg-chelatase has been demonstrated in intact chloroplasts, but is greatly reduced on disruption of the organelles (Castelfranco *et al.*, 1979; Pardo *et al.*, 1980; Richter and Rienits, 1980, 1982; Fuesler *et al.*, 1981; Walker and Weinstein, 1991a). A mixture of stromal and membrane fractions from the chloroplast is necessary for high levels of activity (Walker and Weinstein, 1991b), which suggests that chelation depends on at least one membrane-associated and one soluble protein. Although OLI has an N-terminus which may target it to the chloroplast, its amino acid sequence suggests that it is soluble. The product of the *Arabidopsis thaliana* locus, *cs*, which is located in the stroma (Koncz *et al.*, 1990), has been reported to show homology to that of *bchI* (Armstrong *et al.*, 1989; Orsat, 1992), and may therefore be another soluble subunit of the chelatase.

The sharp boundary between mutant and revertant clones in the *oli*-605 mutant implies that the intermediates of the chlorophyll pathway after the step catalysed by OLI, are unable to pass from wild-type cells to their mutant neighbours. This cell-autonomous behaviour of *oli* supports biochemical evidence which suggests that the Mg-porphyrin precursors of chlorophyll are restricted to the chloroplast (Granick, 1961).

Expression of oli

In situ hybridization shows that expression of *oli* is confined to photosynthetic cells and therefore reflects the distribution of chlorophyll. In green tissue of wild-type plants *oli* expression is inhibited by light, but increases rapidly in darkness. Although expression is reduced in *oli*-605 mutants, it remains responsive to light: mutant plants show higher levels of *oli* transcript when grown in dim light rather than bright light. This effect of light on *oli* expression could explain why mutant plants are able to accumulate higher levels of chlorophyll at lower light intensities. Light is also known to affect two other steps in chlorophyll biosynthesis. In etiolated seedlings of some angiosperms, it may increase production of δ-aminolevulinic acid (ALA), a precursor of both chlorophylls and haem (Harel and Klein, 1972), and it is involved directly in the reduction of protochlorophyllide by protochlorophyllide reductase, late in the pathway (Griffiths, 1974). Mg-chelation occurs between these two stages, and there is evidence to suggest that it is a rate-limiting reaction (Griffiths, 1975) which shows the sigmoidal response to substrate concentration characteristic of controlling steps in other metabolic pathways (Fuesler *et al.*, 1981). The repression of *oli* by light suggests that control

of its mRNA levels may be involved in regulating chlorophyll synthesis in green tissues. The same might also be true of protochlorophyllide reductase activity, later in the pathway, because expression of the gene which encodes this enzyme is inhibited by light in the etiolated seedlings of some species (Apel *et al.*, 1980).

The response of *oli* expression to light may be involved in adapting the amount of chlorophyll to the level of light energy available for photosynthesis. In many plants, a decrease in the intensity of illumination causes an increase in the concentration of chlorophyll and other components of the light reactions, presumably so that light can be harvested more efficiently (reviewed by Anderson, 1986). *Rhodobacter* responds in a similar way, producing more bacteriochlorophyll, and other photosynthetic components (Aagaard and Sistrom, 1972; Zucconi and Beatty, 1988), and an increase in the expression of several genes necessary for photosynthesis, including *bchH*, appears to be involved (Zhu and Hearst, 1986; Sganga *et al.*, 1992; Shimada *et al.*, 1992).

Effects of the oli-605 mutation on chloroplast structure

In addition to chlorophyll synthesis, the *oli*-605 mutation also affects chloroplast structure. One explanation for these pleiotropic effects is that they are the result of photo-oxidative damage, mediated by chlorophyll precursors accumulated in the *oli*-605 mutant. A build-up of the substrate for Mg-chelation, Proto IX, is known to occur in plants treated with diphenyl-ether herbicides, and appears to be responsible for the lethal effects of light on these plants (Becerril and Duke, 1989). However, mutants of maize and barley which are thought to be blocked at the Mg-chelation or methylation steps of the chlorophyll pathway do not accumulate detectable levels of Proto IX, unless fed ALA in the dark, nor do they have the necrotic phenotypes associated with later blocks in the pathway (Gough, 1972; Mascia, 1978), suggesting that photo-oxidation may not make a significant contribution to the effects of the *oli*-605 mutation on chloroplast structure. An alternative explanation is that the pleiotropic effects are a consequence of the reduced levels of chlorophyll in *oli*-605 mutants. The observation that chlorophyll-deficient mutants of barley show abnormal chloroplast ultrastructure has suggested that pigment synthesis is necessary for normal development of the organelle (von Wettstein *et al.*, 1971). The *oli*-605 mutant, which contains chloroplasts with thylakoids but not grana, provides the first confirmation that ultrastructural abnormalities can be the result, rather than the cause, of a block in chlorophyll biosynthesis. Chlorophyll also appears to be necessary either for the translation or stability of the nuclear-encoded apoproteins of the light-harvesting complexes (Herrin *et al.*, 1992), as well as the accumulation of chloroplast-encoded proteins (Apel, 1979; Terao and Katoh, 1989; Mullet *et al.*, 1991). Therefore chlorophyll production may have the potential to influence the wider composition of the organelle. A system of regulation in which the synthesis of chloroplast proteins is coupled to the level of chlorophyll production would have the obvious advantage of preventing accumulation of excess unbound pigment and hence photo-oxidative damage within the organelle.

Materials and methods

Plant material

Production of the *oli*-605 mutant from the wild-type line, JI.98, has been described by Luo *et al.* (1991). Plants used to investigate the effects of light on *oli* expression were clonally propagated as cuttings and maintained at 25°C under illumination from metal-halide lamps. Light intensity was varied by shading plants with muslin and measured as photon flux density in the wavelength range 400–700 nm. Shading had no significant effect on light quality or air temperature. Chlorophyll concentrations in at least 10 fully expanded leaves of each plant were estimated using the method given by Harborne (1973).

DNA and RNA analysis

The methods used for the extraction of DNA and RNA from *Antirrhinum*, and for Southern and Northern hybridizations have been described previously (Coen *et al.*, 1986; Coen and Carpenter, 1988). In order to clone the 8.2 kb *Eco*RI fragment from the *oli*-605 allele, a size fraction of *Eco*RI-digested DNA from a mutant plant was ligated into the *Eco*RI site of λ NM1149 (Murray, 1982), and phages carrying Tam3 sequences were identified by hybridization to an internal *Bst*EII–*Xba*I fragment from the transposon (Sommer *et al.*, 1985). Because the fraction of genomic DNA used to construct the library contained several different Tam3 bands, the sizes of the inserts carried by positive phages was checked by Southern hybridization, using the method described by Bradley *et al.* (1993). Samples of purified phage DNA were added to *oli*-605 genomic DNA, in a ratio of about four λ genomes to one of *Antirrhinum*. The mixtures were digested with *Eco*RI and used in Southern hybridization with the Tam3 probe. This allowed phages which produced a more intense 8.2 kb band to be identified. Two fragments containing *oli* sequences (LHO and RHO in Figure 5) were subcloned in pBluescript (Stratagene), and their sequences determined. The corresponding region of the wild-type *Oli*$^+$ allele was amplified from genomic DNA of line JI.98 by the PCR (Saiki *et al.*, 1988), using oligonucleotide primers representing sequences 241–259 bp upstream and 1163–1181 bp downstream of the Tam3 insertion site. Complementary single-stranded probes for Northern hybridization were made from the subclone of RHO by the prime-cut method of Hudson and Davidson (1984), and purified as described by Coen *et al.* (1986). The cDNA from the pea *petH* gene, used as a probe in Northern hybridization to confirm the light-responsiveness of *Antirrhinum* tissues, was kindly provided by Paul Dupree and John Gray, Department of Plant Sciences, University of Cambridge, UK.

A cDNA library in λ NM1149 was prepared from the poly(A)$^+$ RNA of young wild-type leaves using a Pharmacia cDNA synthesis kit. Two large clones were obtained by screening the library with the RHO probe, and remaining downstream sequences were obtained using the RACE technique of Frohman *et al.* (1988). To map the start site of *oli* transcription, an oligonucleotide complementary to a sequence represented in the upstream end of the longer cDNA clone, at a position 78–93 bp downstream of the Tam3 insertion site, was end-labelled with ^{32}P and used to prime reverse transcription of RNA from mutant and wild-type plants, using the methods described by Sambrook *et al.* (1989).

Nucleotide sequences of plasmid subclones, or deletion derivatives produced by exonuclease III digestion (Henikoff, 1987) were determined using Sequenase enzyme (USB), according to the supplier's instructions. Amino acid sequence comparisons were made with the UWGCG programmes 'Bestfit' and 'Pileup' (Devereux *et al.*, 1984), using penalty values of 3.0 for gaps and 0.1 for gap-length.

Microscopy

In order to detect *oli* mRNA *in situ*, a *Hin*dIII fragment from the *oli* cDNA was subcloned into pBluescript in both orientations. Complementary sense and antisense RNA probes labelled with digoxigenin were produced from these subclones, and hybridized with sections of plant tissue according to the method of Coen *et al.* (1990).

Material for examination of chloroplast structure was prepared as described by Roland (1978). Sections 1 μm thick were stained with toluidine blue for light microscopy. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate.

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