The EMBO Journal vol.15 no.16 pp.4194–4207, 1996

DAG, a gene required for chloroplast differentiation and palisade development in *Antirrhinum majus*

Manash Chatterjee[1], Stefano Sparvoli, Clive Edmunds[2], Paola Garosi, Kim Findlay and Cathie Martin[3]

John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH and [2]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

[1]Present address: Plant Biology Laboratory, The Salk Institute for Biological Studies, San Diego, CA 92186-5800, USA

[3]Corresponding author

We have identified a mutation at the *DAG* locus of *Antirrhinum majus* which blocks the development of chloroplasts to give white leaves with green revertant sectors. The green areas contain normal chloroplasts whereas the white areas have small plastids that resemble proplastids. The cotyledons of dark-grown dag mutant seedlings have plastids which also resemble proplastids. The palisade cells in the white areas of dag mutant leaves also lack their characteristic columnar shape. The *DAG* locus was cloned by transposon tagging: *DAG* encodes a novel protein with a predicted M_r of 26k, which is targeted to the plastids. Cleavage of its predicted transit peptide gives a mature protein of M_r 20k. Screening of databases and analysis of Southern blots gave evidence that DAG belongs to a protein family with homology to several proteins of unknown function from plants. Expression of *DAG* is required for expression of nuclear genes affecting the chloroplasts, such as *CAB* and *RBCS*, and also for expression of the plastidial gene *RPOB* encoding the plastidial RNA polymerase β subunit, indicating that it functions very early in chloroplast development.

Keywords: chloroplast development/*DAG*/palisade development/*RPOB* expression

Introduction

Specialized plastids such as chloroplasts, amyloplasts, leucoplasts and chromoplasts develop from proplastids present in the cells of plant meristems (Kirk and Tilney-Bassett, 1978). The metabolic specialization that a plastid undertakes depends on the tissue in which the cell is located. Functional specialization is accompanied by modification in plastid morphology. The developmental status of a plastid is not necessarily fixed: under appropriate conditions one plastid type may convert to another. Control of the development of specialized form and function in plastids is not well understood, although environmental factors such as light and developmental signals play important roles (Kirk and Tilney-Basset, 1978; Virgin and Egnéus., 1983; Mohr, 1984; Chory, 1992). The development of functional chloroplasts also requires coordinated interaction of plastid and nuclear genomes and, while the plastid genome is not known to play an essential role in the formation of plastids other than chloroplasts (such as amyloplasts or chromoplasts), there is evidence for expression of plastid-encoded genes in these types of plastid (Deng and Gruissem, 1987, 1988; de Pamphilis and Palmer, 1990; Morden *et al.*, 1991; Mullet, 1993).

Stages in chloroplast development have been defined by mutant analysis. Plastid division appears to be separate from the processes controlling chloroplast growth and differentiation, as evidenced by arc (accumulation and replication of chloroplasts) mutants (Pyke and Leech, 1992, 1994; Pyke *et al.*, 1994) which affect plastid proliferation but not their development. Many mutants have been described which inhibit chloroplast development and some have been demonstrated to affect specific stages in thylakoid assembly and production of the photosynthetic apparatus (Miles, 1994). The chloroplasts of these mutants are usually small and contain thylakoids but not stacked grana. Mutations that completely block chloroplast development would give rise to plastids that are very small, lack internal membranes and resemble proplastids. However, mutants of this type are relatively rare, perhaps because they are usually lethal. When mutations of such genes are somatically unstable, such as those caused by transposable elements, the instability may provide sufficient wild-type revertant cells to allow viability. Such mutations can be recognized relatively easily by their variegated leaf pigmentation. We have identified one such gene, *DAG*, from an unstable mutation in *Antirrhinum* caused by the transposon Tam3. The *DAG* gene is essential not only for chloroplast development from proplastids in the leaves of *Antirrhinum* but also for the formation of normal etioplasts in dark-grown cotyledons. Here we describe the phenotype of the *dag* mutation, the molecular isolation of the *DAG* gene and its effects on the expression of other genes involved in chloroplast development.

Results

Phenotype of the dag mutants

The dag mutant arose in a stock *Antirrhinum* line (JI:98) which carries an active copy of Tam3 inserted at the *NIV* locus (*niv^{rec}*:Tam3; Sommer *et al.*, 1985). In greenhouse-grown plants the mutation was first recognized by the white bases of the cotyledons. As the plants grew they produced pale green leaves with white/yellow tips containing green sectors (Figure 1A), suggesting that the mutation was caused by a transposon showing somatic excision in these areas. The growth of dag mutant plants was slow compared with wild-type siblings. When plants were grown in controlled environment cabinets at 15°C, the white mutant tissue was seen over the whole leaf and the somatic sectoring increased compared with plants

Fig. 1. The dag mutant phenotype. (**A**) Sibling plants from the original family (H170) that segregated for the *dag* mutation. The plant on the left is wild-type and the plant on the right shows the dag mutant phenotype; it is slow growing and the tips of its leaves are white with green sectors. (**B**) Leaves from wild-type (left) and dag mutant (right) plants grown at 15°C. The dag mutant leaves show a general background of white mutant tissue surrounding raised revertant sectors that give the leaf its irregular shape.

grown in the greenhouse (on average ~22°C). The increase in somatic reversion at lower temperatures is typical of Tam3-induced mutations (Harrison and Fincham, 1964; Carpenter *et al.*, 1987). With each successive leaf that developed at 15°C the mutant areas became more extensive and there was an increase in the frequency of somatic sectors. Interestingly, the mutant areas of dag leaves were thinner than leaves of wild-type plants grown at the same temperature (15°C) and the revertant green sectors were raised above the mutant tissue (Figure 1B). The flowers had sepals with green revertant sectors on a white background, although within the sepals the green tissue was not raised relative to the white tissue.

In an initial screening of the progeny from the dag mutant line grown in the greenhouse, no germinal revertants were observed among 60 plants, but when plants were grown at 15°C to promote transposition and flowers were self-pollinated at this temperature, germinal revertants were readily obtained among the progeny. The germinal reversion frequency was ~10% (36/343). Thus, a reduction in growing temperature from 22°C to 15°C significantly enhanced the production of revertant somatic sectors on leaves and full-green, germinal revertants amongst the progeny, supporting the view that the instability of *DAG* was enhanced by low temperature and suggesting that the *dag* mutation might be caused by Tam3.

Since dag mutant plants had white leaf sectors this phenotype might have resulted from a mutation in the carotenoid biosynthetic pathway, which can cause destruction of chlorophyll and chloroplasts through photoxidation (Bachmann *et al.*, 1967; Reiss *et al.*, 1983; Mayfield *et al.*, 1986). To check for this, dag mutant plants were grown in a controlled growth cabinet at 15°C, under both high light (184 μmol m^2 s) and low light (44 μmol m^2 s). Lines blocked in carotenoid biosynthesis usually become green under low light where photoxidative damage is avoided. No difference was observed in the phenotype of the dag mutant plants under these conditions, suggesting that the dag phenotype did not result from carotenoid deficiency.

Cellular and ultrastructural analysis of the leaves of dag plants

The effect of the *dag* mutation on chloroplast development was established by ultrastructural analysis of the white areas and the adjacent sectors of green tissue. In transverse sections of dag mutant leaves green regions were found to be confined to discrete areas (sectors) in the mesophyll layer (LII), suggesting that the green cells represented clonal descendents of a wild-type cell in which an excision had occurred (Figure 2). Furthermore, the influence of the *DAG* gene on chloroplast development was cell autonomous, since single green revertant cells occurred as islands among white mutant cells. The cells in the green sectors contained many well-developed chloroplasts, while the mutant white cells completely lacked developed chloroplasts. Indeed, plastids were difficult to identify in the white mutant cells since they were small and unpigmented (Figure 2).

An additional feature caused by the *dag* mutation was that the palisade cells in the white areas of the leaf failed to expand as fully as in the revertant areas, especially along their dorsi-ventral axes (Figure 2). Freeze–fracture scanning electron microscopy of dag mutant leaves grown

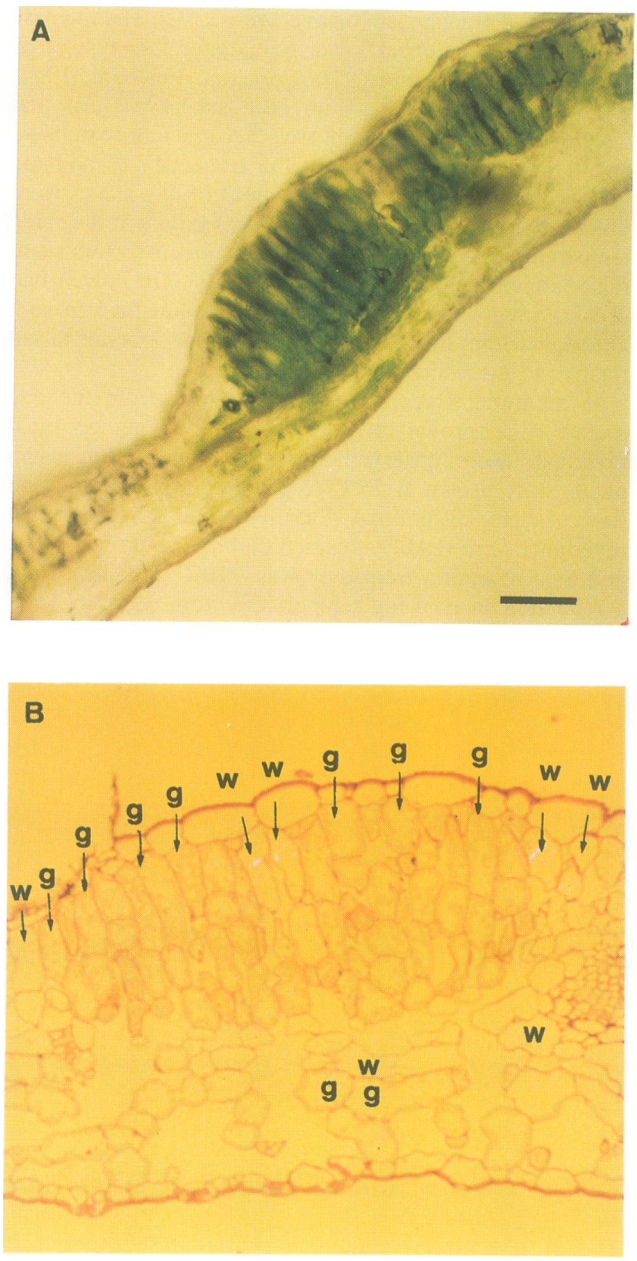

Fig. 2. Light micrographs of leaves from dag mutant plants grown at 15°C. (**A**) A hand section from a dag mutant leaf grown at 15°C. The green areas contain chloroplasts whereas the white areas lack chlorophyll. The expansion of the leaf palisade is normal in the green areas but restricted in the white areas, the cells failing to divide and expand to the extent shown by the revertant sectors. (**B**) Thin section (0.5 μm) stained with toluidine blue showing sectors of green cells (g) containing chloroplasts in the raised areas of the leaf. The white cells (w) fail to divide and expand to the same extent. Cells containing (g) and lacking (w) chloroplasts can also be seen in the spongy mesophyll. White cells lie adjacent to green cells and single green revertant cells were observed in a background of mutant cells, indicating that the function of *DAG* is cell-autonomous.

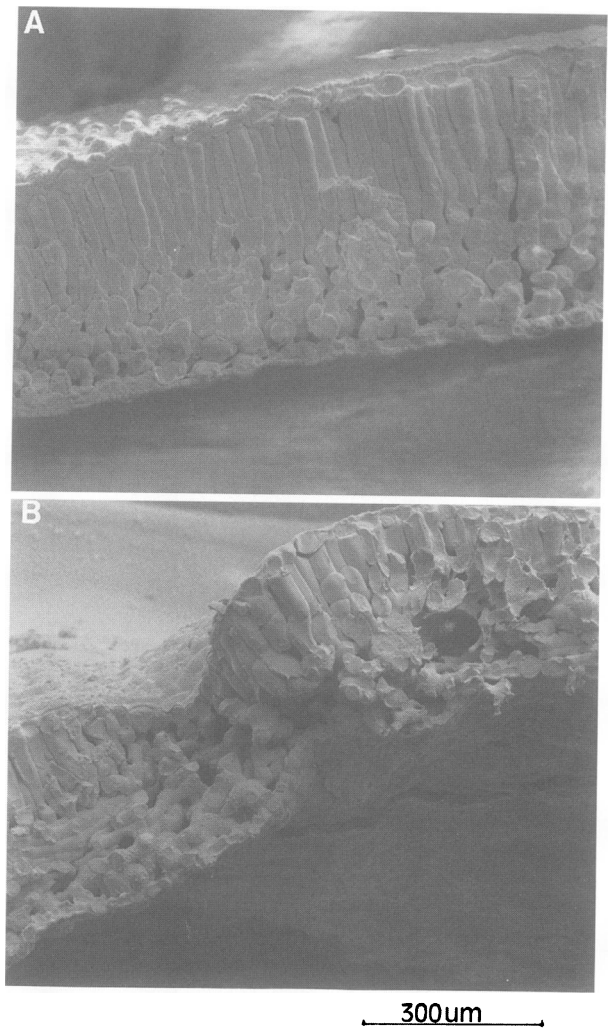

Fig. 3. Scanning electron micrographs of wild-type (**A**) and dag mutant (**B**) leaves from plants grown at 15°C. The cells in the thinner areas, which are devoid of wild-type revertant cells, lack the fully expanded columnar palisade and the cells are more spherical and more closely resemble the cells of the spongy mesophyll.

at 15°C revealed the abnormal growth of the palisade cells more clearly (Figure 3). Two to three layers of palisade cells in the green revertant sectors appeared to expand normally and adopt the column-like appearance characteristic of the palisade cells of wild-type leaves. In the white, mutant areas, although a discrete palisade layer was visible, it was usually only one cell thick; the cells failed to expand to their normal columnar shape and appeared more spherical. This abnormal form of the palisade layer was evident in both young and old dag mutant leaves. The failure of dag palisade cells to divide normally or to expand fully explains why the green revertant sectors appeared as raised bumps on the leaves, and conversely why the mutant areas of a dag leaf were thinner than in wild type. Where individual white cells were found among green cells, the white cells were more elongated than where many white cells were located together (Figure 2B). This may be because of physical constraints, promoting the growth of the mutant cells when they are surrounded by green cells, or because the effect of *DAG* on palisade cell expansion is not strictly cell autonomous, in contrast to its effect on plastid development.

In the sections examined with the electron microscope, there were seven or eight well-developed chloroplasts per cell in the green areas and five or six small defective plastids per cell in the white areas (Figure 4). Given the smaller size of the plastids in the white areas, this indicated that the mutation did not significantly affect plastid

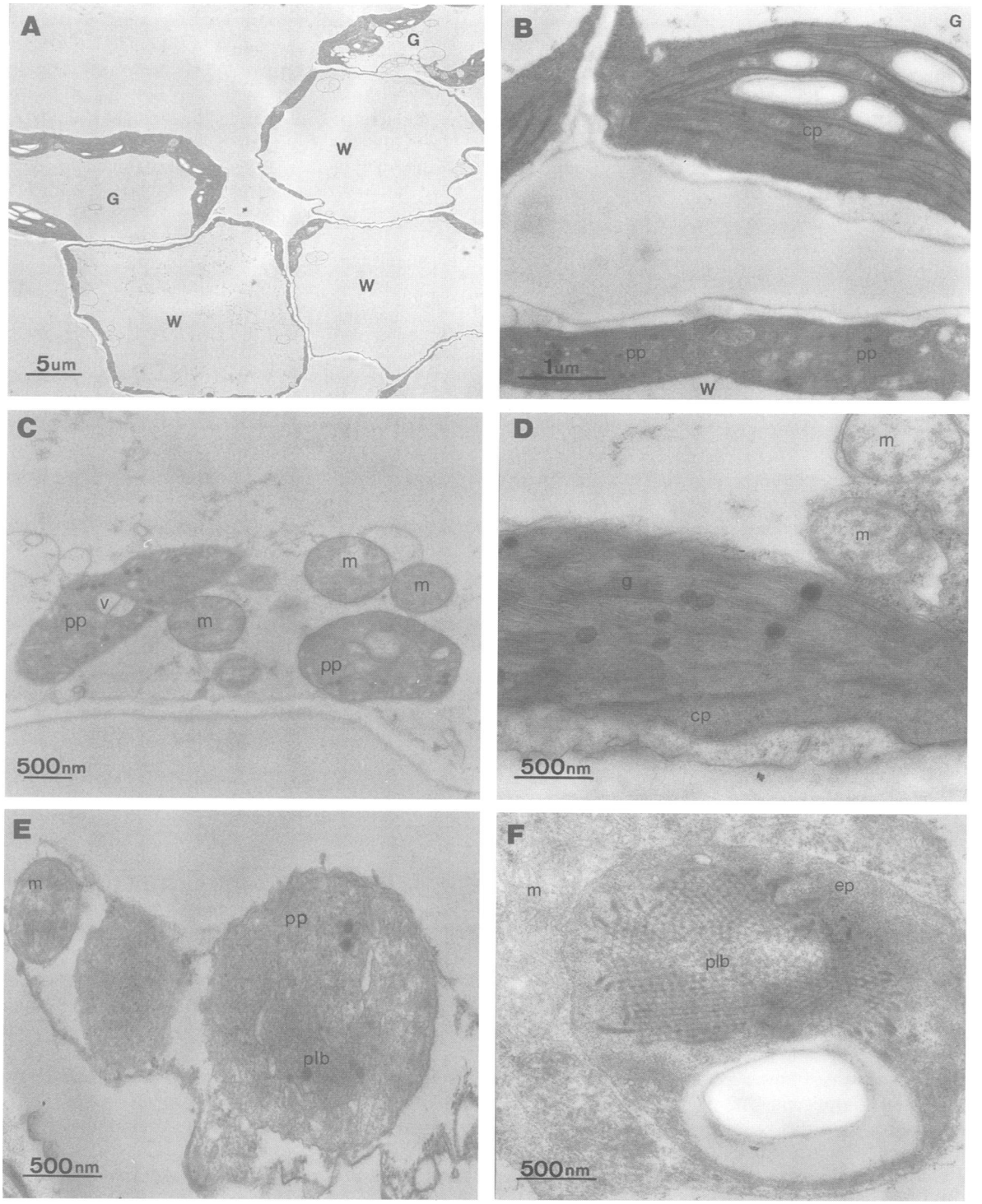

Fig. 4. Electron micrographs of plastids from wild-type and white areas of dag mutant leaves grown at 15°C. The plastids in the green cells (G) are morphologically normal. In the white cells (W) they are small, lack internal membranes and resemble proplastids; m, mitochondrion; v, vesicle; pp, proplastid; plb, prolamellar body; ep, etioplast; cp, chloroplast; g, grana. (**A**) Small scale picture to show adjacent green (G) and white (W) cells. (**B**) Large scale picture of adjacent green (G) and white (W) cells within the palisade layer of the mesophyll. (**C**) Plastid of white cell, showing its small size and very limited internal development of membranes. The plastids frequently contain vesicles. The plastids are two to three times larger than the mitochondria. (**D**) Chloroplast of green cell showing normal internal thylakoid structures and mitochondria for size comparison. (**E**) Etioplast from sector in etiolated dag mutant cotyledon. The plastid structure is very similar to that of the white cells of light-grown dag mutants plants. There is little of the paracrystalline appearance of the normal prolamellar body. (**F**) Wild-type etioplast from cotyledons for comparison with (E), showing well-developed paracrystalline body and a large starch granule which is probably the major carbon store in the *Antirrhinum* seed.

multiplication. The plastids of the green areas were large (5 μm in diameter) and had well-developed thylakoid membrane systems similar to those of wild-type leaves. The cells of white areas contained small plastids (2 μm or less in diameter) and lacked thylakoids (Figure 4). These plastids most closely resembled proplastids since they showed typical small vesicles and invaginations of the inner membranes (Kirk and Tilney-Bassett, 1978). It

appeared that the growth and development of dag mutant plastids was blocked after proliferation and distribution to the daughter cells. Cells in white areas contained only defective plastids and cells in green areas had only wild-type chloroplasts. Mitochondria in both the white and green areas of dag mutant leaves appeared normal and similar to the mitochondria in wild-type plants (Figure 4). There was no indication of any maternal inheritance of *DAG* in crosses, implying that the mutation was in a nuclear gene.

To test whether *dag* presented a block to the photomorphogenic signal inducing plastid development, dag mutant plants were grown in the dark at 15°C. The dag mutant seedlings became etiolated in a manner very similar to wild-type plants, and transfer to light resulted in normal suppression of hypocotyl elongation. On photo-induced greening, variegated plants were observed while wild-type seedlings showed normal greening. These results suggested that dag mutant plants were normal with respect to phytochrome and other photoreceptor functions, although it could be that the cells of the revertant sectors provided adequate phytochrome or other phytoreceptor function to rescue the mutant cells, to give the appearance of normal responses.

The effect of *DAG* on etioplast development was investigated by growing wild-type and dag mutant seedlings in the dark at 15°C and examining sections of the cotyledons in the electron microscope (Figure 4). In the cotyledons of the dag mutant, etioplasts were found that lacked well-developed prolamellar bodies; they were small and resembled proplastids. Thus, dag mutant plastids fail to develop into chloroplasts in the light and normal etioplasts in the dark.

Transposon tagging and cloning of the DAG locus

To examine whether a copy of Tam3 co-segregated with the dag mutant phenotype, genomic DNA from dag mutant and wild-type plants was digested with *Eco*RI, blotted and probed with an internal fragment of Tam3 (Figure 5C, probe A). Among the 50 bands hybridizing to the Tam3 probe, a single band of 6.5 kb co-segregated with the phenotype (Figure 5B). The plants examined included the heterozygous parent of the original dag mutant plants and several wild-type siblings of the plants in which the *dag* mutation was first observed (Figure 5A), so this segregation was significant. The 6.5 kb *Eco*RI fragment was gel-purified from the genomic DNA and cloned in λNM1149. One clone of 6.5 kb, called λJAM232, was analysed further. Southern blotting showed that it contained 3.5 kb of Tam3 with 2 kb and 1 kb of flanking sequences on either side. The entire fragment was subcloned into pBluescript as an *Eco*RI insert and called pJAM 958 (Figure 5D). The flanking sequence, isolated by digestion with *Sma*I and *Eco*RI (2 kb; probe C) was then used as a probe on a fresh Southern blot of genomic DNA (digested with *Eco*RI) from wild types, dag mutant individuals and four independent wild-type revertants. All wild-type (*Dag/Dag* homozygous) plants showed a single 3 kb band, *Dag/dag* heterozygous plants showed both a 3 kb and a 6.5 kb band, all *dag* homozygotes showed a single 6.5 kb band and all first generation, independent revertants showed both the 6.5 kb and the 3 kb band, indicating that they were heterozygous at the locus (Figure 5E). To confirm that these bands represented fragments derived from the *DAG* locus, the segregation of 3 kb and 6.5 kb bands was studied in the progeny of the heterozygous revertants. The four independent revertants were selfed and their progeny segregated for wild-type and dag mutant phenotypes. Seed was collected from all the wild-type plants and the phenotypes of their progeny were scored to identify the *Dag* homozygotes among the parents. Genomic DNA was isolated from these parental homozygotes and then probed with fragment C of pJAM 958. All the plants scored genetically as *Dag/Dag* homozygotes showed only the 3 kb band, demonstrating that this locus co-segregated with the wild-type phenotype and confirming that pJAM 958 contained part of the *DAG* locus.

The existence of transposon-generated DNA footprints at the *DAG* locus in the independent revertants was confirmed by PCR amplification using oligonucleotide primers (G1902 and G1903) which flank the site of Tam3 insertion (Figure5D). Using these primers a band of 300 bp was amplified from the genomic DNA of each revertant. All four revertants showed sequence footprints (Figure 5F) which could be generated via the model for Tam3 excision proposed by Coen *et al.* (1989).

The DAG cDNA and the deduced protein product

To identify the sequences encoding the *DAG* gene product within the *DAG* locus, fragments of DNA from the locus were analysed for hybridization to transcripts on Northern blots. Northern blots containing poly(A)$^+$ RNA from leaves, flower petals and roots were probed with either fragment B or fragment C from pJAM958. A 1 kb transcript was observed only in blots probed with fragment C (Figure 6), indicating that fragment C contained part of the transcribed sequence. The same transcript was also observed in the flowers. cDNA libraries in λgt10 made to mRNA from both petals and leaves were screened with fragment C from pJAM958. The longest cDNA clone from the petal library (pJAM963) and the largest from the leaf library (pJAM1046) were sequenced by the dideoxynucleotide sequencing method on plasmid DNA (Chen and Seeburg, 1985). The nucleotide sequences of the two cDNAs were identical except that 5′ and 3′ end points were slightly different. The nucleotide sequence of the *DAG* cDNA contained one long open reading frame (ORF) with the potential to encode a protein (DAG) of 230 amino acids with a predicted M_r of 26k (Figure 7). The nucleotide sequence around the ATG codon, AAAGAAATGG, conformed well to the consensus for initiation of translation (Kozak, 1981, 1984; Messing *et al.*, 1983; Carvener and Ray, 1991). At the 3′ end of the cDNA the consensus polyadenylation signal AATAAA was not present. However, between positions +888 and +893 a sequence (TTTGTA) was present, which has been reported to be an important upstream element for polyadenylation of plant genes (Rothnie *et al.*, 1994). To determine the start of transcription, 5′ RACE (rapid amplification of cDNA ends) was performed using total RNA from leaves of wild-type plants. The results showed that in wild-type plants the transcripts initiated at a C residue 19 bp upstream of the first ATG in the cDNA. A putative TATA box, TAATAAA, was found 81–87 bp upstream of the transcription start point in the genomic DNA (pJAM958) which is somewhat further upstream

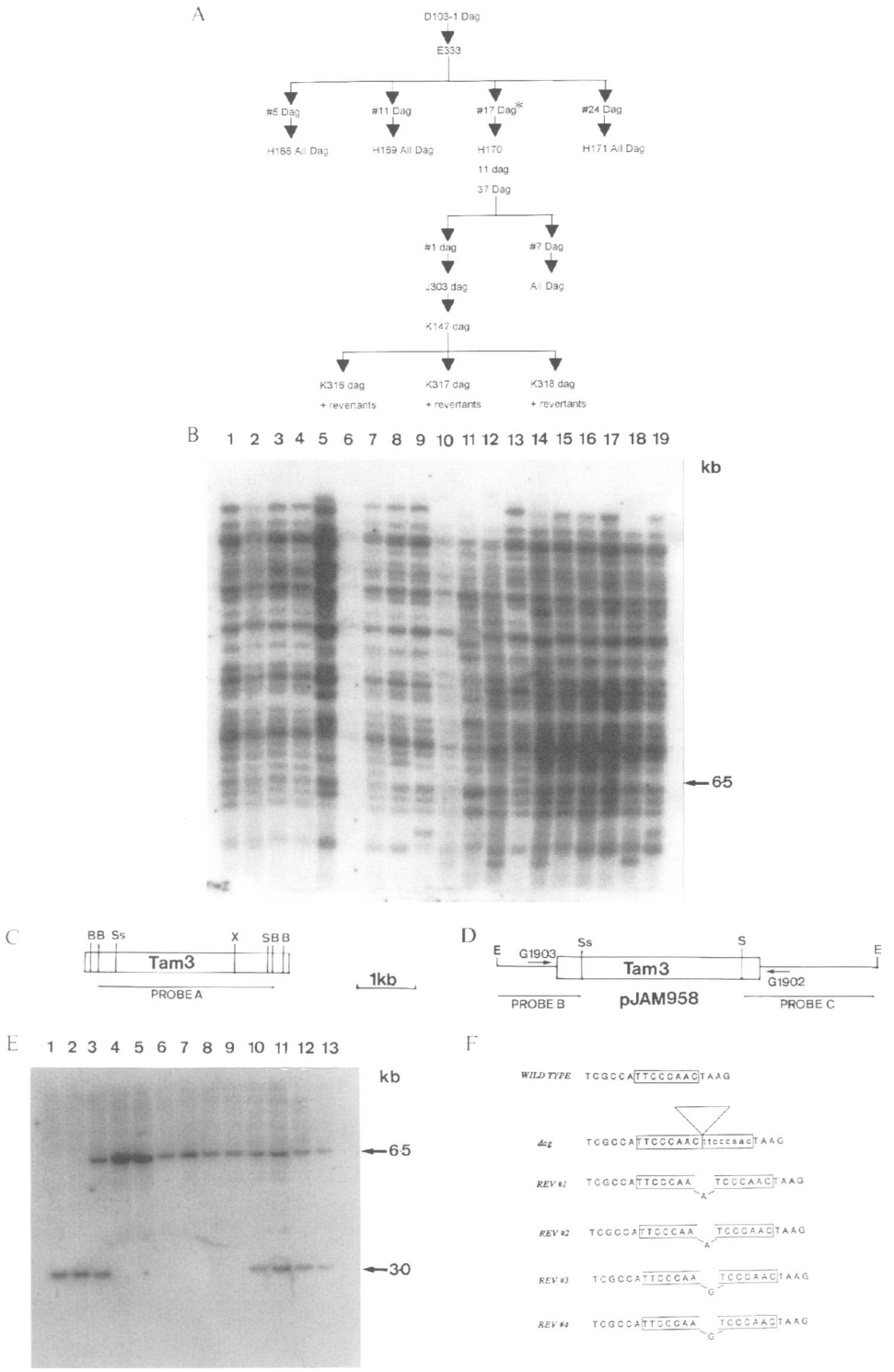

Fig. 5. Cloning the *DAG* locus by transposon tagging. (**A**) Pedigree showing the initial segregation of the *dag* allele in family H170 along with three sibling homozygous wild-type plants. The subsequent generations derived from the original dag mutant plants are shown and their phenotypes are indicated. Individuals from family K147 were grown at 15°C and their progeny contained wild-type revertants among the dag mutant plants. The asterisk indicates the individual in which the original *dag* mutation arose. (**B**) Identification of the Tam3 insertion at the *DAG* locus. Southern blot of genomic DNA digested with *Eco*RI and probed with an internal *Bgl*I fragment (probe A) of Tam 3 (see Table I). (**C**) Restriction map of Tam3 detailing the internal fragment used to probe the filter shown in (B). B, *Bgl*I; Ss, *Sst*II; X, *Xba*I; S, *Sma*I. (**D**) Restriction map of the Tam3 insertion in the *DAG* locus (pJAM958) showing the orientation of the element at the locus. E, *Eco*RI; Ss, *Sst*II; S, *Sma*I. The position of probes B and C are indicated, as are the oligonucleotides used for amplifying the sequences around the point of element excision. (**E**) Southern blot of genomic DNA extracted from wild-type, dag mutant and revertant lines, cut with *Eco*RI and probed with fragment C from pJAM958 (see Table II). (**F**) Sequences of the empty donor sites of DNA from four independent, germinal, wild-type revertants from dag mutant lines, amplified using PCR with primers G1902 and G1903. The footprints left at each locus in the revertants are boxed and the target site in the wild type is boxed.

Table I. Individuals used for Tam3 segregation analysis in Figure 5B showing familial relations and genotypes

Track number	Plant number	Genotype
1	K147-5	*dag/dag*
2	K147-8	*dag/dag*
3	K147-2	*dag/dag*
4	K147-1	*dag/dag*
5	J303-3	*dag/dag*
6	J303-2	*dag/dag*
7	J303-1	*dag/dag*
8	H170-7	*Dag/Dag*
9	H168-11	*Dag/Dag*
10	H169-10	*Dag/Dag*
11	H169-9	*Dag/Dag*
12	H169-1	*Dag/Dag*
13	H170-1	*dag/dag*
14	E333A-13	*Dag/Dag*
15	E333A-8	*Dag/Dag*
16	E333A-3	*Dag/Dag*
17	E333-17	*Dag/dag*[a]
18	E333-11	*Dag/Dag*
19	E333-5	*Dag/Dag*

[a]The individual in which the original *dag* mutation arose.

Table II. Individuls used to relate the insertion of Tam3 at the *DAG* locus to genotype and phenotype

Track	Plant	Genotype	Phenotype
1	E333-11	*Dag/Dag*	wild-type
2	E333A-8	*Dag/Dag*	wild-type
[a]3	E333-17	*Dag/Dag*	wild-type
4	K316-42	*dag/dag*	dag
5	K316-9	*dag/dag*	dag
6	J303-1	*dag/dag*	dag
7	K147-1	*dag/dag*	dag
8	K147-2	*dag/dag*	dag
9	K147-8	*dag/dag*	dag
10	K316-18	*Dag/dag*	revertant
11	K316-70	*Dag/dag*	revertant
12	K317-59	*Dag/dag*	revertant
13	K138-4	*Dag/dag*	revertant

[a]The individual in which the original *dag* mutation arose.

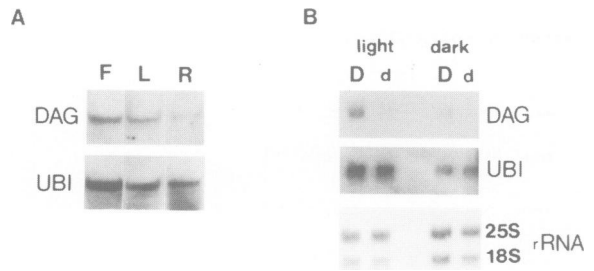

Fig. 6. Northern blots to show expression of *DAG*. (**A**) Poly(A)$^+$ RNA (10 μg) from flowers (F), leaves (L) and roots (R) was probed with the *DAG* cDNA. To check for equal loading the filter was stripped and reprobed with a *UBIQUITIN* cDNA probe from *Antirrhinum*. (**B**) Northern blot to show *DAG* expression in light and dark grown seedlings. Expression in wild-type (D) and dag mutants (d) was also compared. To check for equal loading the filter was stripped and reprobed, first with the *UBIQUITIN* cDNA (*UBI*) and secondly with an rDNA probe (a gift from Noel Ellis).

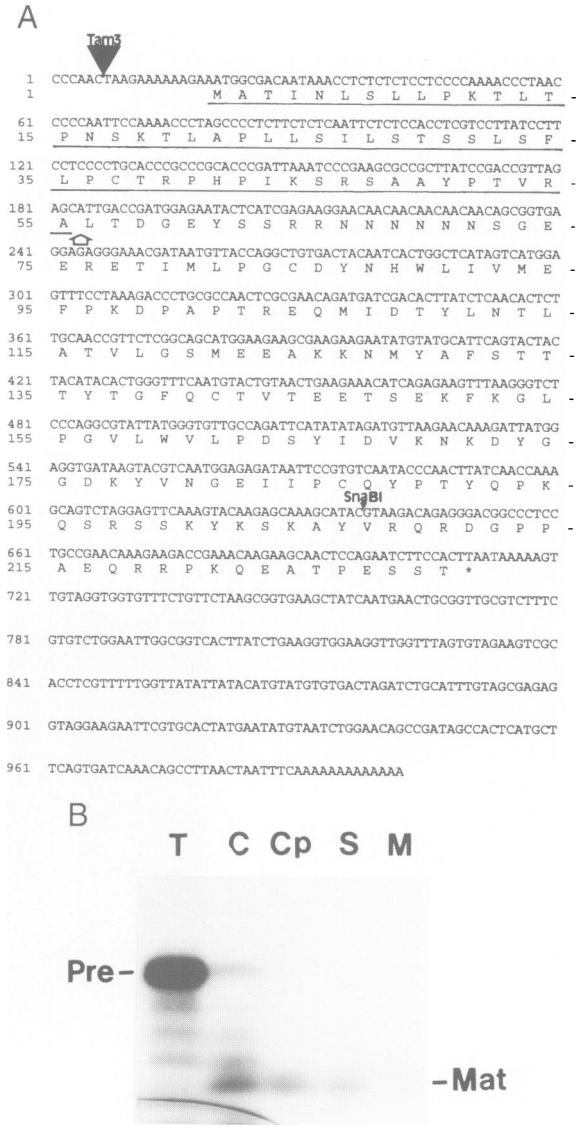

Fig. 7. (**A**) Sequence of *DAG* cDNA and deduced amino acid sequence of DAG. The amino acids comprising the potential transit peptide are underlined and an unfilled arrow marks the most likely point of cleavage of the transit peptide. The position of insertion of Tam3 in the untranslated leader sequence is indicated. The first 10 nucleotides of the *DAG* transcript were determined by 5′ RACE using oligo G1902 to prime the PCR. The site of the *Sna*BI site is indicated. Probes with sequences encoding the N-terminus were constructed using the *Bam*HI site of the adaptor in the cDNA and the *Sna*BI site, and those encoding the C-terminus ran from the *Sna*BI site to the *Bam*HI site in the cDNA adaptor sequence lying beyond the poly(A) tail. The sequence has been submitted to the EMBL database under accession number 95753. (**B**) Analysis of products from chloroplast import assay of DAG protein. Proteins were analysed by SDS–PAGE. T, DAG *in vitro* translation products labelled with [^{35}S]methionine C, proteins from chloroplasts after import showing bound precursor and imported protein (Mat). Cp, protease-treated chloroplasts; S, soluble fraction; M, membrane fraction; Pre, precursor protein; Mat, mature imported protein.

than positions reported for most plant and animal genes (Messing *et al.*, 1983; Lewin, 1994).

The deduced amino acid sequence of DAG showed several interesting features. It contained a domain similar to those of typical transit peptides of chloroplast and mitochondrial proteins (Keegstra *et al.*, 1989). The first 55 amino acids, which comprise the putative transit peptide, were not very rich in arginine, suggesting it to be a chloroplast transit peptide rather than a mitochondrial one (von Heijne *et al.*, 1989). This region was rich in the

hydroxylated amino acids serine (13%) and threonine (13%), and also contained small hydrophobic amino acids such as alanine and valine. These are general features of the primary structure of chloroplast transit peptides (Keegstra *et al*, 1989; von Heijne *et al*., 1989). On the basis of studies of the cleavage sites of chloroplast transit peptides (Gavel and von Heijne, 1990), the proteolytic processing site of the transit peptide was predicted to occur between the amino acids alanine and leucine (positions 55 and 56), as this region matches very well to the consensus proposed for cleavage sites: alanine (A) in position –1, arginine (R) in positions –2 and –10 and valine (V) in position –3 with respect to the cleavage site which lies between –1 and +1 (Figure 7). This cleavage site would make the transit peptide 55 amino acids long, as in other chloroplast transit peptides (Smeekens *et al*., 1986; Keegstra *et al*., 1989). Targeting of the DAG protein to chloroplasts was tested using an *in vitro* import assay. DAG protein synthesized *in vitro* was incubated with isolated, intact pea chloroplasts according to Robinson and Barnett (1988). A sample of this showed both bound precursor and imported mature DAG protein (Figure 6B). Treatment of the chloroplasts with protease showed that the imported peptide was protected from digestion. Separation of the plastids into soluble and membrane fractions revealed the protein to be localized almost exclusively in the stromal fraction.

A hydropathy plot (Jahnig, 1990) showed the mature protein to be largely hydrophilic with no predicted membrane-spanning regions, supporting the view that it is a soluble, globular, plastid protein.

The DAG gene family

To test the possibility that *DAG* was one member of a gene family in *Antirrhinum*, genomic DNA from *Dag/Dag* and *dag/dag* plants was digested with *Eco*RI and *Hin*dIII, fractionated on a 1% agarose gel and Southern blotted. Two sets of filters were made and each one was individually probed at low stringency. Filter 1 (N) was probed with a *DAG* cDNA probe encoding the N-terminus of the DAG protein (a 0.6 kb *Bam*HI–*Sna*BI fragment; Figure 7) and filter 2 (C) was probed with *DAG* cDNA probe encoding the C-terminus of the DAG protein (a 0.4 kb *Sna*BI–*Bam*HI fragment; Figure 7). The N-probe hybridized to more than 22–25 bands, suggesting that this part of the sequence is repeated and that *DAG* is a member of a gene family (Figure 8, tracks labelled N). In contrast, the C-probe hybridized to only three *Eco*RI bands and only two *Hin*dIII bands (Figure 8C). The fragment belonging to the *DAG* gene itself could be identified because it shifted in size between *Dag/Dag* plants and *dag/dag* plants (see arrowed bands in Figure 8), and from isolated genomic clones the *DAG* gene was known to span two *Eco*RI fragments. We suggest, therefore, that the *DAG* gene exists as a member of a gene family in *Antirrhinum majus*, probably with two members very similar to *DAG* and a larger number of sequences sharing homology over the region encoding the N-terminal protein domain.

Analysis of DAG-homologous proteins

A comparison of the predicted DAG amino acid sequence with sequences in current databases was performed using the BLAST program. A BLAST search (NCBI, BLAST network server; Altschul *et al*., 1990) with the DAG sequence revealed significant similarity to a transcript expressed specifically in male flowers of maize, called *MFS* (from pMS 10; Bridges *et al*., 1990) and two *Arabidopsis* expressed sequence tags (ESTs) from the random sequencing project of the *Arabidopsis* genome (accession numbers: T13916 and Z25581). Neither of the *Arabidopsis* cDNA sequences is complete, and so it was impossible to predict whether they have transit peptides. However, there is no predicted transit peptide in the deduced MFS protein, suggesting that one member of the DAG family is not targeted to the plastid. MFS is longer than DAG at its C-terminus, however, and the sequences at the C-termini of these two proteins are not similar, suggesting that these proteins are not orthologues. Interestingly, no homology was found between DAG and animal or yeast proteins in the database, suggesting that DAG-related proteins are unique to plants.

Overall, DAG-related proteins contain a highly conserved N-terminus but have rather variable C-terminal regions. This suggests that these different proteins are functionally distinct members of a gene family, sharing a common domain.

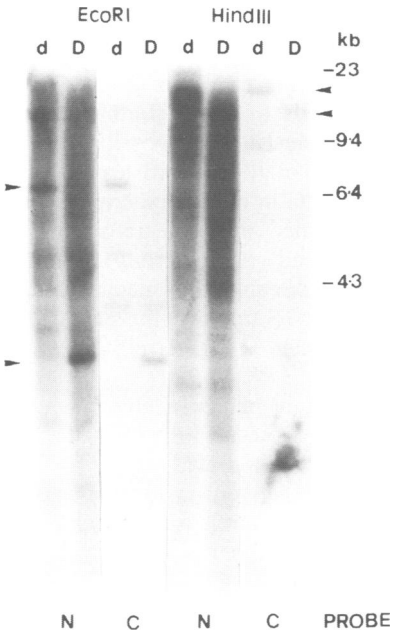

Fig. 8. Estimation of *DAG* gene copy number in the *Antirrhinum* genome. Genomic DNA from either dag mutant (d) or wild-type (D) plants was digested with *Eco*RI or *Hin*dIII and Southern blotted. Filters were probed with the 650 bp N-terminal probe (N, a *Bam*HI–*Sna*BI fragment) or with the 350 bp C-terminal probe (C, a *Bam*HI–*Sna*BI fragment) and washed at low stringency. The N-terminal probe hybridizes to many bands in addition to the bands of the *DAG* locus indicated by arrow heads, whereas the C-terminal probe hybridizes to only one or two additional bands.

Expression of DAG: Northern analysis

Northern blots containing poly(A)$^+$ RNA from leaves, flowers and roots, probed with the *DAG* cDNA fragment revealed that *DAG* was expressed in flowers including petals, in leaves and to a low level in roots (Figure 6). RNA from plants grown at 15°C (Figure 6) revealed a high level of *DAG* expression in leaves of wild-type, while in dag mutant plants expression was greatly reduced.

Since the dag mutant leaves consisted of a mixture of white and green revertant cells (resulting from somatic excision of Tam3), the transcript detected was likely to be produced by cells in which Tam3 had excised from the *DAG* locus. To test for this possibility 5′ RACE was performed using total RNA from leaves of dag mutant plants grown at 15°C in controlled environment cabinets. The results confirmed that the transcripts detected on Northern blots indeed resulted from somatic excision of Tam3 rather than from any other mechanism, since all the transcripts contained footprints at the site of Tam3 insertion similar to those observed for germinal revertants #1 and #2 (see Figure 5F).

Because dag mutant plants failed to develop etioplasts in the dark and chloroplasts in the light, the effect of light on *DAG* gene expression was studied. Northern blots containing total RNA from wild-type and dag mutant seedlings grown in the dark for 2 weeks (at 15°C) and harvested under a dim green safe light were probed with the *DAG* cDNA (Figure 6). In wild-type plants a low level of *DAG* expression was detected in the dark and the expression increased significantly in the light. The *DAG* gene was expressed in all plant tissues examined, but its expression was enhanced by light. When expression of wild-type and dag mutant seedlings grown in the dark was compared, an extremely low level of transcription was observed in dag mutant seedlings. Again, this could result from sectors of revertant tissue following Tam3 excision. To confirm that Northern blots were loaded equally, each of the blots was stripped and re-probed with a *UBIQUITIN* cDNA probe from *Antirrhinum*. There was a small induction of *UBIQUITIN* expression between tissues grown in the dark and those grown in the light when samples with equal amounts of ribosomal RNA were compared (Figure 6). However, this induction was not as great as the induction of *DAG*, suggesting that *DAG* transcript levels do increase specifically in plants grown in the light.

In situ hybridization

Since dag mutant leaves consisted of a mixture of white and green tissues, it was difficult to determine the tissue-specificity of the *DAG* gene expression on Northern blots. We also wished to determine whether the activity of the *DAG* gene had any effect on the expression of nuclear-encoded chloroplast genes such as *CAB* and *RBCS*. The expression of these genes is thought to require the transcriptional and/or translational activity of chloroplasts to provide a positive signal or to remove an inhibitory signal for nuclear gene expression (Taylor, 1989; Susek and Chory, 1992; Susek *et al.*, 1993). The expression of *DAG*, *RBCS* and *CAB* was investigated by *in situ* hybridization to leaves of dag mutant plants grown at 15°C. These leaves showed mutant tissue with a high frequency of wild-type revertant sectors so that the expression of these genes could be compared directly in adjacent wild-type and mutant cells. Young leaves were fixed, sectioned and probed with digoxygenin-labelled antisense probes to *DAG*, *RBCS* and *CAB* from *Antirrhinum*. As controls, antisense probes of these were used on wild-type leaves. After tissue fixation it was impossible to distinguish between the green and white areas on the leaves on the basis of their pigmentation; however, hand sections of the dag leaves revealed that the green areas on tissue sections were largely confined to regions of raised leaf tissue, in contrast to the background of flat white mutant cells (Figure 2A).

Hybridization (Figure 9) clearly showed that the expression of all three nuclear genes was restricted to regions of raised tissue and so was correlated with the formation of green revertant sectors. None of the three genes was expressed in the flat white areas. This indicated that insertion of Tam3 at the *DAG* locus blocked *DAG* gene expression and, when plants were grown at 15°C, the *DAG* transcript observed on Northern blots was derived from the *DAG*-expressing, green revertant sectors. Moreover, in cells lacking *DAG* expression, no expression of *RBCS* or *CAB* was observed although expression of these genes was detected in revertant sectors, indicating that *DAG* activity is required for their expression, and supporting the view that the *dag* mutation provides a very early block in chloroplast differentiation.

Effect of dag on chloroplast gene expression

The effect of *DAG* on expression of genes in the plastid genome was also examined by *in situ* hybridization using part of the plastidial RNA polymerase β subunit gene (*RPOB*) as a probe. In wild-type leaves, expression was observed in all mesophyll cells and was particulate, indicating that the transcript was confined to the chloroplasts (Figure 9G). In the unstable dag mutant leaves, expression of *RPOB* was restricted to the wild-type revertant sectors and absent from the thinner mutant areas (Figure 9I). This result indicates that *DAG* acts very early in chloroplast development, being required for expression of *RPOB* which, in turn, transcribes the plastid-encoded genes required for photosynthetic light harvesting in functional chloroplasts.

Discussion

We have described a leaf-pigmentation mutant, dag (differentiation and greening) from *Antirrhinum* which was caused by insertion of Tam3 at the *DAG* locus. The dag mutant plants have leaves with white/yellow tips; within these, raised, green sectors give the leaves a variegated appearance.

The chloroplasts in the green areas of dag mutant leaves have well-developed thylakoid membrane systems similar to those seen in wild-type leaves, while the cells of the mutant white areas have plastids which are small, contain no or very few internal membranes and resemble proplastids. These structural features suggest that the *DAG* gene acts at an early step in the process of chloroplast development, before membrane proliferation and development of the photosynthetic complex. This is unlike many other genes affecting chloroplast development such as *OLIVE* in *Antirrhinum* (Hudson *et al.*, 1993), *HCF* 106 in maize (Miles, 1994), *ALBINA* and *XANTHA* in barley (Henningsen *et al.*, 1993) and *PAC* in *Arabidopsis* (Reiter *et al.*, 1994). These mutants have partially developed but defective chloroplasts, suggesting that the genes involved are probably concerned with synthesis or assembly of one or more of the components of the photosynthetic complexes as has been shown for *OLIVE* and *HCF* 106 (Hudson *et al.*, 1993; Barkan *et al.*, 1995). Since dag

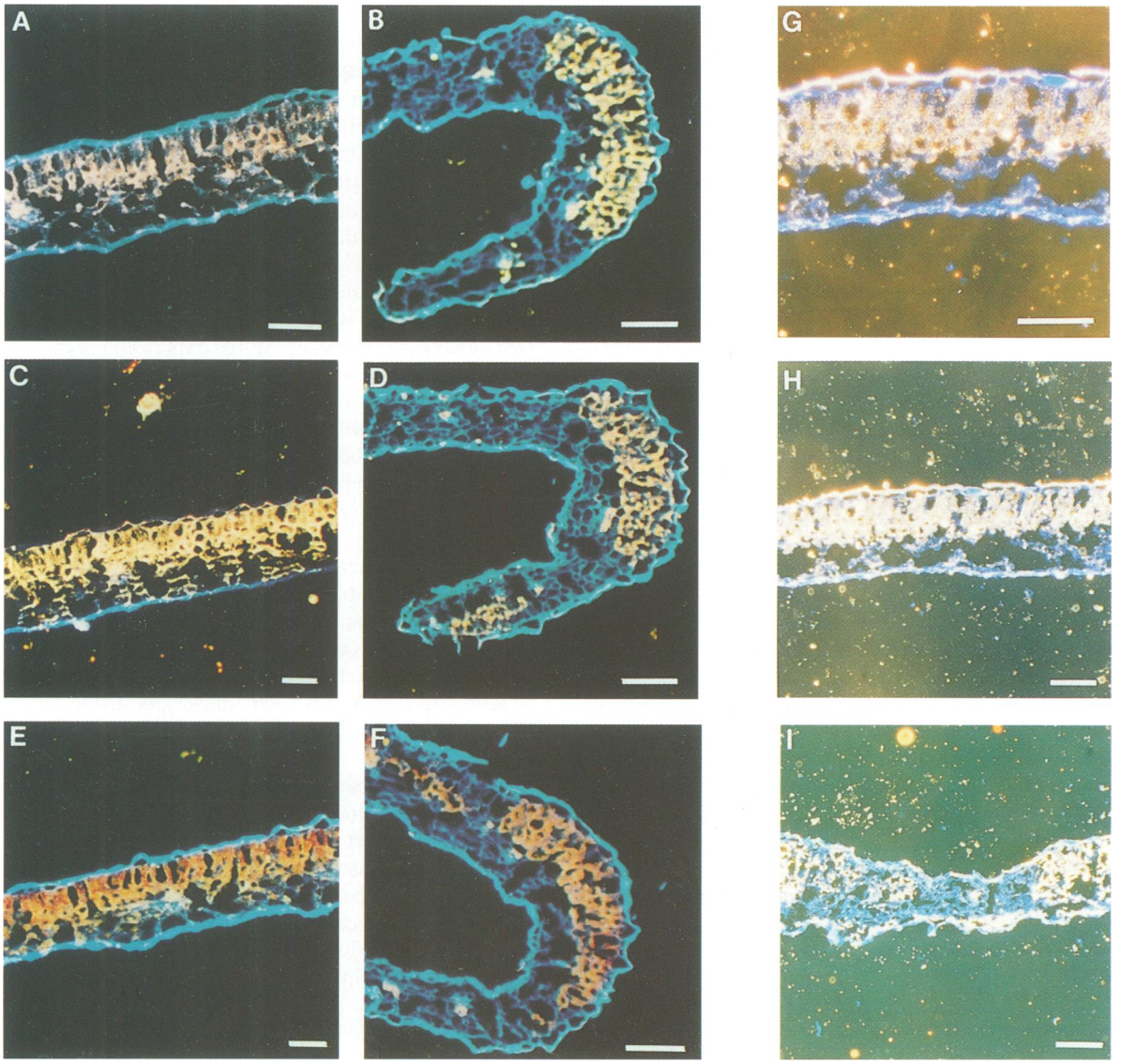

Fig. 9. *In situ* hybridizations of transverse sections of growing leaves from wild-type (**A**, **C**, **E**, **G** and **H**) and dag mutant (**B**, **D**, **F** and **I**) plants grown at 15°C. Sections were probed with digoxygenin-labelled antisense probes to *DAG* (A and B), *CAB* (C and D), *RBCS* (E and F) and *RPOB* (G, H and I). In dark-field the signal appears orange and the background blue because of the staining of the cell walls with calcoflor. In dag mutant leaves, expression of all genes is seen only in the raised revertant cells and not in the thinner areas of the leaf with predominantly mutant cells. (G) shows a close up of a wild-type leaf hybridized with the *RPOB* probe. Here the signal is restricted to small circular structures, (presumably chloroplasts) unlike the signal seen for *DAG*, *CAB* or *RBCS*. Bar = 100 μm.

mutant cells have plastids lacking thylakoid membranes which look like proplastids, it seems that the loss of *DAG* function blocks the biogenesis of all the components of the photosynthetic apparatus rather than causing a block in the synthesis of a single complex.

Some mutations in other plant species resemble the dag mutant in their phenotypes. The *iojap* mutation of maize (Jenkins, 1924; Coe *et al*, 1988; Han *et al*, 1992, 1993, 1995) results in small, undifferentiated plastids with very little internal membrane production. Iojap plastids lack plastidial ribosomes (Walbot and Coe, 1979), and so the mutation causes an early block in expression of the plastidial genome during chloroplast development. *IOJAP* has no reported effects on development of maize leaf palisade however (Coe *et al*, 1988), possibly because monocots such as maize do not rely on a plastid-based signal for the final stages of palisade division and cell expansion. In this respect it is interesting that leaves of dicotyledonous plants require high light for full leaf expansion, whereas in monocots these final stages of cell division and expansion are not light-stimulated (Vince-Prue and Tucker, 1983; van Volkenburgh *et al*, 1985). The effects of the *dag* mutation suggest that the light stimulation of leaf expansion in dicots works through events occurring in the early stages of chloroplast differentiation.

The ghost mutant of tomato (Rick *et al*, 1959) is phenotypically very similar to dag. It is unstable but does not revert germinally, so the basis of its somatic instability may be epigenetic. In mutant sectors, *ghost* causes a complete block in chloroplast development, its plastids remaining small and showing no internal membrane proliferation (Scolnik *et al*, 1987). Revertant sectors are raised compared with mutant areas on leaves, indicating a similar effect of *GHOST* to *DAG* on palisade development.

GHOST has been suggested to cause a block in carotenoid biosynthesis because phytoene accumulates in mutant areas (Scolnik *et al.*, 1987). However, it is not a mutation in the gene-encoding phytoene desaturase (Giuliano *et al.*, 1993), and more recent evidence suggests that in ghost mutants plastids never differentiate, rather than developing and subsequently suffering photodestruction. The similarities between the phenotypes of the ghost mutant and dag mutant suggest that the genes may function at similar stages in plastid differentiation.

Several other mutations that block chloroplast development and also affect palisade formation in dicots have been reported, for example in *Phaseolus* (Zaumeyer, 1938, 1942; Wade, 1941) and *Arabidopsis* (Kirk and Tilney-Bassett, 1978; Wetzel *et al.*, 1994). These mutants collectively provide strong evidence for a signal, produced very early in chloroplast development in dicot leaves, that stimulates the final stages of leaf palisade division and expansion. Interestingly, in dag mutants green revertant sectors on the sepals are not raised, indicating that the signal is only functional in leaves and not in sepals (which lack a distinct palisade layer). Another gene, *PALE CRESS* (*PAC*) in *Arabidopsis* has also been reported to affect palisade cell development (Reiter *et al.*, 1994). However, young leaves of pac mutants have normal palisade cells while the older leaves lose their palisade cell layer (Reiter *et al*, 1994). No difference was observed with respect to palisade cell arrangement between young and old leaves of dag mutants (M.Chatterjee, unpublished) and *DAG* appears to be required for chloroplast differentiation at an earlier stage than *PAC* since no thylakoid membranes are produced in dag mutant sectors (whereas some are in pac mutants), dag mutants are affected in etioplast development whereas pac mutants are not, and *DAG* is required for *CAB* and *RBCS* expression whereas *PAC* is not. Therefore the effect of loss of *PAC* activity on palisade development may be distinct from the effect of loss of *DAG* activity. Also in contrast to dag mutants, olive mutants of *Antirrhinum*, which are blocked in the formation of chlorophyll (Hudson *et al.*, 1993), do not show any morphogenetic effects on palisade development, implying that only signals generated very early in the development of chloroplasts are required for stimulating the final divisions and expansion of the cells of the palisade mesophyll.

The effects of *DAG* on plastidial *RPOB* expression also imply that the *DAG* gene product operates very early in chloroplast development, being required for the expression of the plastidial genome. Further analysis is required to determine whether the function of DAG involves the activation of plastidial RNA polymerase production directly, although nuclear factors are believed to be involved in this process. These include a nuclear-encoded RNA polymerase required for the transcription of plastidial genes, such as those encoding components of the plastidial RNA polymerase (*RPOB*, *RPOC1*, *RPOC2*) and genes encoding ribosomal proteins (Hess *et al.*, 1993; Mullet, 1993). The expression of *DAG* in non-photosynthetic tissues such as roots might argue against such a role, although expression of the plastidial genome is thought to occur at a low level in all plastid types (Baumgartner *et al.*, 1993) despite there being little direct evidence to support the view that activity of the chloroplast genome is necessary for development of any plastid type except chloroplasts. However, *DAG* gene expression is induced strongly by light, suggesting that its function is intimately linked to chloroplast development and the massive stimulation of expression of the chloroplast genome that occurs concomitantly with chloroplast development. If *DAG* does play such a role, its effect on etioplast development could imply that formation of etioplasts also requires activity from the chloroplast genome.

The *in situ* results showed that the expression of *DAG*, *RPOB* and the nuclear-encoded genes *CAB* and *RBCS* was restricted to the green, revertant wild-type sectors and did not occur in the white, mutant dag areas, showing that *DAG* activity is necessary for the expression of these genes. Thus, in the hierarchy of control leading to chloroplast development, *DAG* expression appears to be required early for plastidial RNA polymerase production and hence, indirectly, for subsequent expression of *CAB* and *RBCS* and perhaps other photosynthetic genes. These results also indicate that the signal transduction pathway from the chloroplast to the nucleus that induces expression of nuclear genes encoding chloroplast proteins is probably normal in dag mutant plants, since the expression of *CAB* and *RBCS* does not occur in the cells that do not develop chloroplasts. In contrast, gun mutants of *Arabidopsis*, which are affected in the transduction of such signals, accumulate *RBCS* and *CAB* transcripts in the absence of functional chloroplasts (Susek and Chory, 1992; Susek *et al.*, 1993).

Since DAG does not show any homology to proteins of known function in the databases it is difficult to elucidate the exact nature of the DAG protein and its role in controlling plastid development. It appears to function very early in the pathway leading to chloroplast differentiation and its effect on *RPOB* expression suggests that it may be concerned with the control of expression of those plastidial genes involved in plastidial transcription and translation. While the DAG product may be too small to represent the nuclear-encoded polymerase itself (suggested to be a monomeric protein of M_r 110 000; Lerbs-Mache, 1993), its activity might be associated with this early step in the differentiation of plastids. Its action is required to initiate the first steps of chloroplast biogenesis which in turn signal to the cells of the leaf to undertake the final rounds of cell division and expansion associated with development of the palisade mesophyll.

Materials and methods

Antirrhinum strains

The *dag* mutation was identified in line JI:98 which carries a *nivea* mutation caused by Tam3 insertion (Sommer *et al.*, 1985; Carpenter *et al.*, 1987). Growing conditions were similar to those described by Carpenter *et al.* (1987). The light intensity in the cabinet was 184 μmol m^2 s. In some cases, the plants were grown at low light intensity of 44 μmol, m^2 s at 15°C by shading with muslin.

Fixation of leaves and cotyledons (grown in light or dark) for light and electron microscopy

Leaves were harvested and cut into 1 mm^3 pieces in the fixative solution (2.5% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2), and placed in universal bottles. These were then vacuum infiltrated until the tissues were completely submerged in fixative and left overnight at 4°C. The tissues were rinsed in cacodylate buffer, dehydrated through an ethanol series at −20°C and then passed through a mixture of resin/ethanol (1:3, then 1:2, then 1:1; L.R.White, Agar Scientific Ltd) at −20°C (1 h each) before being left in 100% resin at −20°C overnight. The resin was

changed the next day and incubated for 12 h. The resin was polymerized in Beem capsules at –20°C under UV light for 24 h, followed by 16 h under UV light at ambient temperature. For light microscopy, 0.5 μm thin sections were stained with toluidine blue (0.5% toluidine blue, 0.5% Borax) and observed in white light. For electron microscopy, 0.1 μm thin sections were stained with uranyl acetate (2%) and lead citrate (1%) and micrographs were taken with a Jeol 1200 EX electron microscope. The cotyledons of dark grown seedlings were fixed according to the method of Henningsen *et al.* (1993). For all dark experiments seedlings were harvested under a dim green safe light. Sections were made and observed as described above.

Scanning electron microscopy

Scanning electron microscopy of wild-type and dag leaves grown at 15°C was performed by mounting leaves on an aluminium stub in a vertical position, freezing them in nitrogen slush at –210°C and fracturing using a scalpel blade. The samples were sputter coated with gold for 12 min and observed with a scanning microscope (Camscan mark IV with an exland cryo-stage).

Hand sections

Hand sections of leaves from dag mutant plants grown at 15°C were cut using razor blades and mounted in a drop of water for observation and photography.

Isolation of DNA from A.majus

DNA was isolated from leaves as described by Martin *et al.* (1985). Frozen or fresh leaves were used for this purpose.

Isolation of total and poly(A)$^+$ RNA

Total RNA was isolated from leaves according to the method described in Napoli *et al.* (1990) except that the RNA was precipitated by adding equal volumes of 4 M LiCl instead of lithium acetate. Poly(A)$^+$ RNA was extracted from leaves, flowers and roots using an mRNA purification kit (Pharmacia) following the manufacturer's instructions.

Cloning of plant genomic DNA into λNM1149

Cloning of plant genomic DNA into λNM1149 was done as described by Martin *et al.* (1985) and Sommer *et al.* (1985) except that after electro-elution the DNA was purified using ion-exchange chromatography on a resin column (NACS PREPAC, Bethesda Research Laboratories Inc.). For detailed analysis the positive clone was subcloned into pBluescript SK$^+$ to give clone pJAM958.

Southern and Northern hybridization

Southern and Northern hybridization was performed as described by Martin *et al.* (1985). Filters were washed twice at 65°C in 0.1× SSC, 0.5× SDS for 30 min for high stringency washing or 3× SSC, 0.5× SDS at 50°C for 1 h for low stringency washes.

Chloroplast import assays

pJAM963 containing the *DAG* cDNA transcript was capped and translated in a wheat germ cell-free system in the presence of [^{35}S]methionine. The protein was incubated with intact pea chloroplasts as described by Robinson and Barnett (1988). Following import the chloroplasts were treated with protease and then fractionated into soluble and membrane fractions according to Robinson and Barnett (1988).

Sequencing

Fragments to be sequenced were ligated into pBluescript SK$^+$ and sequencing was performed according to Chen and Seeburg (1985) using Sequenase version 2 (United States Biochemical Corporation).

In situ hybridization

In situ hybridization was based on the method of Jackson (1991). The non-radioactive (digoxygenin) labelling of RNA probes was according to methods described in Coen *et al.* (1990) and Bradley *et al.* (1993). cDNA clones for *RBCS* and *CAB* were isolated from a cDNA library made from mRNA from *Antirrhinum* leaves in λgt10. Clones were identified by screening with a fragment of the genomic clone for *RBCS* from tobacco (a gift from Christine Raines) and a cDNA clone of *Lhcb*I from tobacco (a gift from Angel Merida) respectively. cDNA clones were subcloned into pBluescript and their identity was confirmed by sequencing.

A fragment of the *RPOB* gene was cloned from *Antirrhinum* DNA from leaves using PCR amplification with primers G3235 (5′-GGG-AAA-AAC-GTA-TTA-GTA-GCT-TAT-ATG-CCG-TGG-3′) and G3236 (5′-CCC-TAG-TGA-ACA-TTC-AAA-TAT-CTG-TCC-TAC-3′) from the C-terminal region of the tobacco *RPOB* gene (Shinozaki *et al.*, 1986). PCR conditions used 200–500 ng DNA, 2 μM each primer and an amplification programme of 35 cycles of 94°C (30 s), 50°C (30 s) and 72°C (1.5 min), followed by 10 min at 72°C and soak at 4°C. The amplified fragment was cloned into pBluescript prior to probe preparation.

5′ RACE (Rapid Amplification of cDNA Ends)

5′ RACE was performed using the RACE kit (Gibco BRL) following the manufacturer's instructions except that the first strand cDNA was made at 52°C rather than at 42°C to give more specific products. First strand cDNA was made using oligo G2062 (5′-CTA-TGA-GCC-AGT-GAT-TGT-AGT-CAC-AGC-CTG-GTA-ACA-TT-3′; 5 μm, specific for *DAG*). After C-tailing, PCR amplification used oligo G1902 (5′-GAA-GAA-TTC-GGA-CGA-GGT-GGA-GAG-AAT-TGA-GAG-AAG-AGG-GGC-TAG-3′; specific for *DAG* with an *Eco*RI site, 10 μM) and anchor primer (5′-CTA-CTA-CTA-CTA-GGC-CAC-GCG-TCG-ACT-AGT-ACG-GGI-IGG-GII-GGG-IIG-3′; 10 μm). The PCR reaction was: 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min. This was followed by an incubation at 72°C for 10 min to allow for final extension of the DNA fragments followed by a final soak cycle at 4°C. After PCR, products were purified using the Wizard PCR purification kit (Promega) following the manufacturers instructions and the products were cloned into pBluescript SK$^+$ and sequenced as described before.

PCR amplification of footprints from the revertants

PCR amplification was done following standard procedures, including 20 μM of each primer (G1902: 5′-GAA-GAA-TTC-GGA-CGA-GGT-GGA-GAG-AAT-TGA-GAG-AAG-AGG-GGC-TAG-3′; and G1903: 5′-CTA-TGG-ATC-CGC-TGG-CAA-CAA-AAT-TAT-TGG-GCC-AAC-TGA-TCA-3′) which flank the Tam3 insertion on either side at the *DAG* locus. The program consisted of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min over 35 cycles. This was followed by an incubation at 72°C for 10 min to allow for final extension of the DNA fragments followed by a final soak at 4°C. The products were purified and subcloned into pBluescript SK$^+$ and sequenced as described before.

Analysis of the predicted protein sequences of the DAG gene

Gel readings were entered into the UWGCG (University Wisconsin Genetics Computer Group) database using the GEL ASSEMBLE program and sequence data were analysed using UWGCG computer programs (Devereux *et al.*, 1984). The BLAST program was used to search for homology with other sequences present in GenBank, EMBL and other databases (Altschul *et al.*, 1990). GAP and BESTFIT programs were used to produce optimal alignments between peptide sequences using the UWGCG default option for Gap weights and lengths. The most homologous sequences were later compiled using the 'PRETTY BOX' function in the UWGCG package. UWGCG PEPTIDESTRUCTURE and PLOTSTRUCTURE programs were used to analyse peptide secondary structure. Hydrophilicity was calculated according to the alogarithm of Kyte and Doolittle (1982) using a window of 19 residues according to Jahnig (1990).

Acknowledgements

We are extremely grateful to Drs Christine Raines and Angel Merida for provision of clones, and to Drs Tristam Dyer and Nic Harberd for their support and encouragement of this project. We would also like to thank David Hopwood and James Keddie for their constructive comments on the manuscript which have led to its improvement, and Theresa Warr for preparation of the manuscript. M.C. was supported by a John Innes Foundation Studentship and an ORS award.

References

Altschul,S.R., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.

Bachmann,M.D., Robertson,D.S., Bowen,C.C. and Anderson,I.C. (1967) Chloroplast development in pigment deficient mutants of maize. *J. Ultrastruct. Res.*, **21**, 41–60.

Barkan,A., Voelker,R., Mendel-Hartvig,J., Johnson,D. and Walker,M. (1995) Genetic analysis of chloroplast biogenesis in higher plants. *Physiol. Plant.*, **93**, 163–170.

Baumgartner,B.J., Rapp,J.C. and Mullet,J.E. (1993) Plastid genes encoding the transcription/translation apparatus are differentially

transcribed early in barley (*Hordeum vulgare*) chloroplast development. *Plant Physiol.*, **101**, 781–791.

Bradley,D., Carpenter,R., Sommer,H., Hartley,N. and Coen,E. (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the *plena* locus of *Antirrhinum*. *Cell*, **72**, 85–95.

Bridges,I.G., Bright,S., Greenland,A.J., Schuch,W. and Reid,G. (1990) Inhibition of plant respiration. Patent No. WO 9008831-A/1.

Carpenter,R., Martin,C. and Coen,E.S. (1987) Comparison of genetic behaviour of the transposable element Tam3 at two unlinked loci in *Antirrhinum majus*. *Mol. Gen. Genet.*, **207**, 82–89.

Carvener,D.R. and Ray,S.C. (1991) Eukaryotic start and stop translation sites. *Nucleic Acids Res.*, **19**, 3185–3192.

Chen,E.Y and Seeburg,P.H. (1985) Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA*, **4**, 165–170.

Chory,J. (1992) A genetic model for light-regulated seedling development in *Arabidopsis*. *Development*, **115**, 337–354.

Coe,E.H., Neufer,M.G. and Hoisington,D.A. (1988) The genetics of corn. In Sprague G.F. (ed.), *Corn and Corn Improvement*. 3rd edn. Agronomy. Am. Soc. Agron. Inc./Crop. Sci. Am. Inc./Soil Sci. Soc. Am. Inc. Madison. Vol. 18, pp. 81–258.

Coen,E.S., Robbins,T.P., Almeida,J., Hudson,A. and Carpenter,R. (1989) Consequences and mechanisms of transposition in *Antirrhinum majus*. In Berg,D.E. and Howe,M.H. (eds), *Mobile DNA*. American Society for Microbiology, Washington DC, pp. 413–436.

Coen,E.S., Romero,J.M., Doyle,S., Elliott,R., Murphy,G. and Carpenter,R. (1990) *Floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. *Cell*, **63**, 1311–1322.

Deng,X.-W. and Gruissem,W. (1987) Control of plastid gene expression during development: the limited role of transcriptional regulation. *Cell*, **49**, 379–387.

Deng,X.-W. and Gruissem,W. (1988) Constitutive transcription and regulation of gene expression in non-photosynthetic plastids of higher plants. *EMBO J.*, **7**, 3301–3308.

de Pamphilis,C.W. and Palmer,J.D. (1990) Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant. *Nature*, **348**, 337–339.

Devereux,J., Haeberi,P. and Smithies,O. (1984) A comprehensive set of sequence analysis for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Gavel,Y. and von Heijne,G. (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.*, **261**, 455–458.

Giuliano,G., Bartley,G.E. and Scholnik,P.A. (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell*, **5**, 379–387.

Han,C.-d., Coe,E.H. and Martienssen,R.A. (1992) Molecular cloning and characterization of *iojap* (*ij*), a pattern striping gene of maize. *EMBO J.*, **11**, 4037–4046.

Han,C.-d., Patrie,W., Polacco,M. and Coe,E.J. (1993) Aberrations in plastid transcripts and deficiency of plastid DNA in striped and albino mutants in maize. *Planta*, **191**, 552–563.

Han,C.-d., Derby,R.J., Schnable,P.S. and Martienssen,R.A. (1995) Characterization of the plastids affected by class II albino mutations of maize at the morphological and transcript levels. *Maydica*, **40**, 13–22.

Harrison,B.J. and Ficham,J.R.S. (1964) Instability at the *pallida* locus in *Antirrhinum majus*. *Heredity*, **19**, 237–258.

Henningsen,K.W., Boynton,J.E. and von Wettstein,D. (1993) *Mutants at Xantha and Albina Loci in Relation to Chloroplast Biogenesis in Barley (Hordeum vulgare L.)*. Royal Danish Academy of Sciences and Letters, Munksgaard, Copenhagen, Denmark.

Hess,W.R., Prombona,A., Fieder,B., Subramanian,A.R. and Borner,T. (1993) Chloroplast *rps*15 and the *rpo*B/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase. *EMBO J.*, **12**, 563–571.

Hudson,A., Carpenter,R., Doyle,S. and Coen,E.S. (1993) *Olive*: a key gene required for chlorophyll biosynthesis in *Antirrhinum majus*. *EMBO J.*, **12**, 3711–3719.

Jackson,D. (1991) *In situ* hybridization in plants. In Bowles,D.J., Gurr,S.J. and McPhereson,M. (eds), *Molecular Plant Pathology: A Practical Approach*. Oxford University Press, Oxford, UK, pp. 163–174.

Jahnig,F. (1990) Structure predictions of membrane proteins are not that bad. *Trends Biochem. Sci.*, **15**, 93–95.

Jenkins,M.T. (1924) Heritable characters of maize XX; iojap-striping, a chlorophyll defect. *J. Hered.*, **15**, 467–472.

Keegstra,K., Olsen,L.J. and Theg,S.M. (1989) Chloroplastic precursors and their transport across the envelope membranes. *Annu. Rev. Plant Physiol. Mol. Biol.*, **40**, 471–501.

Kirk,J.T.O. and Tilney-Basset,R.A.E. (1978) *The Plastids. Their Chemistry, Structure, Growth and Inheritance*. 2nd edn. Elsevier North Holland, Amsterdam, The Netherlands.

Kozak,M. (1981) Possible role of flanking nucleotides in recognition of the AUG codon by eukaryotic ribosomes. *Nucleic Acids Res.*, **9**, 5233–5252.

Kozak,M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.*, **12**, 857–872.

Kyte,J. and Doolittle,R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.*, **157**, 105–132.

Lerbs-Mache,S. (1993) The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: single-subunit enzyme or catalytic core of multimeric enzyme complexes? *Proc. Natl Acad. Sci. USA*, **90**, 5509–5513.

Lewin,B. (1994) *Genes V*. Oxford University Press, Oxford, UK.

Martin,C., Carpenter,R., Sommer,H., Saedler,H. and Coen,E.S. (1985) Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the *pallida* locus by transposon tagging. *EMBO J.*, **4**, 1625–1630.

Mayfield,S.P., Nelson,T., Taylor,W.-C. and Malkin,R. (1986) Carotenoid synthesis and pleoitropic effects in carotenoid deficient seedlings of maize. *Planta*, **169**, 23–32.

Messing,J., Greaghty,D., Heidecker,F., Hu,N.-T., Krid,I.J. and Ruberstein,I. (1983) Plant gene structure. In Kosuge,T., Meredith,C.P and Hollaender,A. (eds), *Genetic Engineering of Plants*. Plenum Press, New York, pp. 211–227.

Miles,D. (1994) The role of high chlorophyll fluroscence photosynthesis mutants in the analysis of chloroplast thylakoid membrane assembly and function. *Maydica*, **39**, 35–45.

Mohr,H. (1984) Phytochrome and chloroplast development. In Baker N.R. and Barber,J. (eds), *Chloroplast Biogenesis*. Elsevier, Amsterdam, The Netherlands, pp. 305–347.

Morden,C.W., Wolfe,K.H., de Pamphilis,C.W and Palmer,J.D. (1991) Plastid translation and transcription genes in a non-photosynthetic plant: intact, missing and pseudo genes. *EMBO J.*, **10**, 3281–3288.

Mullet,J.E. (1993) Dynamic regulation of chloroplast transcription. *Plant Physiol.*, **103**, 309–313.

Napoli,C., Lemieux,C. and Jorgensen,R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell*, **2**, 279–289.

Pyke,K.A. and Leech,R.M. (1992) Chloroplast division and expansion is radically altered by nuclear mutations in *Arabidopsis thaliana*. *Plant Physiol.*, **99**, 1005–1008.

Pyke,K.A. and Leech,R.M. (1994) A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana*. *Plant Physiol.*, **104**, 201–207.

Pyke,K.A., Rutherford,S.M., Robertson,E.J. and Leech,R.M. (1994) *arc6*, a fertile *Arabidopsis* mutant with only two mesophyll cell chloroplasts. *Plant Physiol.*, **106**, 1169–1177.

Reiss,T., Bergeld,R., Link,G., Thein,W. and Mohr,H. (1983) Photooxidative destruction of chloroplasts and its consequences for cytosolic enzyme levels and plant development. *Planta*, **159**, 518–528.

Reiter,R.S., Coomber,S.A., Bourett,T.M., Bartley,G.E. and Scholnik,P.A. (1994) Control of leaf and chloroplast development by the *Arabidopsis* gene *pale cress*. *Plant Cell*, **6**, 1253–1264.

Rick,C.M., Thompson,A.E. and Brauer,O. (1959) Genetics and development of an unstable chlorophyll deficiency in *Lycopersicon esculentum*. *Am. J. Bot.*, **46**, 1–11.

Robinson,C. and Barnett,L.K. (1988) Isolation and analysis of chloroplasts. In Shaw,C.H. (ed.), *Plant Molecular Biology: A Practical Approach*. IRL Press, Oxford, UK, pp. 67–78.

Rothnie,H.M., Reid,J. and Hohn,T. (1994) The contribution of AAUAAA and the upstream element UUUGUA to the efficiency of mRNA 3′-end formation in plants. *EMBO J.*, **13**, 2200–2210.

Scolnik,P.A., Hinton,P., Greenblatt,I.M., Giuliano,G., Delanoy,M.R., Spector,D.L. and Pollock,D. (1987) Somatic instability of carotenoid biosynthesis in the tomato *ghost* mutant and its effect on plastid development. *Planta*, **171**, 11–18.

Shinozaki,K. *et al.* (1986) The complete nucleotide sequence of the tobacco chloroplast genome: Its gene organization and expression. *EMBO. J.*, **5**, 2043–2049.

Smeekens,S., Bauerie,C., Hagman,J., Keegstra,K. and Weisbeek,P. (1986) The role of the transit peptide in the routing of precursors toward different chloroplast compartments. *Cell*, **46**, 365–375.

Sommer,H., Carpenter,R., Harrison,B.J. and Saedler,H. (1985) The transposable element Tam3 of *Antirrhinum majus* generates a novel

type of sequence alterations upon excision. *Mol. Gen. Genet.*, **199**, 225–231.
Susek,R.E. and Chory,J. (1992) A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. *Aust. J. Plant Physiol.*, **19**, 387–399.
Susek,R.E., Ausubel,F.M. and Chory,J. (1993) Signal transduction mutants of *Arabidopsis* uncouple *CAB* and *RBCS* gene expression from chloroplast development. *Cell*, **74**, 787–799.
Taylor,W.C. (1989) Regulatory interactions between nuclear and plastid genomes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 211–233.
van Volkenburgh,E., Cleland,R.E. and Schmidt,M.G. (1985) The mechanism of light-stimulated leaf cell expansion. In Baker,N.R., Davies,W.J. and Ong,C.K. (eds), *Control of Leaf Growth SEB Seminar Series*. Cambridge University Press, Cambridge, UK, pp. 223–238.
Vince-Prue,D. and Tucker,D.J. (1983) Photomorphogenesis in leaves. In Dale,J.E. and Milthorpe,F.L. (eds), *The Growth and Functioning of Leaves*. Cambridge University Press, Cambridge, UK, pp. 233–270.
Virgin,H.I. and Egnéus,H.S. (1983) Control of plastid differentiation in higher plants. In Shropshire,W. and Mohr,H. (eds), *Photomorphogenesis, Encyclopaedia of Plant Physiology*. Springer Verlag, Berlin, Germany, Volume 16A, pp. 289–311.
von Heijne,G., Steppuhn,J. and Herrmann,R.G. (1989) Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.*, **180**, 535–545.
Wade,B.L. (1941) Genetic studies on variegation in snap beans. *J. Agric. Res.*, **63**, 661–669.
Walbot,V. and Coe,E.J. (1979) Nuclear gene *iojap* conditions: a programmed change to ribosome-less plastids in *Zea mays*. *Proc. Natl Acad. Sci. USA*, **76**, 940–950.
Wetzel,C.M., Jiang,C.-Z., Meehan,L.J., Voytas,D.F. and Rodermel,S.R. (1994) Nuclear-organelle interactions: the *immutans* variegation mutant of *Arabidopsis* is plastid autonomous and impaired in carotenoid biosynthesis. *Plant J.*, **6**, 161–175.
Zaumeyer,W.J. (1938) A heritable abnormality of beans resembling moseic. *Phytopathology*, **28**, 520–522.
Zaumeyer,W.J. (1942) Inheritance of leaf variegation in beans. *J. Agric. Res.*, **64**, 119–127.

Received on February 19, 1996

Note added in proof

The accompanying paper by Keddie *et al.* describes a gene (*DCL1*) from tomato which, when mutated by *Ds* insertion, gives a phenotype very similar to the dag mutant. DAG and DCL1 are not orthologous proteins and represent different steps in the pathway for chloroplast biogenesis and leaf palisade development in dicotyledonous plants.