The *DCL* gene of tomato is required for chloroplast development and palisade cell morphogenesis in leaves

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The defective chloroplasts and leaves-mutable (*dcl-m*) mutation of tomato was identified in a *Ds* mutagenesis screen. This unstable mutation affects both chloroplast development and palisade cell morphogenesis in leaves. Mutant plants are clonally variegated as a result of somatic excision of *Ds* and have albino leaves with green sectors. Leaf midribs and stems are light green with sectors of dark green tissue but fruit and petals are wild-type in appearance. Within dark green sectors of *dcl-m* leaves, palisade cells are normal, whereas in albino areas of *dcl-m* leaves, palisade cells do not expand to become their characteristic columnar shape. The development of chloroplasts from proplastids in albino areas is apparently blocked at an early stage. *DCL* was cloned using *Ds* as a tag and encodes a novel protein of ~25 kDa, containing a chloroplast transit peptide and an acidic α -helical region. *DCL* protein was imported into chloroplasts *in vitro* and processed to a mature form. Because of the ubiquitous expression of *DCL* and the proplastid-like appearance of *dcl*-affected plastids, the *DCL* protein may regulate a basic and universal function of the plastid. The novel *dcl-m* phenotype suggests that chloroplast development is required for correct palisade cell morphogenesis during leaf development.

Keywords: cell morphogenesis/chloroplast development/*dcl/Ds* mutagenesis/plastid factor

Introduction

In higher plants, plastids differentiate from proplastids in apical meristems to acquire cell-specific morphology and function. These essential functions range from photosynthesis in leaves to amino acid, lipid and starch biosynthesis in all cells. The temporal and spatial differentiation of the different plastid types is regulated by communication between the organelle and the nucleus and is tightly co-ordinated with plant development. Moreover, the primary function of several tissue types is typically correlated with the type of plastid in the tissue (Kirk and Tilney-Bassett, 1978). Despite the importance of plastids, little is understood about the regulation of their differentiation, their role in plant development and the molecular basis of plastid–nuclear communication.

An essential role for the nucleus in regulating plastid development has been demonstrated clearly by investigations into abnormalities of chloroplast ultrastructure in several chlorophyll-deficient nuclear mutants (reviewed in Somerville, 1986). Biochemical, ultrastructural and genetic studies in barley have also ordered several regulatory genes within the framework of plastid biogenesis (Wettstein *et al.*, 1971; Henningsen *et al.*, 1993). However, little is known about the molecular nature of the genes that regulate chloroplast development or how regulation is achieved. Only a few mutations in nuclear genes that regulate chloroplast development have been subjected to molecular analysis. The mutants are affected in a variety of chloroplast functions, including protein targeting to the thylakoid (*hcf106* of maize; Martienssen *et al.*, 1989; Voelker and Barkan, 1995), maintenance of ribosomes within the plastid (*iojap* of maize; Walbot and Coe, 1979; Han *et al.*, 1992) and communication between the chloroplast and the mitochondrion (*CHM* of *Arabidopsis*; Martinez-Zapater *et al.*, 1992).

The mesophyll layer is the primary site of photosynthesis within a plant, and in leaves of a C3 plant, it is comprised of two basic cell types. Palisade cells are columnar in shape and stacked together to optimize light capture whereas spongy mesophyll cells are specialized for light scattering. Chloroplast development is tightly co-ordinated with mesophyll cell differentiation and leaf development. The expansion of cells in the mesophyll layer is accompanied by an increase in plastid size and number per cell (Maksymowych, 1963; Holowinsky *et al.*, 1965), and a nuclear mutation has been reported that uncouples this process (Pyke and Leech, 1992). This co-ordinated developmental program is dependent on light as well as other external and endogenous factors. Some mutants, in which leaf development is uncoupled from light regulation, develop leaves and chloroplasts in the dark (Chory *et al.*, 1989; Deng *et al.*, 1991) while others develop leaves in the dark in the absence of chloroplast development (Cabrera y Poch *et al.*, 1993). Together, these mutants clearly demonstrate that leaf and chloroplast development are co-ordinated, but separable processes. Although a definite role for the nucleus has been established in co-ordinating these events, very little is known about the role of the chloroplast in regulating leaf development and, in particular, mesophyll differentiation.

Signaling between the nucleus and plastids has an important role in the temporal and spatial regulation of plastid gene expression and chloroplast development (Mullet, 1988; Gruissem, 1989). For example, plants in which plastid carotenoid biosynthesis is inhibited display not only an albino phenotype but also a decrease in the transcription of nuclear *CAB* and *RBCS* genes (Harpster *et al.*, 1984). *CAB* and *RBCS* gene expression is therefore at least partially regulated by the plastid. This phenomenon

led to the postulation of a plastid signal (reviewed in Taylor, 1989) which regulates nuclear gene expression. Whether similar nuclear-plastid signaling pathways operate to co-ordinate the development of the chloroplast with the differentiation and morphogenesis of mesophyll cells of the leaf is unknown.

Here we report on a new tomato gene, *DEFECTIVE CHLOROPLASTS AND LEAVES* (*DCL*), which is required for both chloroplast development and palisade cell morphogenesis in leaf mesophyll. Mesophyll cells in the upper side of the internal tissues of the leaf that contain *dcl*-affected plastids do not expand to form cells typical of the palisade layer. The *dcl-m* mutation, caused by *Ds* insertion, resides in a nuclear gene which encodes a novel type of chloroplast protein. We propose that this protein regulates an early step in chloroplast development, and that the developing chloroplast generates a cell-autonomous signal which is necessary for the correct morphogenesis of palisade cells.

Results

Transposon mutagenesis

A total of 150 independent lines containing a transposed *Ds* (transposants) was generated by selection for excision and/or re-insertion of *Ds* (Carroll *et al.*, 1995). Transposants were self-pollinated and screened for visible seedling mutant phenotypes. Two families that contained mutants were identified. One family (J45) contained plants with an unstable defective chloroplasts and leaves-mutable (*dcl-m*) phenotype and is the subject of this report. The other family, containing plants with a defective embryo and meristem (*dem*) phenotype, will be reported elsewhere (B. Carroll, J. Keddie and J.D.G. Jones, in preparation).

Of 15 plants screened in family J45, three had a *dcl-m* phenotype, a result that is consistent with *dcl-m* being a single recessive lesion. When these *dcl-m* mutants were self-pollinated, ~50% of the progeny had reverted to wild-type, indicating that *dcl-m* is both germinally and somatically unstable. Genomic analysis of mutants and revertants using the insert from pMC20 as a probe identified a 3.0 kb *Bst*YI restriction fragment that co-segregated with the *dcl-m* phenotype (data not shown). The mutant allele is therefore designated *dcl::Ds-mutable* (*dcl::Ds-m*).

To determine whether the *dcl-m* phenotype is cytoplasmically inherited, reciprocal test crosses were performed between *dcl-m* and wild-type plants. All plants in this TC1 generation ($n = 100$ for each cross) were wild type in appearance, which demonstrates that *dcl-m* is not cytoplasmically inherited. All plants tested in the TC1 generation contained both a stabilized *Ac* (*sAc*; $n = 20$) and *dcl::Ds-m* ($n = 6$, analyzed by DNA gel blot), showing that the *dcl-m* mutant selected for the reciprocal crosses was homozygous for both *sAc* and *dcl::Ds-m*.

Three plants heterozygous for both *dcl::Ds-m* and *sAc* were self-pollinated. The number of *dcl-m* plants recovered in this generation (12 of 200 plants scored) is below that expected for a simple recessive mutation. However, because *dcl-m* is germinally unstable, fewer mutant plants would be expected. Our results are therefore consistent with the hypothesis that *dcl-m* is an unstable, recessive, nuclear mutation. In this F2 generation *sAc* and *dcl::Ds-m* segregated independently (by PCR analysis and GUS

testing of 140 plants) demonstrating that the *sAc* and *dcl::Ds-m* are genetically unlinked (data not shown). Mutant plants in this F2 generation were test-crossed to wild type and the progeny stained for GUS activity to determine whether the mutants were homozygous or hemizygous for *sAc*. A positive effect of *Ac* dosage on *Ds* excision (Jones *et al.*, 1989) was observed: mutants containing only one *sAc* exhibited fewer sectors than mutants containing one copy of *sAc* (Figure 1). All *dcl-m* plants tested in this and previous generations contained *sAc*. The phenotype of plants which are homozygous for *dcl::Ds-m* in an *sAc*⁻ background (designated *dcl-stable*) will be reported elsewhere.

DCL is required for palisade cell morphogenesis

Clonal analysis has shown that the dicot leaf is comprised of five independent lineages of cells (Poethig, 1989); the upper and lower L1 layers correspond to epidermis on the upper or lower surfaces of the leaf; the L3 occupies a central layer and contains vascular tissue and some mesophyll cells; and the upper and lower L2, which consist of the mesophyll cells between L3 and the two L1 layers.

dcl-m leaves are variegated, displaying a wide range of sector size in all three internal layers (Figure 1). In the palisade layer, the upper L2, sectors are dark green and protrude from the surface of the albino leaf to restore the wild-type thickness to the leaf. In the L3 mesophyll cells, sectors are lighter green and never reach the outer margins of the leaf. In the lower L2, sectors form light green areas which are easily viewed on the lower surface of the leaf. The epidermis, represented by the upper and lower L1 layers, is non-photosynthetic and is not sectorized for a visible phenotype. Sepal variegation is similar to leaves except that the surface is not distorted. Leaf midribs and stems in mutant plants are light green and sectorized with dark green areas. No visible variegated color or structural phenotype is observed in either green or red fruit or petals. Roots appear normal in *dcl-m* plants. In general, *dcl-m* plants are smaller than wild type but leaf shape, phyllotaxy and plant architecture are unaltered. Self pollinations of *dcl-m* plants always resulted in ~30–60% aborted seed.

Initial observations indicated that *dcl*-affected leaves are thinner than wild-type leaves and L2 revertant sectors restore the leaf to a thickness that is similar to wild type. When the rough upper surface of *dcl-m* leaves was investigated by scanning electron microscopy (Figure 2A and B), elevated areas always corresponded to dark green sectors in the L2. To determine which cells in the interior of the leaf contributed to the rough surface phenotype, variegated parts of the leaf were sectioned and examined by light microscopy (Figure 2C and D). In white sectors the palisade layer was missing and replaced with smaller, spherical-shaped cells similar to those observed in spongy mesophyll. The sharp boundary between a wild-type L2 sector and mutant areas in a mature leaf was defined by the presence of a typical palisade layer. In contrast, six layers of cells were observed in leaves of young *dcl-m* mutant and wild-type leaves of 3–5 mm in length (Figure 2E and F), indicating that *DCL* does not affect the early phase of palisade cell differentiation.

Normal chloroplast development is disrupted in young and mature *dcl-m* leaves

Light-grown *dcl*-affected mesophyll cells contain plastids which are irregularly shaped and 1–3 µm in size (Figure

Fig. 1. Leaves of *dcl-m* plants are clonally sectoried. A representative leaf of a *dcl-m* mutant (A). The variegation pattern is dependent on *sAc* dosage: leaflets of *dcl-m* mutants which contain two *sAc* elements (B) have more sectors than leaflets of plants with one *sAc* element (C). Sectors in the palisade layer appear dark green whereas sectors limited to the underlying spongy mesophyll layers appear lighter green.

3A). These plastids contain a largely homogeneous matrix with 5–15 small spherical vesicles and up to six incomplete primary lamellae (Figure 3A). No organized membrane system or starch granules were observed in *dcl*-affected plastids. Plastids in revertant sectors of *dcl*-affected leaves had a morphology identical to chloroplasts from the mesophyll of wild-type plants (Figure 3B). To examine the effect of the *dcl-m* mutation on chloroplast development in young leaves, young *dcl-m* leaves examined by light microscopy as described above were also processed for TEM. Plastids in young leaves have a morphology identical to plastids in mature *dcl-m* leaves (data not shown). Mitochondria appeared identical between *dcl-m* and wild-type plants.

Carotenoids play a fundamental role as accessory pigments for photosynthesis. Their main function in dissipating excess light energy prevents formation of reactive triplet-state chlorophyll. In high light conditions, mutants in carotenoid biosynthesis suffer severe photo-oxidative damage to chloroplasts and their leaves bleach. Under low light conditions, photo-oxidative damage is reduced and these mutants can develop chloroplasts and accumulate chlorophyll. To investigate the possibility that *DCL* is primarily involved in regulating carotenoid biosynthesis, mutant plants were grown in low light conditions and compared to plants grown in high light. Plastid and leaf morphology were identical in *dcl-m* plants grown under these two conditions (data not shown). Also mutant areas of *dcl-m* plants grown under low light conditions did not green, suggesting that *DCL* does not encode or regulate a component of the carotenoid biosynthesis pathway.

The *DCL* gene is disrupted by *Ds*

Genetic analysis of somatic and germinal instability implicated an unstable *Ds* insertion as the cause of the *dcl-m* mutant phenotype. DNA gel blot analysis of *Bst*YI digested genomic DNA confirmed that *dcl-m* plants contain *sAc* and a single *Ds* element (Figure 4A). DNA sequences flanking the right border of this *Ds* were amplified by inverse PCR, cloned and the resultant plasmid was called pMC27. This flanking sequence hybridized to the same 3.0 kb *Bst*YI restriction fragment as the *sAc/Ds* probe from pMC20 (Figure 4B). In wild-type controls, the pMC27 flanking sequence probe hybridized to a *Bst*YI fragment of 6.0 kb (Figure 4B, lane 1). Confirmation that pMC27 contained the correct flanking sequences was obtained from further DNA gel blot analysis using *dcl-m* genomic DNA digested with *Eco*RI, *Xba*I, *Hind*III and *Eco*RV (data not shown). Analysis of genomic DNA from *dcl-m* plants ($n = 10$), wild-type sectors of *dcl-m* plants ($n = 6$) and independent germinal revertants ($n = 5$) indicated that *dcl-m* plants were homozygous for an unstable *Ds* insertion and germinal revertants were heterozygous (Figure 4B). Subsequent genetic and molecular analysis of plants in lanes 17, 18, 19, 20 and 22 confirmed that the germinal revertants were heterozygous for *dcl::Ds-m*. Genomic DNA analysis of the wild-type sectors showed that somatic instability of the mutant phenotype was due to *Ds* excision from *dcl::Ds-m*.

The cloned flanking sequence was used to screen a genomic DNA library and four *Hind*III fragments (3.6 kb, 1.5 kb, 0.9 kb and 2.0 kb) surrounding the cloned flanking sequence were isolated. Since the 1.5 kb *Hind*III fragment

Fig. 2. Differentiation of palisade cells in *dcl-m* leaves. (A) SEM of the upper surface of a *dcl-m* variegated leaf $\times 60.3$. Notice the irregular surface in comparison with wild type. (B) SEM of the upper surface of a wild-type tomato leaf $\times 61.7$. (C) Transverse section ($\times 20$) through a variegated area of a mature *dcl-m* leaf. Dark green sectors correspond to the regions where the palisade layer has a wild-type morphology. (D) Transverse section through a wild-type leaf ($\times 20$). (E) Transverse section ($\times 40$) through a young (3–5 mm) *dcl-m* leaf. Six layers of densely packed cells are clearly visible. (F) Transverse section ($\times 40$) through a young (3–5 mm) wild-type leaf. g; green sector; w; white area; P, palisade mesophyll; S, spongy mesophyll.

hybridized to a transcript of ~ 1.2 kb in an RNA blot analysis, it was used to screen a tomato leaf cDNA library. The longest cDNA clone was sequenced and found to be contained completely within the cloned genomic region (Figure 5A). The *Ds* insertion in *dcl::Ds-m* is located in the second intron of the gene. The sequence of the entire genomic region of the *DCL* gene (accession No.U55278) and the longest cDNA (1147 bp; U55219) were determined (Figure 5B).

An 8 bp target site duplication is typical of *Ds* insertion and many *Ds* excision alleles retain this duplication or have deletions/substitutions of one or two nucleotides (Saedler and Nevers, 1985). To confirm that the *dcl-m* mutation was caused by the *Ds* insertion, DNA from wild-type revertants of four independent *dcl-m* plants was prepared and the 'footprints' expected from *Ds* excision were analyzed. A 200 bp region spanning the *Ds* insertion site was amplified by PCR, cloned and sequenced (Figure 5C). All *DCL* revertant alleles contained sequence alterations consistent with *Ds* excision. These data confirm that

the *dcl-m* mutation is the result of a *Ds* insertion at the *DCL* locus.

To analyze the effect of *Ds* insertion on *DCL* gene expression, total RNA was prepared from wild type and *dcl-m* leaves and subjected to RNA blot analysis using the *DCL* cDNA as a probe. An additional transcript of ~ 1.1 kb in size was found in *dcl-m* but not in wild-type leaves (Figure 6A). The 1.2 kb transcript observed in RNA prepared from *dcl-m* leaves probably represents correct transcription and processing of wild-type *DCL* gene in revertant sectors. The sequence of the aberrant transcript observed in *dcl-m* leaves was not determined, although it is possible that it is either a prematurely terminated transcript or a product of incorrect splicing.

The *DCL* gene was mapped and localized between RFLP markers CT50 and CT133 on the long arm of chromosome 4 (Tanksley *et al.*, 1992). The 1561E T-DNA insertion mapped precisely to the same location (Thomas *et al.*, 1994). The *dcl::Ds-m* mutation was therefore created by a linked transposition of *Ds* from the 1561E T-DNA

Fig. 3. Plastid ultrastructure in mesophyll cells of light grown *dcl-m* (A) and wild-type (B) leaves. *dcl*-affected mesophyll cells contain plastids that contain a largely homogeneous matrix with small vesicles and up to seven incomplete primary lamellae. No organized membrane system typical of wild-type chloroplasts is seen in *dcl*-affected plastids. The ultrastructure of plastids in wild-type sectors of *dcl-m* leaves is identical to wild type (data not shown). Size bar = 1 μ m; M = mitochondrion; P = *dcl*-affected plastid; C = chloroplast.

into *DCL*. Because no mutants in chloroplast development have been genetically mapped to this region, *dcl-m* defines a new locus.

The *DCL* gene is expressed ubiquitously and encodes a novel, plastid-localized protein

RNA gel blot hybridization showed that the *DCL* gene is expressed at similar levels in a wide range of photosynthetic and non-photosynthetic tissues (Figure 6B). Total RNA from light-grown and dark-grown 12 day-old tomato seedlings was also examined and no light regulation of the *DCL* mRNA was apparent.

The open reading frame in the *DCL* cDNA starts from the ATG at position 140 because several in-frame stop codons are found upstream. This open reading frame encodes a protein of 225 residues with a predicted molecular weight of 25 421 Da. Database searches identified no regions of homology to any known protein. A specific search for known genes involved in pigment biosynthesis in plants or bacteria also failed to find a significant match. However, we found that the first 50 amino acids encode a segment which fulfills all criteria for a chloroplast transit peptide (Heijne and Nishikawa, 1991), with a cleavage site at the AAKAR cleavage site consensus sequence (Gavel and von Heijne, 1990). Immediately C-terminal to the transit peptide is a highly acidic region, which is

Fig. 4. *Ds* is inserted into the *DCL* gene. (A) Genomic DNA gel blot analysis using the insert of plasmid pMC20, which recognizes both the *sAc* and the 1561E *Ds*, as a probe. Genomic DNA from a 10512A plant containing the *sAc* source (lane 1), variegated *dcl-m* plants (lanes 2, 4, 6, 8, 9, 11, 13, 14, 15 and 16), wild-type sectors of *dcl-m* plants (lanes 3, 5, 7, 10, 12 and 21) and germinal revertants (lanes 17, 18, 19, 20 and 22) was prepared, digested with *Bst*YI and analyzed. Two bands are observed in DNA prepared from *dcl-m* plants, corresponding to *sAc* (1.9 kb) and *dcl::Ds* (3.0 kb). The 3.0 kb band was not present in 10512A plants. (B) The same filter used in (A) was stripped and re-probed with DNA fragments flanking the 3'-end of the *Ds* from plasmid pMC27. A superimposable 3.0 kb band was identified in *dcl-m* lanes. A 5.9 kb band corresponding to the wild-type allele of *DCL* was identified in 10512A and *dcl-m* plants. This 5.9 kb band was always the least intense in *dcl-m* plants, and its intensity always increased in germinal revertants and wild-type sectors of *dcl-m* plants, consistent with the idea that a somatically and germinally unstable *Ds* insertion was the cause of the *dcl-m* phenotype.

strongly predicted to form an α -helix using two standard secondary structure predictions (Robson and Suzuki, 1976; Chou and Fasman, 1978). The remaining portion of the protein is predicted to form predominantly β -sheet structure with no transmembrane or amphipathic regions that could anchor the protein to a membrane. Thus, we predict that *DCL* is a compact, globular, soluble and plastid-localized protein.

Washing DNA blots at low and high stringency demonstrated that *DCL* is a single-copy gene in tomato (Figure 6C and data not shown). The absence of faint restriction fragments after over-exposure of a DNA blot which was hybridized and washed at low stringency suggested that there were no sequences similar to *DCL* which could encode closely related proteins.

To examine the effects of the *dcl-m* mutation on the expression of the plastid-encoded RNA polymerase, an *rpoB* probe was hybridized against RNA prepared from mature leaves of *dcl-m* and wild-type plants. A processed *rpoB* transcript of ~3 kb in size appears to accumulate in *dcl-m* leaves (Figure 6D).

To investigate the localization of *DCL* within the cell, a chloroplast import assay was performed (Figure 7). *In vitro* transcription and translation of the major *DCL* open reading frame produced a protein with an apparent molecular weight of 32 kDa that was specifically imported into isolated chloroplasts. Protease-treated and washed chloroplasts contained a protein of ~27 kDa, consistent with the proposal that *DCL* is processed during import and resides in the chloroplast. The discrepancy between the predicted and apparent molecular weights of *DCL* is

Fig. 5. Structure of the *DCL* gene and its transcript. (A) Genomic structure of the *dcl-m* locus. The *DCL* gene contains three exons, E1, E2 and E3, represented by filled boxes. *Ds* is inserted into the second intron. Restriction enzyme sites *Bs*: *BsrYI* and *H*: *HindIII* are indicated. (B) The cDNA and deduced amino acid sequence of *DCL*. A putative chloroplast transit peptide and a highly acidic domain with predicted α -helical structure are boxed. Intron positions are marked by filled triangles. (C) Excision footprint analysis of *DCL* revertant alleles. DNA was prepared from four independent revertants and sequences encompassing the *Ds* insertion were amplified by PCR, cloned and sequenced.

likely due to the lower mobility of proteins with highly acidic domains during SDS-PAGE (J.Keddie and W.Gruissem, unpublished observations).

Discussion

We have defined a new nuclear locus, *DCL*, involved in both chloroplast development and palisade cell morphogenesis in tomato leaves. The mesophyll cells of *dcl-m* mutants contain plastids with none of the characteristic features of chloroplasts. Cells in the upper side of the mesophyll layer containing these *dcl*-affected plastids do not expand correctly to form typical columnar palisade cells. *DCL* is expressed ubiquitously and encodes a novel protein which is imported into chloroplasts *in vitro*.

At the ultrastructural level, proplastids in apical meri-

Fig. 6. DNA and RNA blot analysis of the *DCL* gene and transcript. (A) Northern blot analysis of transcripts from a *dcl-m* mutant. Total RNA was prepared and 20 μ g analyzed using the *DCL* cDNA as a probe. Lane 1, wild type; lane 2, wild-type sector of a *dcl-m* mutant and lane 3, variegated sector of a *dcl-m* mutant. An extra *DCL* transcript of 1.1 kb is observed in *dcl-m* plants. (B) Northern blot analysis of *DCL* transcript during plant development. Twenty micrograms of total RNA from light grown seedlings (LGS), dark grown seedlings (DGS), dissected meristems (AM), young leaves (YL), mature leaves (ML), 1 cm fruit (1cm), red fruit (RF), roots (R), stems (S), flowers (F) and callus (C) were analyzed using the *DCL* cDNA as a probe. Exposure time of this filter was 3 h. As a control for loading and transfer, the blot was stripped and re-hybridized with an 18S/28S rRNA probe. (C) Southern analysis of DNA prepared from wild-type plants using the *DCL* cDNA as a probe. Low stringency hybridization yielded an identical pattern (data not shown) consistent with the proposal that *DCL* is represented by a single-copy gene. X, *XbaI*; RI, *EcoRI*; H, *HindIII*; B, *BamHI*; RV, *EcoRV*. (D) Northern blot analysis of *rpoB* transcript levels. A total of 20 μ g of total RNA prepared from *dcl-m* and wild-type plants was analyzed using *rpoB* as a probe. An *rpoB* transcript of ~3 kb in size was observed in *dcl-m* plants. Over exposure of this blot revealed a weakly expressed *rpoB* transcript in wild-type leaves.

stems of light-grown wild-type seedlings are small, irregular in shape and contain a homogeneous matrix which can develop primary lamellae and become vesiculated (Kirk and Tilney-Bassett, 1978; Newcomb, 1990; Reiter *et al.*, 1994). Plastids in the mesophyll cells of *dcl-m* plants therefore possess several of the structural characteristics of proplastids. The proplastid-like appearance of *dcl*-affected plastids suggests that *DCL* affects an early step of chloroplast development. Further support for this proposal is obtained from the comparison of plastids in mutant areas of *dcl-m* leaves with plastids affected by the nuclear mutation *iojap* (*ij*) in maize (Thompson *et al.*,

Fig. 7. Chloroplast import experiments show that DCL is specifically imported and processed by isolated pea chloroplasts *in vitro*. Labeled protein products of an *in vitro* translation reaction (lane 1) were incubated with purified chloroplasts and either not treated (lane 2) or treated (lane 3) with thermolysin protease. A processed peptide of 27 kDa which is resistant to protease treatment is observed in lane 3.

1983). *Ij*-affected plastids suffer a programmed loss of ribosomes (Walbot and Coe, 1979) and are therefore blocked early in development. Plastids in mutant sectors of *ij* plants also resemble proplastids throughout leaf development. The *Ij* gene encodes a novel chloroplast protein (Han *et al.*, 1992) that is associated with the 50S plastid ribosomal subunit, suggesting a role for the *Ij* protein in protein translation required for normal chloroplast development (Han and Martienssen, 1994). We do not yet know the localization of DCL within the plastid, but its function is probably different from *Ij* because *ij* does not disrupt cell differentiation. The observation that *dcl*-affected plastids are proplastid-like in appearance indicates that *DCL* acts as an early regulator of chloroplast development.

To address a possible role of carotenoid-induced photo-oxidative damage in the development of the *dcl*-m phenotype, leaf and plastid morphology were compared between *dcl*-m plants grown in low and high light conditions. Plastids and leaf structure were identical in plants grown under both conditions. Chloroplast development can usually proceed in carotenoid-deficient mutants when grown under low light conditions (Bachmann *et al.*, 1967). However, mutant sectors of *dcl*-m plants, remained albino under low light and did not become green. Thus, it is unlikely that *DCL* is required for, or regulates, carotenoid biosynthesis within the plastid. In support of the idea that photo-oxidation plays no role in the generation of the *dcl*-m phenotype, several single green cell sectors were identified in mature *dcl*-affected leaves grown in high light. Severe photo-oxidative damage to chloroplasts is irreversible and permanent. Single cell sectors, representing late *Ds* excision events in the expansion phase of leaf development, indicate that the *dcl*-m phenotype is reversible at any stage of leaf development.

Plastids which are blocked in the synthesis of chlorophyll, the other main pigment of the chloroplast, accumulate a highly disorganized membrane system (Hudson *et al.*, 1993). Because *dcl*-affected plastids have no distinctive membrane system and *DCL* appears to be expressed constitutively, it is unlikely that *DCL* is involved in chlorophyll biosynthesis. These observations, in combination with the similarity of *dcl*-affected plastids to proplastids, suggest that *DCL* acts at an early stage of

chloroplast development, before chlorophyll biosynthesis is activated.

The precise role of *DCL* in palisade cell morphogenesis is difficult to predict, although our combined molecular and genetic data allow us to present two alternative hypotheses: either (i) *DCL* is required for both chloroplast development and palisade cell morphogenesis independently; or (ii) *DCL* is primarily required for chloroplast development and the effect seen on palisade cell expansion is the result of the incomplete development of chloroplasts in the leaf. Because *DCL* is a plastid-localized protein, the latter hypothesis appears more likely. Our results provide circumstantial evidence for a model in which a signal is generated from the chloroplast at an early stage of development that is necessary for correct palisade cell expansion and morphogenesis.

A signal from the plastid known as 'the plastid factor' has long been postulated to provide the nuclear genome with information about the developmental status of the plastid in each cell (Bradbeer *et al.*, 1979; reviewed by Taylor, 1989). The plastid factor is proposed to co-ordinate gene expression in the nucleus with the needs of the chloroplast. Mutants of *Arabidopsis* termed *gun* (for genomes uncoupled) express *CAB* and *RBCS* mRNA when chloroplast development is blocked by treatment with carotenoid biosynthesis inhibitor, norflurazon (Susek *et al.*, 1993). *GUN* genes are predicted to participate in the plastid factor signal transduction pathway. Palisade cell morphology appears unaffected in *gun* mutants (Susek *et al.*, 1993), in other mutants in chloroplast biogenesis (Hudson *et al.*, 1993), in photomorphogenic mutants (Deng *et al.*, 1991) and in plants grown in the presence of norflurazon (Tonkyn *et al.*, 1992; Susek *et al.*, 1993; J.Tonkyn and W.Gruissem, unpublished observations). Thus, the plastid signal which may participate in palisade cell shape changes is separable from the plastid factor involved in the regulation of expression of nuclear *CAB* and *RBCS* genes. As plants grown on norflurazon have a palisade layer but reduced *CAB* gene expression (Susek *et al.*, 1993), it is likely that the proposed plastid signal which is required for palisade cell morphogenesis appears earlier in chloroplast development. Because a sharp boundary between mutant and wild-type cells is evident, we propose that this novel plastid signal is cell autonomous.

A role for organelles in cellular differentiation has previously been established in humans, where mutations in the mitochondrial genome have been shown to induce a broad spectrum of maternally inherited neurological and neuromuscular diseases (reviewed in Wallace, 1989). Mitochondrial mutations can also have pronounced effects on plant development: severe non-chromosomal stripe mutants of maize can exhibit a twisted, highly distorted morphology (Newton and Coe, 1986). These mutations in plants and animals generally reduce the ATP-generating potential of the mitochondria. Their effects are thought to be a direct consequence of tissue-specific ATP starvation and not through specific signaling from the mitochondria. The nature of the factor produced by the developing chloroplast which may participate in palisade cell differentiation is unknown, but because it appears to be produced at an early stage of chloroplast development, it is unlikely to be a product of photosynthesis.

The predicted *DCL* sequence shows no significant

homology to any protein sequence in the database although it contains a serine-rich N-terminal domain characteristic of chloroplast-targeted proteins (Gavel and von Heijne, 1990). DCL protein is imported into isolated chloroplasts *in vitro*, demonstrating its probable localization in the plastid *in vivo*. Immediately C-terminal to the putative transit peptide cleavage site is a highly charged, acidic domain which is strongly predicted to form an α -helical structure. Negatively charged domains are often involved in regulatory protein-protein interactions (Luna and Hitt, 1992) suggesting that DCL may interact with other proteins within the chloroplast. A screen of the EST database identified castor and *Arabidopsis* homologs of DCL (accession numbers T14885 and N38199). The predicted proteins terminate at a similar position to DCL and are ~50% identical over the sequenced regions. Similar mechanisms of control of chloroplast development are therefore likely to be conserved in a variety of plant species.

It is interesting that even though *DCL* mRNA is expressed in all tissues, chloroplast development is severely affected only in leaf mesophyll cells in *dcl-m* mutants. A less severe phenotype is seen in leaf midribs and stem tissue (dark green sectors over a background of light green) and no visible mutant phenotype is observed in green fruit. Because *DCL* is represented by a single gene in tomato, a redundant function may be performed by a different protein in tissues other than the leaf. An alternative explanation is that the *dcl-m* phenotype is leaky in tissue types other than the leaf, possibly as a result of tissue-specific splicing to remove the entire second intron of *dcl::Ds-m*, including the *Ds* element. The function of DCL in non-leaf tissues is presently under study.

Plants containing the *dcl-m* mutation appear affected only in the development of chloroplasts and no other plastid type. This is difficult to reconcile with the apparent ubiquitous and constitutive expression of *DCL*. The plastid genome primarily encodes proteins required for photosynthesis and plastid gene expression (Mullet, 1988). Mutations affecting plastid gene expression might be predicted to affect solely the biogenesis of chloroplasts. It is possible, therefore, that DCL is involved in the regulation of plastid gene expression. However, *rpoB* transcription is thought to be accomplished by an RNA polymerase encoded by the nuclear genome (Hess *et al.*, 1993). Because *rpoB* transcription is activated in *dcl-m*, it is unlikely that DCL encodes a subunit of this polymerase. The plastids of the barley mutant, *albostrians*, are ribosome-deficient and exhibit an increase in the expression of *rpoB* mRNA (Hess *et al.*, 1993). *dcl-m* leaves also display an increase in the expression of *rpoB* mRNA, although at this point we do not know if *dcl*-affected plastids are ribosome-less. Without a detailed study of gene expression in *dcl*-affected plastids and data concerning the expression and localization of the DCL protein, the biochemical function of DCL within the plastid is difficult to predict.

In summary, we have shown that *DCL* functions at an early stage in the development of chloroplasts from proplastids in mesophyll cells of the tomato leaf. This disruption of chloroplast development is correlated with an arrest in the expansion of palisade cells. Because DCL is a plastid localized protein and chloroplast development

is blocked before mesophyll cells start to undergo characteristic cell shape changes, it seems possible that the developing chloroplast creates a signal which is required for palisade cell morphogenesis. Efforts to confirm and elucidate this novel signaling system are in progress.

Materials and methods

Plant material

Transgenic tomato (*Lycopersicon esculentum* Miller) var. Moneymaker carrying maize transposable elements were used for all experiments described. A total of 150 transposants was generated from a single *Ds* T-DNA line (1561E) by selection for excision and re-insertion of *Ds* after a test cross of a 1561E/10512A double heterozygote to wild type (Carroll *et al.*, 1995). Line 10512A contains a *sAc* linked to a β -glucuronidase (GUS) reporter gene. The presence of *sAc* was monitored by staining leaf or cotyledon explants with the chromogenic substrate X-gluc (Carroll *et al.*, 1995). Seedlings carrying a transposed *Ds* were self-pollinated and the progeny screened for mutations.

Preparation and analysis of RNA and DNA

DNA was extracted from plants as described (Tai and Tanksley, 1989), except that a 2 M lithium chloride precipitation step was substituted for the ammonium acetate precipitation. RNA extraction was performed as described (Martienssen *et al.*, 1989). Low stringency hybridization as described in Tai and Tanksley (1989) was performed at 58°C and filters were washed in 2× SSC, 0.1% SDS for 30 min. High stringency hybridization was performed at 65°C and washes were in 0.2× SSC, 0.1% SDS for 30 min.

Isolation of sequences flanking *Ds*, genomic clones and cDNAs

Sequences flanking *Ds* were isolated by inverse PCR using oligos BAR1, BAR2, Ds1 and Ds2 and cloned into plasmid pKRV using previously described methods (Thomas *et al.*, 1994). To confirm the correct identity of the flanking sequences, DNA blot analysis was performed to show that the cloned flanking sequences hybridized to the DNA fragment identified by probe to the 3' end of *Ac* contained in the plasmid pMC20 (Carroll *et al.*, 1995). The probe from pMC20 hybridizes to both *Ds* and the *sAc*, so the presence of both elements can be monitored using one probe. Genomic DNA from germinal revertants, mutants and wild-type sectors from variegated mutants was digested with a variety of restriction enzymes, blotted and hybridized using the cloned flanking sequences as a probe.

Using the cloned flanking sequences as a probe, 12 genomic clones were isolated by screening 5×10^5 clones of a λ Charon35 library. This library was prepared from a *Sau3a* partial digest of 15–20 kb fragments of VFNT Red Cherry genomic DNA. A total of 5×10^5 clones of a λ ZAPII cDNA library constructed from leaf poly(A)⁺ was screened and 24 hybridizing plaques were obtained.

The *DCL* cDNA was mapped as a polymorphic band using DNA prepared from a *Lycopersicon esculentum*/*Lycopersicon pinellii* F2 population (Tanksley *et al.*, 1992) and digested with *EcoRV*. Data were processed using the Map Manager 2.5 program (Manly, 1993).

The tomato *rpoB* probe was amplified by PCR from tomato using the oligonucleotides *rpoB* (CTGAATTCATATTTCCGTATGTGAA) and *rpoR* (GGAAGTTTAACATAGACTAATTT).

Standard molecular biology techniques were performed as described (Sambrook *et al.*, 1989). Sequencing was performed on a Pharmacia ALF sequencer using the manufacturer's instructions. Analysis of DNA sequence was performed using GCG software.

Microscopy

Healthy leaves of 6 to 8 week-old greenhouse-grown plants were used for microscopy. For low light intensity experiments plants were grown for 4–6 weeks in growth chambers using an incandescent bulb as a light source supplying 0.2–1.0 $\mu\text{E}/\text{m}^2$. Leaf pieces covering the boundary between wild-type and mutant areas were fixed and embedded in LR white plastic (Polyscience, Inc.) following the manufacturer's recommendations. A total of ~100 sections of 3–4 μm was prepared from six leaf samples and stained for 2 min in 0.1% toluidine blue O at room temperature then observed through a Zeiss Axiophot microscope using bright field illumination.

For scanning electron microscopy (SEM) samples were fixed in FAA (5% formalin, 5% acetic acid and 50% ethanol), dehydrated through an

ethanol series, dried in a critical point dryer, sputter-coated with palladium and viewed on a ISI DS130 scanning electron microscope.

For transmission electron microscopy (TEM), samples from wild-type and mutant leaves were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer pH 7.2 and post-fixed in 2% osmium tetroxide. Samples were stained *en bloc* in 1% aqueous uranyl acetate, dehydrated in acetone and embedded in epon araldite. Thin sections of 60 nm were viewed with a JOEL 100CX transmission electron microscope.

Chloroplast import experiments

The open reading frame encoding the entire DCL protein was amplified by PCR and cloned as an *NcoI*–*Bam*HI fragment into plasmid pET14B (Novagen). [³⁵S]Met-labeled DCL protein was expressed using the Promega TNT coupled reticulocyte lysate system and *in vitro* import of the DCL protein into pea chloroplasts was performed exactly as described (Lamppa, 1995).

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Note added in proof

An accompanying paper by Chatterjee *et al.* (this issue) describes a mutant of *Antirrhinum* called *dag* which displays a similar phenotype to *dcl-m*. *DAG* and *DCL* are different genes, although both encode plastid-localized proteins. The idea that chloroplast development is required for palisade cell morphogenesis is also a conclusion of their research.