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The tobacco plastid *acc*D gene is essential and is required for leaf development

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Summary

Angiosperm plastid genomes typically encode approximately 80 polypeptides, mainly specifying plastid-localized functions such as photosynthesis and gene expression. Plastid protein synthesis and expression of the plastid *clp*P1 gene are essential for development in tobacco, indicating the presence of one or more plastid genes whose influence extends beyond the plastid compartment. The plastid *acc*D gene encodes the β-carboxyl transferase subunit of acetyl-CoA carboxylase and is present in the plastids of most flowering plants, including non-photosynthetic parasitic plants. We replaced the wild-type *acc*D gene with an *aad*A-disrupted mutant allele using homologous recombination. Persistent heteroplasmy in the presence of antibiotics indicated that the wild-type *acc*D allele was essential. The phenotype of the *acc*D knockout was revealed in plastid transformants grown in the absence of antibiotics. Leaves contained pale green sectors and lacked part or all of the leaf lamina due to arrested division or loss of cells. Abnormal structures were present in plastids found in mutant plants, indicating that *acc*D might be required to maintain the plastid compartment. Loss of the plastid compartment would be expected to be lethal. These results provide genetic evidence showing the essential role of plastid ACCase in the pathway leading to the synthesis of products required for the extra-plastidic processes needed for leaf development.

Keywords: evolution, essential organelle gene, chloroplast ultrastructure, fatty acid synthesis, leaf lamina loss.

Introduction

Plastids contain their own genetic system which, in most flowering plants, codes for around 110 different genes (Wakasugi *et al.*, 2001). The majority of plastid genes code for photosynthesis-related proteins, or protein and RNA components of the plastid expression apparatus (Wakasugi *et al.*, 2001). Plastid transformation is well established in tobacco, enabling reverse-genetic approaches to be used to study the functions of plastid genes. Knockouts of 21 plastid genes encoding polypeptides have revealed loss-of-function phenotypes that specifically affect plastids (Maliga, 2004). Mutants deficient in photosynthesis-related functions grow and develop on media containing sucrose. In addition to genes with plastid-limited functions, the observations that inhibition of plastid protein synthesis arrests development of tobacco seedlings (Zubko and Day, 1998) and leaves (Ahlert *et al.*, 2003) suggest that the expression of one or more plastid genes is required for plant development.

The tobacco plastid genome is known to encode a number of essential genes including *ycf*1, *ycf*2 (Drescher *et al.*, 2000) and *clp*P1 (Shikanai *et al.*, 2001). Knockouts of these genes result in persistent heteroplasmy where both wild-type (WT) and *aad*A disrupted-mutant alleles are required for survival on selection media containing antibiotics (Drescher *et al.*, 2000; Shikanai *et al.*, 2001). The regulatory *clp*P1 gene is required for tobacco development (Kuroda and Maliga, 2003) and encodes a subunit of a protease which must act on essential plastid polypeptide(s), but these might be encoded by nuclear or plastid genes. Plastid protein synthesis is dispensable in cereals (Hess *et al.*, 1993; Walbot and Coe, 1979; Zubko and Day, 2002) and *Brassica napus* (Zubko and Day, 1998), demonstrating the absence of essential plastid-encoded proteins in these species. In cereals and *B. napus*, genes located in the nucleus could encode plastid-targeted proteins that replace the functions of the essential

plastid genes present in tobacco. Tobacco plastid genes that are absent in cereal plastid genomes are candidates for essential tobacco genes. The *clp*P1 gene is present in cereal plastid DNA, but the list of absent genes in cereal plastid genomes includes *ycf*1, *ycf*2 and *acc*D (Wakasugi *et al.*, 2001). Both *clp*P and *acc*D genes are transcribed by a nucleus-encoded plastid RNA polymerase, which acts early in development (Hajdukiewicz *et al.*, 1997; Hanaoka *et al.*, 2005; Liere *et al.*, 2004). The *acc*D, *ycf*1 and *ycf*2 genes are present in the minimal plastid genome of the non-photosynthetic holoparasite *Epifagus virginiana* (Wolfe *et al.*, 1992), indicating their importance.

The *acc*D gene encodes the β-carboxyl transferase subunit of acetyl-CoA carboxylase (Wakasugi *et al.*, 2001). Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) catalyses the formation of malonyl-CoA from acetyl-CoA, and is considered to be the regulatory enzyme of fatty acid synthesis (Alban *et al.*, 2000; Ohlrogge and Browse, 1995; Sasaki and Nagano, 2004). The plastid *acc*D product is part of a eubacteria-like multisubunit ACCase attached to the plastid envelope (Ferro *et al.*, 2003; Thelen and Ohlrogge, 2002a), which also contains three nuclear encoded subunits encoded by the *Acc*A, *Acc*B and *Acc*C genes (Sasaki and Nagano, 2004). In *Escherichia coli*, *acc*D is an essential gene (Nagano *et al.*, 1991). Plants also contain a cytosolic ACCase comprised of a single multifunctional polypeptide (Konishi and Sasaki, 1994; Sasaki and Nagano, 2004). Biochemical evidence indicates that plastid ACCase is responsible for providing most, if not all, the malonyl CoA required for *de novo* synthesis of fatty acids (Ohlrogge and Browse, 1995; Sasaki and Nagano, 2004). Reverse genetics provides a tool for determining the roles of cytosolic (Baud *et al.*, 2003, 2004) and plastid ACCases (Shintani *et al.*, 1997; Thelen and Ohlrogge, 2002b) in cellular metabolism and development. Anti-sense and sense expression of *Acc*C encoding biotin carboxylase (Shintani *et al.*, 1997), and *Acc*B encoding biotin carboxylase carrier protein (Thelen and Ohlrogge, 2002b), did not drastically change the overall appearance of plants grown in normal light. Expression of the plastid *acc*D gene, encoding the β-subunit of carboxyl transferase, might be the key regulatory step determining plastid ACCase levels (Madoka *et al.*, 2002; Sasaki and Nagano, 2004). Here we use a reverse-genetics approach to show that the tobacco *acc*D gene is an essential gene required for leaf development.

Results

*Isolation of Δacc*D *plastid transformants*

To inactivate *accD*, we removed 198 bp of *acc*D coding sequences and 473 bp of upstream DNA in plastid transformation vector pUM86 (Figure 1a). This removes bases 59 320–59 990 of the 155 939-bp tobacco plastid genome (Wakasugi *et al.*, 1998). The pUM75 control vector (Figure 1b) contains an intact *acc*D gene. Both transformation vectors contain *aadA* marker gene- and *uidA* reporter gene-expression cassettes (Iamtham and Day, 2000; Zubko *et al.*, 2004). The plastid DNA sequences flanking the foreign genes target integration by homologous recombination, allowing the isolation of antibiotic-resistant plastid transformants. Plastid genomes are present in multiple copies per cell. Following transformation, transgenic plastid genomes represent a small fraction of the WT plastid

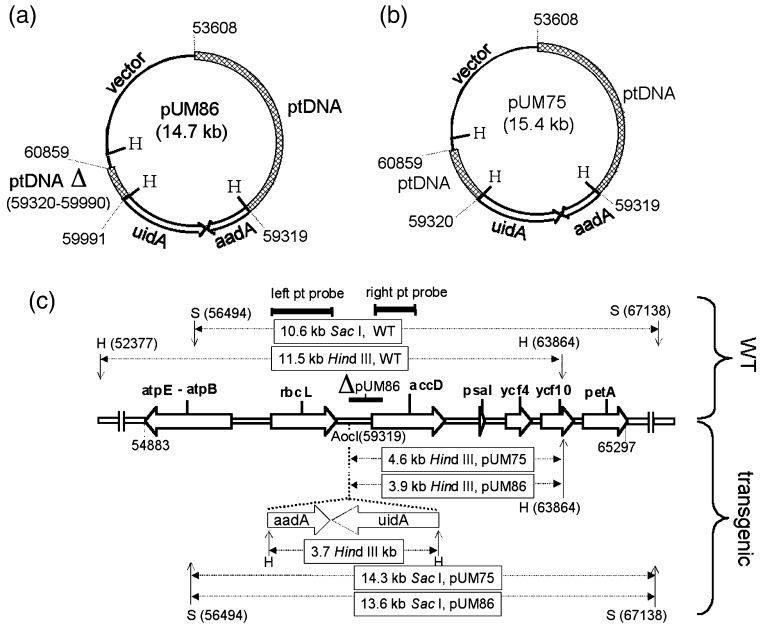

Figure 1. Maps of plastid transformation vectors and plastid genomes.
Plastid transformation vectors: (a) pUM86 contains a deleted *acc*D gene; (b) pUM75 control vector; (c) wild-type (WT) and transgenic plastid DNA following integration of foreign genes. Expression cassettes are shown with *uidA* encoding GUS, *aadA* conferring spectinomycin and streptomycin resistance, and plastid genes *atpE*, *atpB*, *rbcL*, *accD*, *psaI*, *ycf4*, *ycf10* and *petA*; arrows indicate transcript direction. Restriction sites *Aoc*I, *Hin*dIII (H), *Sac*I (S) are shown. Restriction fragment sizes are indicated in WT, and in pUM75 and pUM86 transformants. Left (1.4 kbp) and right (0.74 kbp) plastid (pt) probes used for blot hybridization are indicated. Map coordinates of restriction sites, genes and the deletion in ΔpUM86 refer to tobacco pt DNA (Wakasugi *et al.*, 1998).

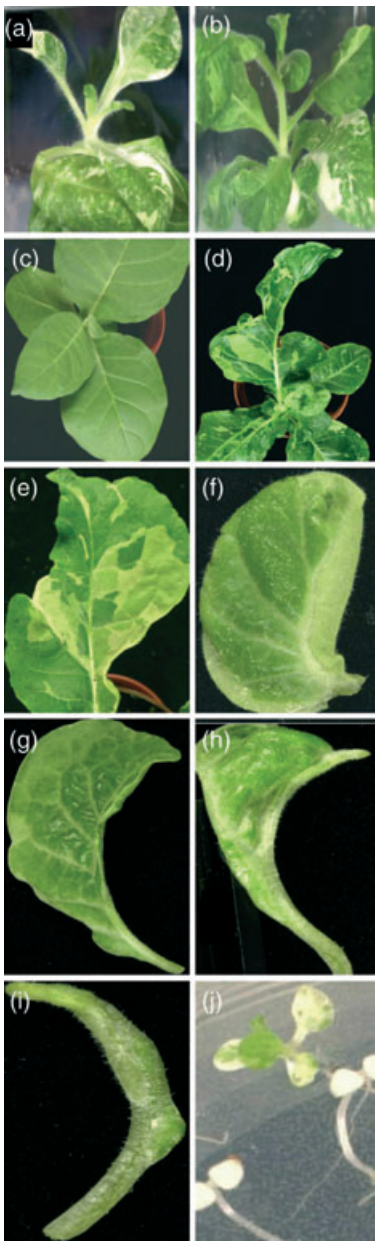

Figure 2. Phenotype of pUM86 Δ*acc*D transplastomic plants.
(a) Δ*acc*D plant on MS medium with spectinomycin (200 mg l^{-1}); (b) new growth after transfer of plant in (a) to antibiotic-free MS medium; (c) wild-type plant (control); (d) Δ*acc*D plant in soil.
(e–i) Leaves of Δ*acc*D plants grown in the absence of antibiotics; (j) Δ*acc*D T_1 seedlings germinated on MS with spectinomycin (100 mg l^{-1}). *In vitro* (a–b, g–j) and soil-grown (c–f) plants and leaves.

genomes in a cell. Transgenic plastids are selected with antibiotics until they replace all WT plastids, resulting in homoplasmic plants containing only transgenic plastid genomes. All 18 independently isolated pUM75 plastid transformants were homoplasmic and were indistinguishable in appearance from green WT plants (Figure 2c).

Four pUM86 transformants (clones 4, 5, 8 and 10), which contain a deleted *acc*D gene, were isolated from independent transformation events. The leaves of all four pUM86 Δ*acc*D transformants were variegated with white sectors (Figure 2a) resulting from spectinomycin-induced bleaching. Both the first T_0 and second T_1 generations (Figure 2j) exhibited this phenotype on spectinomycin medium. When white leaf areas were regenerated into plants on antibiotic-free media, only green shoots resembling WT shoots were isolated (not shown). On transfer of variegated plants to antibiotic-free medium, the new upper leaves lacked white sectors (Figure 2b).

*Analysis of Δ*accD *plastid transformants*

Green–white (GW) variegation indicates a heteroplasmic mixture of transgenic and WT plastid genomes. This was confirmed by DNA blot analysis. Hybridization with left and right plastid (pt) DNA probes (Figure 1c) detected transgenic 13.6-kb *Sac*I and 3.9-kb *Hin*dIII bands, and WT 10.6-kb *Sac*I and 11.5-kb *Hin*dIII bands in digests of variegated GW leaf DNA from pUM86 Δ*acc*D plants grown on spectinomycin medium (Figure 3a, lanes 4 and 6). In contrast, control pUM75 transformants, with an intact *acc*D gene, lacked WT plastid DNA and gave rise to predominant 14.3-kb *Sac*I and 4.6-kb *Hin*dIII transgenic bands in DNA digests (Figure 3a, lane 2). A 3.7-kb *Hin*dIII band in digests probed with *aadA* confirmed the presence of foreign genes in pUM75 and pUM86 plants (Figure 3a, lanes 2–6) and their absence in WT plants (Figure 3a, lane 1). Persistent heteroplasmy in the presence of antibiotics indicates that both WT and *aadA*-disrupted *acc*D alleles are required for viability, and is indicative of a knockout in an essential plastid gene (Drescher *et al.*, 2000; Fischer *et al.*, 1996; Shikanai *et al.*, 2001).

*Leaf-loss phenotype of Δ*accD *plants grown in the absence of antibiotics*

The phenotype of the *acc*D knockout was revealed when plants were grown in the absence of antibiotics. New leaves exhibited a striking pattern of pale green sectors and lacked large sections of the leaf lamina (Figure 2d–i). In some cases, one (Figure 2g,h) or both halves of the leaf lamina (Figure 2i) were missing. This leaf-loss phenotype in the absence of antibiotics has not been reported previously with knockouts in 28 plastid genes (Maliga, 2004). In tobacco, removal of spectinomycin normally results in regreening of new, correctly shaped leaves (Iamtham and Day, 2000; Zubko and Day, 1998). Pale green sectors and leaf lamina loss were visible in new leaves long after withdrawal of antibiotics, and are unlikely to represent any residual effects of antibiotics. Pale green sectors and leaf lamina loss were visible in new leaves of one Δ*acc*D clone right up to flowering. Seeds

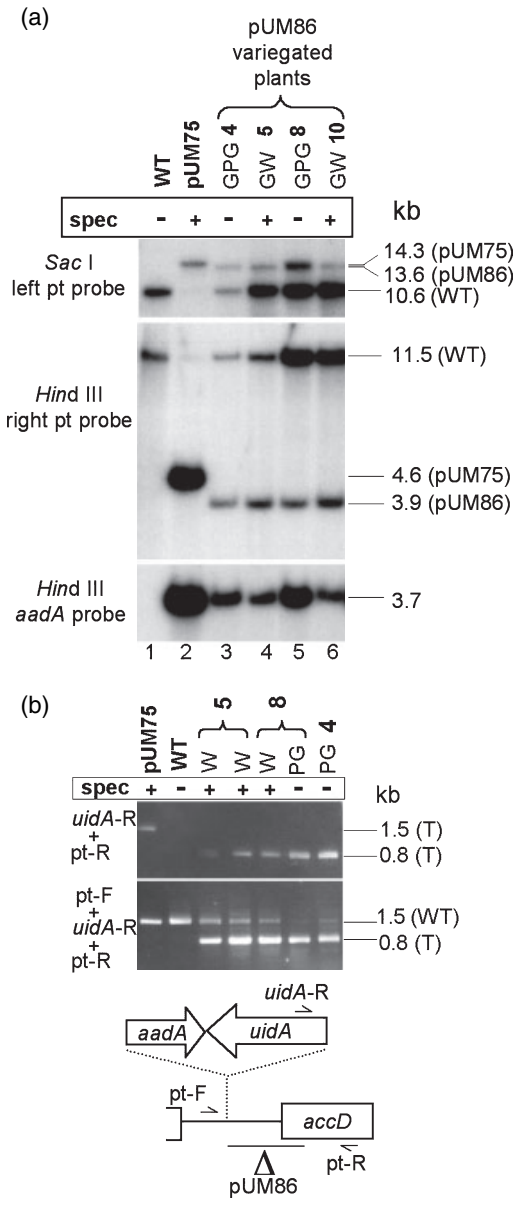

Figure 3. Presence of transgenic and wild-type (WT) plastid genomes in pUM86 Δ*acc*D plants.
(a) Blot analysis with left and right plastid (pt) DNA probes, and *aad*A probe on *Sac*I or *Hin*dIII digests of DNA from variegated green-pale green (GPG) or green-white (GW) leaves from clones 4, 5, 8 and 10 grown in the presence (+) or absence (–) of spectinomycin.
(b) PCR analysis on DNA extracts from white (W) and pale green (PG) leaf sectors from pUM86 Δ*acc*D clones 4, 5 and 8 grown in the presence (+) or absence (–) of spectinomycin. Control WT and pUM75 lanes are indicated. 1.5-kbp WT, 0.8-kbp pUM86 Δ*acc*D, and 1.5-kbp pUM75 transgenic bands (T) are indicated.

collected following selfing of this plant gave rise to 39 variegated spectinomycin-resistant seedlings (Figure 2j) among 439 progeny seedlings. The inheritance of Δ*acc*D transgenic plastid genomes further confirms their persistence in soil-grown plants.

DNA blot analysis of leaf areas containing pale green sectors from Δ*acc*D plants grown in the absence of antibiotics revealed the presence of both transgenic and WT plastid genomes using the right and left plastid DNA probes (Figure 1c). Transgenic 13.6-kb *Sac*I and 3.9-kb *Hin*dIII bands, and WT 10.6-kb *Sac*I and 11.5-kb *Hin*dIII bands were detected in digests of DNA from variegated green/pale green leaves from pUM86 Δ*acc*D plants (Figure 3a, lanes 3 and 5) grown in the absence of antibiotics. PCR analysis was used to study plastid genomes in pale green leaf sectors from soil-grown plants (Figure 2d–f) and white leaf sectors from plants grown on spectinomycin (Figure 2a). DNA extracts (Klimyuk *et al.*, 1993) from small leaf pieces were taken to minimize contamination of sectors with green cells. Transgenic Δ*acc*D plastid genomes were detected in white and pale green leaf sectors with primers *uid*A-R and pt-R, which amplified a 0.8-kb product spanning the *acc*D deletion from pUM86 Δ*acc*D plant extracts and a larger 1.5-kb PCR product from pUM75 control plants (Figure 3b, upper panel). When primer pt-F was added, an additional 1.5-kb WT plastid band was amplified from all white and pale green sectors in pUM86 Δ*acc*D plants (Figure 3b, lower panel). We were unable to detect leaf explants with only Δ*acc*D mutant genomes by DNA blot and PCR analysis.

*Microscopic analysis of pale green sectors in Δ*acc*D plants*

Light microscopic examination of transverse leaf sections revealed a narrowing of the leaf lamina in pale green sectors. Wild-type leaves contain an upper epidermis, palisade mesophyll, spongy mesophyll and lower epidermis (Figure 4a). In pale green leaf sectors a clear palisade layer, which requires functional plastids for its development (Keddie *et al.*, 1996; Pyke *et al.*, 2000), was not visible in the mesophyll (Figure 4b). Epidermal cells and mesophyll cells were irregular in shape and had a flattened appearance in pale green sectors (Figure 4b). Sections spanning the transition across a sector from normal green to pale green revealed a thinning of the leaf lamina (Figure 4c). The transition was gradual in comparison with the sharp sector boundaries found in leaves where cells with fully functional chloroplasts lie adjacent to cells with debilitated plastids (Pyke *et al.*, 2000). This is consistent with sectoring in pUM86 leaves resulting from a progressive decrease in functional plastids from green to pale green areas. Unlike normal green leaves, where chloroplasts are the most abundant plastid type (Figure 4d), pale green sectors contained a variety of plastid forms. A feature of chloroplasts in mutant leaves was the presence of unusual aberrant structures that disrupted the outer regions of chloroplasts (Figure 4e), which were not found in chloroplasts in WT plants (Figure 4d). Vesicle-like structures were found within the stroma of chloroplasts from mutant leaves (Figure 4e): these

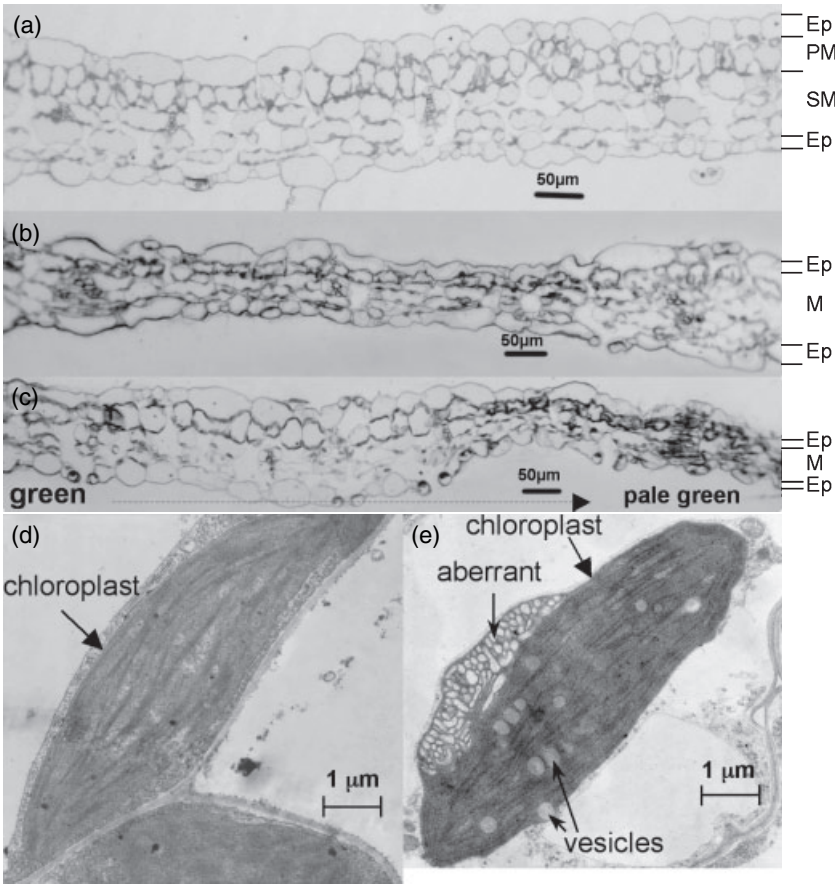

Figure 4. Leaf and plastid sections of pUM86 Δ*acc*D and wild-type (WT) plants grown on MS medium without antibiotics.
Sections visualized by light microscopy (×40) of (a) normal WT leaf; (b) pale green mutant leaf sector, (c) green to pale green sector boundary. Scale bar, 50 μm. Epidermis (Ep), palisade mesophyll (PM), spongy mesophyll (SM) and mesophyll (M) are indicated. Electron microscope images of chloroplast in (d) WT leaf (×11 000); (e) mutant leaf (×13 000). Scale bar, 1 μm.

resemble vesicles found in chloroplasts in senescent leaves (Chory *et al.*, 1991).

Discussion

Replacement of the WT *acc*D gene with an *aad*A disrupted-mutant allele resulted in persistent heteroplasmy, despite strong selection pressure with two antibiotics and multiple rounds of regeneration. Heteroplasmic Δ*acc*D plants exhibited a leaf-loss phenotype and contained pale green sectors when grown either *in vitro* or in soil in the absence of antibiotics. A control construct containing an intact *acc*D gene gave rise to homoplasmic plastid transformants with a uniform population of transgenic plastid genomes and normal leaves. Other insertions in the *rbc*L-*acc*D intergenic region (Iamtham and Day, 2000; Svab and Maliga, 1993; Zubko *et al.*, 2004) including our pUM75 control plants; or an insert just upstream of the *psa*I coding region (Zhang *et al.*, 2001) and knockouts of the *rbc*L (Kanevski and Maliga, 1994), *ycf*10 (Swiatek *et al.*, 2003) and *pet*A (Klaus *et al.*, 2003; Monde *et al.*, 2000) genes, that leave the *acc*D gene intact, have also led to the isolation of homoplasmic tobacco plants. A knockout of a *ycf*4 homologue in *Chlamydomonas reinhardtii* gave rise to homoplasmic mutant algae unable to accumulate the photosystem I complex (Boudreau *et al.*, 1997). Inactivation of the *psa*I gene of *Synechocystis* led to a small defect in photosystem I function, which did not affect photoautotrophic growth at 25°C (Xu *et al.*, 1995). These results indicate that the *rbc*L, *psa*I, *ycf*4, *ycf*10 and *pet*A genes flanking *acc*D are dispensable. Heteroplasmy in pUM86 Δ*acc*D plants is specific to the deletion in *acc*D and appears unlikely to result from any long-range effect of the Δ*acc*D deletion on neighbouring genes.

Persistent heteroplasmy under selection is characteristic of an *aad*A disruption in an essential plastid gene. In a unicellular organism such as *C. reinhardtii*, homoplasmy of a mutant essential gene is cell- and organism-lethal (Fischer *et al.*, 1996). In a multicellular tobacco plant, homoplasmy of a mutant essential gene within a fraction of cells would be lethal to these cells, but the plant would survive provided there are sufficient viable heteroplasmic cells present. Homoplasmic cells would result from segregation of WT and Δ*acc*D plastid genomes during the cell divisions accompanying growth and development. Cells containing a homoplasmic population of mutant *acc*D plastid genomes would not be viable, resulting in a loss of leaf cells. This absence of leaf cells would indicate that *acc*D is an essential gene. Consistent with this idea, we were unable to detect

homoplasmic mutant leaf sections. Homoplasmy of the Δ*acc*D mutation resulting in a deficiency of plastidic malonyl CoA might be expected to lead to cell loss in a number of organs. The observation that the phenotype was most noticeable in leaves is probably because loss of leaf lamina in a fraction of leaves is not lethal to the plant. Loss of cells in other organs might account for the observations that Δ*acc*D plants grow more slowly than WT plants, and some mutant plants had weak, twisted stems.

The multicopy state of plastid DNA enables visualization of the phenotype of an essential gene at different developmental stages. The variation in sector sizes and positions, and irregular pattern of leaf lamina loss in pUM86 Δ*acc*D plants, is consistent with random segregation of mutant and WT plastid genomes. The variable pattern of leaf lamina loss suggests that *acc*D is required at all stages of leaf development. In contrast, a homozygous plant for an essential nuclear gene would arrest at the first developmental stage at which its product was needed (Baud *et al.*, 2003). This is also true of a *Cre-Lox*P method that excises all copies of the essential *clp*P1 plastid gene simultaneously resulting in early arrest of shoot development (Kuroda and Maliga, 2003).

Variation in leaf thickness, and a variety of plastid developmental forms resulting from different mutant-to-WT *acc*D allele ratios, probably underlie the pale green sectors found in our Δ*acc*D knockout plants. A striking feature of plastids in mutant leaves was the presence of unusual aberrant structures that disrupted the outer regions of chloroplasts. These lesions might be explained by the idea that a reduction in malonyl CoA levels could lead to an accumulation of 8-, 10- and 12-carbon fatty acids, which have detergent-like properties resulting in membrane distortion and eventual plastid lysis (Bungard, 2004). Reduced malonyl CoA levels within plastids would result from depletion of plastid-localized ACCase due to inactivation of *acc*D. Plastids are widely considered to house essential metabolic pathways, and disruption of the compartment, which provides the correct environment for their functionality, might be expected to be lethal.

Evolution of plastids from endosymbiotic bacteria involved gene transfer to the nucleus and plastid genome compaction. The essential role of *acc*D shows that plastids have not relinquished complete control of leaf development to the nucleus. The presence of *acc*D in plastids enables gene expression in light-harvesting organelles to influence the development of leaves, which are the light-harvesting organs of plants. The *acc*D gene is also present in the plastids of non-photosynthetic plants (Bungard, 2004; Wolfe *et al.*, 1992). In an extension of the redox control hypothesis (Pfannschmidt *et al.*, 1999) to explain the selective retention of plastid genes, the presence of *acc*D in plastids rather than the nucleus would allow each plastid to influence ACCase activity according to its needs (Bungard, 2004). Our work suggests that *acc*D is an influential gene required to maintain plastid structure, which is consistent with a requirement for regulating its expression in individual plastids.

The presence of essential plastid-encoded proteins in tobacco plastids contrasts with the dispensability of plastid protein synthesis in cereals and *Brassica* species. These contradictory findings in different species require an explanation. Cereal plastids lack the plastid *acc*D gene (Wakasugi *et al.*, 2001) and eubacterial-type multisubunit ACCase (Konishi and Sasaki, 1994; Sasaki and Nagano, 2004), but contain a eukaryotic-type ACCase comprised of multimers of a single multifunctional polypeptide, which is encoded by the nucleus (Gornicki *et al.*, 1997; Sasaki and Nagano, 2004). *Brassica napus* does contain a eubacterial-type multisubunit plastid ACCase (Elborough *et al.*, 1996), but also contains a second eukaryotic-type ACCase in plastids, which is encoded by the nucleus (Schulte *et al.*, 1997). These alternative nucleus-encoded plastid ACCases would allow plastids lacking ribosomes to synthesize malonyl CoA. This idea suggests that the presence of a functional plastid ACCase is the only requirement for the dispensability of protein synthesis in plastids. It would predict the apparent absence or limited expression of a nuclear-coded eukaryotic-type ACCase in tobacco plastids, and identifies *acc*D as an essential plastid gene. Our results are also consistent with the suggestion that the plastid envelope is impermeable to malonyl-CoA (Harwood, 1991; Sasaki and Nagano, 2004) because malonyl-CoA cannot be supplied to plastids from the cytosol to rescue the Δ*acc*D mutation. The indispensability of multisubunit ACCase in tobacco has provided us with genetic evidence to demonstrate the importance of plastid ACCase for maintaining plastid structure and the development of leaves.

Experimental procedures

Vector construction

Expression cassettes contained the *aad*A gene (Goldschmidt-Clermont, 1991) flanked by the 16S rrnBn promoter/*rbc*L ribosome-binding site (EMBL accession no. AJ276677) (Iamtham and Day, 2000) and BnpsbC 3′ UTR (EMBL accession no. AJ578474) (Zubko *et al.*, 2004), and *uid*A gene flanked by 16S rrnHv promoter/*rbc*L ribosome-binding site (EMBL accession no. AJ276676) and NtpsbA 3′ UTR (Iamtham and Day, 2000). A linker with *Apa*I and *Not*I sites was inserted into the *Aoc*I site of pTB27 to make pTB27-link (Zubko *et al.*, 2004). The *aad*A and *uid*A expression cassettes were excised from Bluescript vectors (Short *et al.*, 1988) with *Apa*I and *Not*I, and inserted into the *Apa*I site of pTB27-link to construct pUM75. The *acc*D deletion in pUM86 was made by replacing the *Not*I to *Xho*I fragment in pTB27-link (removes bases 59320–60493) with a *Hin*fI to *Xho*I fragment (bases 59 991–60 493). The *Not*I and *Hin*fI ends were treated with Klenow enzyme and nucleoside triphosphates to allow blunt-end ligation. The deletion was confirmed by sequencing. The *aad*A and *uid*A expression

cassettes were then inserted into the *Apa*I site of the deleted pTB27-link vector to make pUM86.

Isolation of transplastomic plants

Plastid transformants were generated by particle bombardment as described (Day *et al.*, 2004; Svab and Maliga, 1993) using *Nicotiana tabacum* (cv. Wisconsin 38). Green shoots and cell lines were selected on RMOP medium (Svab and Maliga, 1993) containing spectinomycin dihydrochloride pentahydrate plus streptomycin sulphate, each at 500 mg l^{-1}. After three cycles of regeneration on RMOP media containing spectinomycin and streptomycin, shoots were rooted on Murashige and Skoog (MS) medium containing 200 mg l^{-1} spectinomycin. Plants with roots were propagated *in vitro* in the presence or absence of spectinomycin, or transferred to soil. The clone number identifies the bombardment from which it was obtained. Plants *in vitro* were propagated in a growth cabinet at 25°C in a 12-h day/12-h night cycle with light intensities of 40–100 μE m^{-2} sec^{-1}. Plants in soil were grown in a walk-in growth room at 25°C in a 16-h day/8-h night cycle with light intensities of 80–200 μE m^{-2} sec^{-1}. Seeds from self-pollinated Δ*acc*D plants were germinated on MS medium containing 80 or 100 mg l^{-1} spectinomycin.

DNA manipulations

Total DNA extractions and DNA blot analyses were carried out as described (Zubko and Day, 2002). [α-^{32}P]dCTP hybridization probes prepared with High Prime (Roche Applied Science, Lewes, UK) were comprised of a 0.8-kbp *Nco*I–*Pst*I *aad*A fragment from pUC-atpX (Goldschmidt-Clermont, 1991), the left 1.4-kbp probe (co-ordinates 57 595–59 028) containing the *rbc*L gene prepared from pTB27 using primers rbcl-F (5′-ATGTCACCACAAACAGAGACTA) and rbcl-R (5′-TTACTTATCCAAAACGTCCACT), and the right 0.74-kbp probe (co-ordinates 59 793–60 533) containing the *acc*D gene prepared from pTB27 with primers accD-F (5′- ATGACTATTCATCTATTGTA-TTTTC) and pt-R (5′-GCATTGAACCCACAAATGCCTG). Blots were washed in 0.1 × saline sodium citrate, 0.1% (w/v) sodium dodecyl sulphate at 60°C. DNA extracts from small samples of tissue were prepared according to Klimyuk *et al.* (1993) for PCR analysis. Aliquots of 1 μl DNA extract were used in a final volume of 25 μl 1 × ReadyMix Taq with $MgCl_2$ (Sigma-Aldrich, Poole, UK). PCR utilized primers pt-F (5′-GCAGTGGACGTTTTGGATAAG, 59 005–59 025), pt-R (60 512–60 553) and uidA-R (5′-CACAGTTTTCGCGATC-CAGACTGAATG-3′) using 25 cycles of 95°C for 25 sec, 58°C for 45 sec and 72°C for 2 min.

Microscopy

Microscopy was carried out as described by Zubko and Day (1998). Leaf pieces (1–2 mm) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate pH 7.3, post-fixed in cacodylate buffer containing 0.01 mg ml^{-1} osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr's resin. Sections were stained in 1% toluidene blue for light microscopy and 2% uranyl acetate, 0.3% lead citrate for transmission electron microscopy using a Phillips/FEI Tecnai 12 Biotwin transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

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