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ORIGINAL ARTICLE

Ronelle Roth · Ruairidh J.H. Sawers · Helen L. Munn
Jane A. Langdale

plastids undifferentiated, a nuclear mutation that disrupts plastid differentiation in *Zea mays* L.

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Abstract Photosynthetic development in any plant requires the intracellular co-ordination of chloroplast and nuclear gene expression programs. In this report, we investigate the role of a nuclear gene in photosynthetic development by examining C_4 photosynthetic differentiation in a yellow mutant of maize (*Zea mays* L.). The *plastids undifferentiated* (*pun*) mutation disrupts plastid biogenesis in both bundle sheath and mesophyll cells, at an early developmental stage and in a light-independent manner. Chloroplast thylakoids are disrupted in the mutant and both membrane-associated and soluble chloroplast-encoded proteins accumulate at much reduced levels. The observed plastid morphology is consistent with a general defect in chloroplast biogenesis that is most likely exerted at the post-translational level. Despite aberrant chloroplast development, nuclear photosynthetic genes are expressed normally in *pun* mutants. Thus, neither functional chloroplasts nor the *Pun* gene product are required to establish nuclear photosynthetic gene expression patterns in maize.

Keywords Cellular differentiation · Chloroplast biogenesis · C_4 photosynthesis · Mutant maize (*pun*) · *Zea* (plastid differentiation)

R. Roth and R.J.H. Sawers contributed equally to the work reported.

R. Roth[1] · R.J.H. Sawers[3] · H.L. Munn[2] · J.A. Langdale (✉)
Department of Plant Sciences, University of Oxford,
South Parks Road, Oxford, OX1 3RB, UK
E-mail: jane.langdale@plants.ox.ac.uk
Fax: +44-01865-275147

Present addresses:
[1]Department of Phytopathology,
Wageningen Agricultural University,
PO Box 8025, 6700 EE, Wageningen, The Netherlands

[2]University of Edinburgh,
King's Buildings, Mayfield Road,
Edinburgh, EH9 3JL, UK

[3]Boyce Thompson Institute for Plant Research,
Ithaca, NY 14583-1801, USA

Abbreviations LHCPII: chlorophyll *a*/*b*-binding protein of photosystem II · Lsu: large subunit of Rubisco · MDH: NADP-dependent malate dehydrogenase · ME: NADP-dependent malic enzyme · PEPCase: phosphoenolpyruvate carboxylase · PPDK: pyruvate phosphate dikinase · RuBPCase: ribulose bisphosphate carboxylase · SBPase: sedoheptulose bisphosphatase · Ssu: small subunit of Rubisco

Introduction

The development of photosynthetic competence in any plant relies on intracellular signalling mechanisms. Nuclear, chloroplast and mitochondrial genomes all contribute to photosynthetic differentiation but the way in which gene activity is co-ordinated is not entirely understood. Analysis of nuclear mutations that disrupt photosynthesis has shown that nuclear genes can regulate chloroplast gene expression (reviewed in Taylor 1989). Conversely, studies of carotenoid-deficient seedlings in which chloroplast development is disrupted (induced either by mutation or by norflurazon application) have suggested that there are factors synthesised in the chloroplast required for nuclear photosynthetic gene expression (reviewed in Oelmuller 1989; Taylor 1989). For example, photooxidative destruction of chloroplast function is correlated with decreased expression of the nuclear ribulose bisphosphate carboxylase (RuBPCase) small subunit (*RbcS*) genes and also of the nuclear *Cab* genes that encode the chlorophyll *a*/*b*-binding protein of photosystem II (LHCPII) (Batschauer et al. 1986; Mayfield and Taylor 1984, 1987; Oelmuller 1989).

In plants that utilise the C_4 photosynthetic pathway, intercellular signalling mechanisms are also required during photosynthetic development because two photosynthetic cell types co-operate to fix carbon (reviewed in Edwards and Walker 1983). The most extensively studied C_4 plant, maize, exhibits a classical Kranz-type anatomy where veins are surrounded by concentric files of photosynthetic bundle sheath and mesophyll cells

(Brown 1975). Mesophyll cells develop small chloroplasts with stacked grana that are arranged randomly within the cell and contain the majority of photosystem II activity present in the leaf (Meierhoff and Westhoff 1993; Wrischer 1989). The C_4 carbon-shuttle enzymes phospho*enol*pyruvate carboxylase (PEPCase), NADP-dependent malate dehydrogenase (NADP-MDH) and pyruvate phosphate dikinase (PPDK) accumulate and act specifically in mesophyll cells. In contrast, the thick-walled bundle sheath cells develop agranal chloroplasts that accumulate the C_4 decarboxylating enzyme NADP-dependent malic enzyme (NADP-ME) and enzymes of the Calvin cycle. C_4 physiology thus requires the spatial restriction of Calvin cycle enzymes to the bundle sheath and the cell-specific accumulation of enzymes that catalyse the reactions involved in the intercellular carbon-shuttle. Although C_4 differentiation patterns have been characterised extensively (reviewed in Nelson and Langdale 1992) the signals required for specialisation are still unidentified.

To identify nuclear genes that are required for the development of photosynthetic competence in maize, we have screened transposon-containing lines for photosynthetically defective mutants. Work reported here describes a phenotypic characterisation of the *plastids undifferentiated* (*pun*) mutant. In mutant plants, plastid morphology is aberrant throughout development in both the light and the dark. Internal chloroplast membranes are not apparent and thylakoid-associated proteins do not accumulate. Despite perturbed chloroplast development, nuclear photosynthetic gene expression is essentially normal in light-grown *pun* leaves. The reported observations are consistent with a general defect in *pun* mutants that disrupts the accumulation of chloroplast-encoded proteins at the post-translational level.

Materials and methods

Plant material and growth conditions

The *plastids undifferentiated* (*pun*) allele was isolated from a maize (*Zea mays* L.) line containing active *Spm* transposable elements and was previously named *bundle sheath defective3* (*bsd3*) (Langdale et al. 1995). *pun* plants are yellow, do not exhibit a variegated phenotype and die after the endosperm reserves are depleted (normally approx. 13 days after planting). *Pun*/*pun* heterozygous plants were crossed and backcrossed into the inbred line W22. Heterozygous progeny of the second cross into W22 were self-pollinated, and the resultant progeny were used for the experiments described in this report. In all cases, *pun* mutants segregated one in four, indicating the recessive nature of the mutation.

Seedlings were grown in soil in a growth chamber maintained at 28 °C with a 16 h light/8 h dark cycle. Plants were grown under moderate (100 μmol photons m^{-2} s^{-1}) or low (10 μmol m^{-2} s^{-1}) irradiance levels. Plastochron 1–5 (P1–5) leaf primordia were harvested 6 days after planting when seedlings were 1.5–2 cm tall and all leaves were still enclosed in the coleoptile. For all experiments, the young shoots were excised 2–3 mm above the shoot apical meristem, after which the seedlings were transferred to the growth chamber and grown until mutant phenotypes could be scored. Two 2- to 3-mm transverse sections were taken from the base of the immature shoot for in situ experiments, thus comprising P3–P5 leaf primordia enclosed within an outer coleoptile. For transmission electron microscopy (TEM), the coleoptile and all internal leaf primordia were removed before harvesting 2-mm^2 tissue samples across the base of leaf 1 (plastochron 5). Third leaves were harvested 11 days after sowing, as the fourth leaf was emerging. For in situ and TEM analyses, tissue samples were taken 1 cm from the base and 1 cm from the tip of the leaf.

Etiolated seedlings were grown in vermiculite in complete darkness at 28 °C for 6 days until the first 1 cm of the unexpanded first leaf was visible above the coleoptile. Tissue was harvested by excising the seedling 1 cm above the shoot apical meristem (under a green safelight), after which plants were transferred to the growth chamber and grown until mutant phenotypes could be scored. For RNA and immunoblot analyses, the coleoptile was removed prior to harvesting. Tissue samples for TEM analysis were taken within 1 cm of the tip of leaf 1. Light-shifted seedlings were grown in complete darkness, as described above, and then shifted to the growth chamber for 24 h. Seedlings were shifted to either moderate (100 μmol m^{-2} s^{-1}) or low (10 μmol m^{-2} s^{-1}) light intensities.

Measurement of photosynthetic pigments

Photosynthetic pigments in light-grown leaves were measured as described in Lichtenthaler and Wellburn (1983). Briefly, pigments were extracted in 80% acetone (v/v) at 4 °C and the slurry cleared by centrifugation at 10,000 *g* for 5 min. The absorbance was read in a SP8-100 UV/VIS PYE UNICAM Spectrophotometer. Pigment concentrations were estimated according to the absorbance coefficients calculated by Lichtenthaler and Wellburn (1983) and expressed as μg of pigment per cm^2 of leaf area.

Antibody and cDNA probes

Antisera raised against Rubisco holoenzyme (*Triticum aestivum*), Rubisco large subunit (Lsu) (*Flaveria bidentis*), Rubisco small subunit (Ssu) (maize), PEPCase (*Amaranthus hypochondriacus*), PPDK (maize), NADP-ME (maize), and NADP-MDH (maize) have been described previously (Langdale et al. 1987; Langdale and Kidner 1994). Sedoheptulose bisphosphatase (SBPase) antibody (wheat) was a gift from T. Dyer (John Innes Institute, Norwich, UK). Vacuolar ATPase antibody (*Kalanchoe*) was a gift from J.A.C. Smith (University of Oxford). eIF4A antibody (tobacco) was a gift from C. Kuhlemeier (University of Bern, Switzerland). With the exception of Lsu and eIF4A antisera, all antibodies used were polyclonal.

Ribulose bisphosphate carboxylase (RuBPCase) small subunit (*RbcS*), RuBPCase large subunit (*rbcL*), PEPCase (*Ppc1*), NADP-ME (*Mod1*), NADP-MDH (*Mdh1*), and PPDK (*Ppdk1*) cDNA clones (pJL10, pJL12, pTN1, pTN5, C30, and pH2, respectively) were as described in Roth et al. (1996). LHCPII (*Cab*) cDNA clone pZLH5 was a gift from Bill Taylor (CSIRO, Canberra, Australia). *Ubiquitin* cDNA (pSkuB1) was a gift from Peter Quail (Plant Gene Expression Center, Albany, Calif., USA).

Preparation of total leaf protein and immunoblot analysis

The isolation of total leaf protein and immunoblot analysis was carried out as in Roth et al. (1996).

Preparation of RNA and RNA gel blot analysis

RNA was purified, electrophoresed and blotted as reported previously (Roth et al. 1996).

Preparation of polysomes

A modification of the procedure of Klaff and Gruissem (1991) was used to isolate total polysomes from leaf tissue. Light-grown

seedling tissue (0.5 g) was ground in liquid nitrogen to a fine powder and added to 1.5 ml buffer. Homogenate was purified and adjusted to 0.5% sodium-deoxycholate as described previously (Klaff and Gruissem 1991). Aliquots of 0.5 ml were layered onto 10.5 -ml 15% to 45% sucrose gradients in 40 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 0.5 mg/ml heparin, 100 μg/ml chloramphenicol and centrifuged for 150 min at 40,000 rpm in a Sorvall (DuPont, Wilmington, Del., USA) Ti 41 rotor at 4 °C. Ten fractions of 650 μl were collected corresponding to the central region of the gradient. Fractions were adjusted to 0.5% SDS, 20 mM EDTA and 80 mM Tris-HCl (pH 9.0). RNA was extracted with 1:1 (v/v) phenol:chloroform and then 24:1 (v/v) chloroform: *iso*amyl alcohol prior to precipitation with 2-propanol. RNA was fractionated on agarose gels and hybridised with a fragment of maize chloroplast DNA (pZMC 460) containing both *rbcL* and *atpB* sequences (kindly provided by A. Barkan, University of Oregon, USA).

In situ localisation of C4 gene products

Plant tissues were fixed in 3:1 (v/v) ethanol/acetic acid for 30 min at room temperature and stored at 4 °C in 70% ethanol. Samples were embedded in Paraplast Plus (Sherwood Medical Co., St Louis, Mo., USA) and sectioned to 8 μm as reported previously (Langdale et al. 1987). RNA in situ hybridisation assays, were carried out using ^{35}S-labelled riboprobes as described in Langdale and Kidner (1994).

Electron microscopy

All tissues were cut under and fixed in Karnovsky's fixative (3% paraformaldehyde; 3% glutaraldehyde; 0.025 M phosphate buffer, pH 7.2) for 2 h at room temperature. All plant tissues that needed to be scored for a mutant phenotype, e.g. etiolated and P1–5 leaves, were stored in 0.025 M phosphate buffer (pH 7.2) after pre-fixing (for a maximum of 7 days). Post-fixation treatments, embedding in TAAB resin (TAAB Laboratory Equipment, Reading, UK), sectioning and staining were carried out as previously reported (Roth et al. 1996). Sections were examined on a JEOL JEM-2000EX transmission electron microscope and photographed using AGFA Scientia EM film 23 D 56 (Leverkusen, Germany).

Results

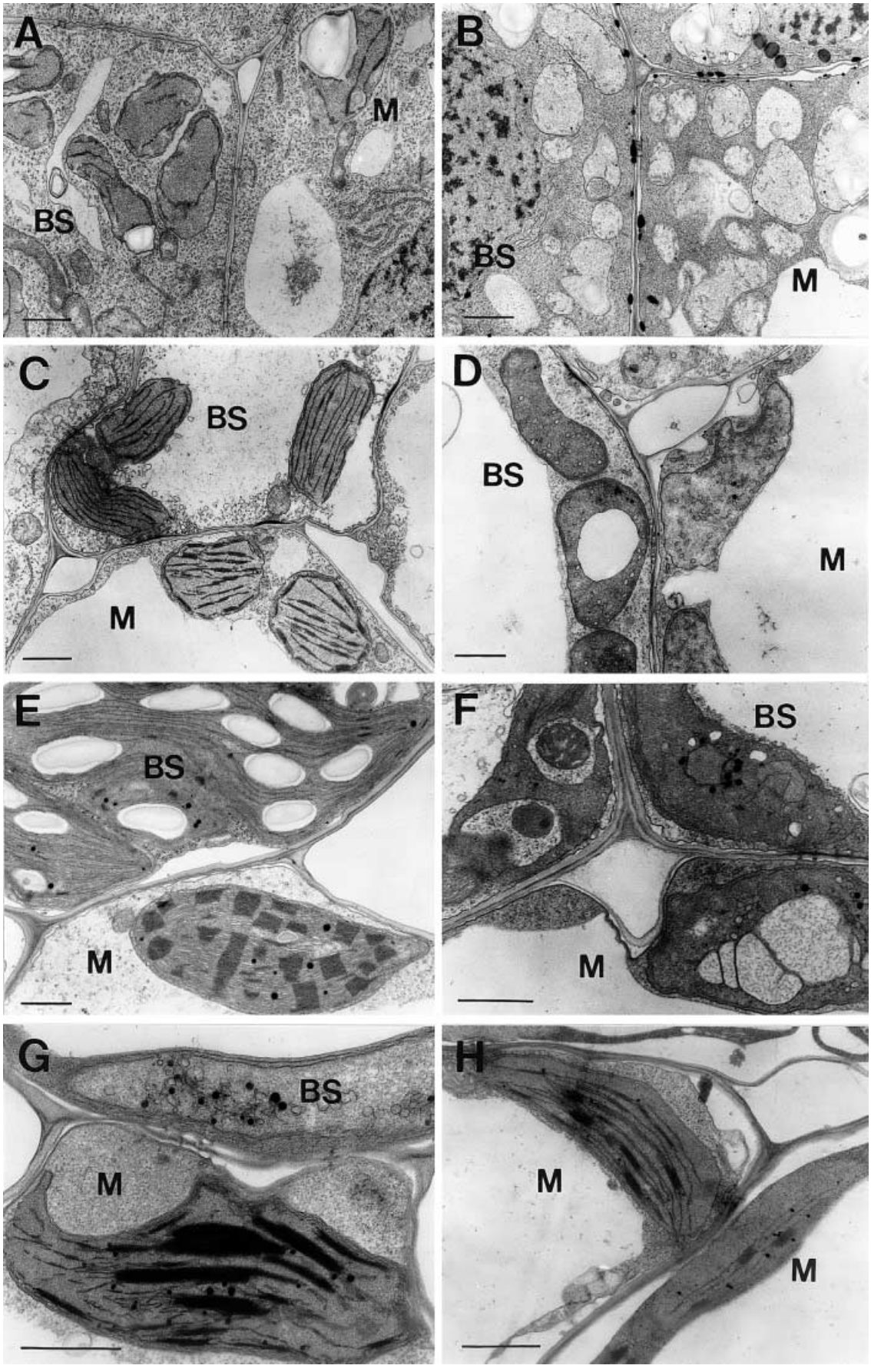
The *pun* mutation disrupts chloroplast biogenesis in both bundle sheath and mesophyll cells

Segregation analyses indicated that the *pun* mutant phenotype is caused by a single recessive nuclear mutation. Leaves of *pun* plants are yellow and do not green at any stage of development unless plants are grown in very dim light (data not shown). Mutant plants die once the endosperm reserves have been depleted. To investigate the extent to which the *pun* mutation disrupts pigment biosynthesis, dark-grown seedlings were shifted to low (10 μmol m^{-2} s^{-1}) or moderate (100 μmol m^{-2} s^{-1}) light for 24 h. Unlike wild-type (*Pun*) siblings, which greened rapidly after exposure to light, *pun* leaves remained yellow and failed to accumulate any visibly detectable chlorophyll (data not shown). This suggested that the *Pun* gene might have a role in synthesising or maintaining normal chlorophyll levels. To quantify this further, pigment concentrations were measured in low- and moderate-light-shifted leaves. As shown in Table 1, when *pun* mutant leaves were exposed to low light conditions, chlorophyll *a*, chlorophyll *b* and carotenoids accumulated to 24%, 29% and 48% of wild-type levels, respectively. Under moderate light conditions, chlorophylls *a* and *b* were reduced by 94% and 92%, respectively, while carotenoid levels were reduced by 65% compared to the wild type. These data suggest that any chlorophyll that is synthesised in mutant leaves is rapidly degraded under moderate light. We cannot exclude the possibility that the *Pun* gene is directly involved in pigment biosynthesis and that the low levels of chlorophyll and carotenoids observed are due to incomplete penetrance of the *pun* mutation. However, the pigment levels seen in plants grown in low light suggest that there is not a major biosynthetic block in either pathway.

To investigate the extent to which chloroplast development is disrupted in *pun* mutant seedlings, chloroplast ultrastructure was examined in young leaf primordia and in seedling leaves grown in moderate light (100 μmol m^{-2} s^{-1}). At plastochron 5 (P5), bundle sheath and mesophyll progenitor cells were cytoplasmically dense and could be distinguished only by their position relative to vein sites; plastids in both cell types were identical (Fig. 1A). (A plastochron is the time interval between the initiations of successive leaf primordia.) Figure 1B shows that in *pun* mutant P5 leaf primordia, plastid structure was aberrant in both bundle sheath and mesophyll progenitor cells. Plastids were reduced in size, were highly vesiculated and lacked the primary thylakoid lamellae characteristic of wild-type plastids at this early developmental stage. Interestingly, electron-dense particles that resemble osmiophilic

Table 1 Concentrations of photosynthetic pigments in leaves of wild-type (*Pun*) and mutant (*pun*) *Zea mays*. Three replicates of each measurement were carried out and standard errors (in parentheses) were determined to account for biological differences between leaves and inherent errors in spectrophotometric procedures

Material	Chl *a* (μg cm^{-2})	Chl *b* (μg cm^{-2})	Chl *a*/*b*	% Normal		Carotenoids (μg cm^{-2})	% Normal
				Chl *a*	Chl *b*		
Moderate light shift							
Pun	17.31 (6.55)	2.85 (0.97)	6.07	100	100	5.22 (1.72)	100
pun	1.05 (0.54)	0.24 (0.83)	4.45	6	8	1.83 (0.14)	35
Low light shift							
Pun	11.96 (1.66)	1.93 (0.48)	6.20	100	100	3.89 (0.46)	100
pun	2.90 (1.34)	0.56 (0.23)	5.16	24	29	1.88 (0.13)	48


A
M
BS
B
BS
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C
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D
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Fig. 1 Transmission electron microscopy of wild-type (**A, C, E**) and *pun* mutant (**B, D, F–H**) leaf tissue of *Zea mays* grown in moderate light (100 μmol m^{-2} s^{-1}). **A, B** Plastochron 4/5 (P4/5) primordia; **C, D** base of third leaf blades; **E–H** tip of third leaf blades. *BS* bundle sheath, *M* mesophyll. Bar = 1 μm

particles were present along the plasma membrane of both cell types in *pun* mutant leaf primordia. Photooxidative damage is unlikely to occur at this stage in development since leaves are enclosed within the coleoptile and are therefore not exposed to a substantial amount of light. Thus, the chloroplast morphology observed at P5 most likely reflects a primary effect of the *pun* mutation.

As a consequence of normal maturation patterns, the strap-shaped maize leaf presents a developmental gradient with immature cells at the base and the most mature cells at the tip of the leaf blade (Sharman 1942; Kirchanski 1975; Sylvester et al. 1990). To determine the effect of the *pun* mutation on plastid biogenesis, chloroplast ultrastructure was examined in leaf sections taken at the base and tip of wild-type (Fig. 1C, E) and mutant (Fig. 1D, F) third leaf blades grown in moderate light (100 μmol m^{-2} s^{-1}). During all stages of leaf development, chloroplast structure in both bundle sheath and mesophyll cells was aberrant. At the base of the *pun* mutant leaf, chloroplasts were vesiculated and lacked the internal lamellae present in the wild type (Fig. 1D). Vesiculation was more pronounced at the leaf tip and was observed in both bundle sheath and mesophyll cell chloroplasts (Fig. 1F). Occasionally, mesophyll cells were observed with chloroplasts that exhibited rudimentary thylakoid stacking (Fig. 1G, H). The existence of these more-normal-looking mesophyll chloroplasts at the leaf tip suggests either that the *pun* mutation is a partial-loss-of-function allele, such that some PUN protein accumulates in older cells, or that a second gene can compensate for loss of PUN activity. In mature cells at the tip of the leaf, it is possible that some aspects of the chloroplast phenotype are due to photooxidative damage and hence represent secondary effects of the mutation. However, aberrant plastid morphologies seen at the base of mutant leaves demonstrate that the wild-type *Pun* gene is essential for normal chloroplast biogenesis.

To distinguish between primary and secondary effects of the *pun* mutation on chloroplast ultrastructure, we examined ultrathin sections of etiolated and light-shifted leaves. In etiolated wild-type leaves, both the bundle sheath and mesophyll cell plastids contained a crystalline prolamellar body as shown in Fig. 2A. In *pun* mutant leaves, however, etioplasts appeared highly vesiculated, lacked primary thylakoid lamellae and contained only rudimentary prolamellar bodies (Fig. 2B). The failure of *pun* mutant plastids to differentiate normally in the dark suggests that PUN plays a light-independent role in plastid biogenesis. Upon exposure to light, wild-type etioplasts differentiate rapidly into chloroplasts (Fig. 2C; Bradbeer 1981). However, after *pun* mutant seedlings were exposed to moderate light (100 μmol m^{-2} s^{-1}) for 24 h, etioplasts did not differentiate into chloroplasts insofar as rudimentary prolamellar bodies could still be observed (Fig. 2D). To determine whether the observed defect in etioplast-to-chloroplast conversion seen in *pun* mutant seedlings was due to photooxidative damage, chloroplast ultrastructure was also examined in seedlings shifted to low light (10 μmol m^{-2} s^{-1}) (Fig. 2E, F). No differences in chloroplast morphology could be observed between seedlings that were shifted to moderate versus low light. Thus, aberrant chloroplast structure in light-shifted *pun* mutant leaves is primarily due to developmental defects.

Thylakoid-associated proteins accumulate to reduced levels in *pun* mutants

Electron micrographs suggested that internal membranes in *pun* mutant chloroplasts are severely disrupted (Figs. 1, 2). To characterise this disruption further, we compared accumulation levels of thylakoid-associated proteins in wild-type and mutant seedlings. Leaf membrane proteins were extracted from etiolated seedlings, seedlings shifted to low light for 24 h (10 μmol m^{-2} s^{-1}) and seedlings shifted to moderate light for 24 h (100 μmol m^{-2} s^{-1}). That is, proteins were isolated from leaves exhibiting the plastid phenotypes shown in Fig. 2. Immunoblots were reacted with antibodies raised against proteins that represent three of the major photosynthetic complexes (PSII, ATPase and Cyt *f*/*b*6). Figure 3A shows that nuclear-encoded LHCPII accumulates to lower levels in *pun* mutant plants than in wild-type siblings, while the chloroplast-encoded proteins CF1a and Cyt *f* are undetectable in mutant tissue. Subunits of the membrane-localised vacuolar ATPase were detected in mutant samples at levels at least equivalent to those in the wild type (Fig. 3B). Thus, there is a specific decrease in the level of chloroplast membrane proteins in *pun* mutants.

The accumulation profile of LHCPII reveals three important points about the *pun* mutation. Firstly, since decreased levels of LHCPII are most apparent in plants shifted to moderate light intensity, some of the perturbations seen in mutant chloroplasts may be due to photooxidative damage of the thylakoid membranes. Secondly, as LHCPII protein is undetectable in dark-grown mutant plants but accumulates within 24 h of exposure to low light, light-signalling pathways are intact in *pun* mutant plants. Thirdly, since nuclear-encoded LHCPII accumulates within *pun* plastids (albeit at reduced levels), chloroplast import pathways are at least partially functional in the mutant.

Our failure to detect CF1a or Cyt *f* protein in mutant leaves (Fig. 3A) suggests that transcription and/or translation of the chloroplast genes *atpA* and *petA* is perturbed in *pun* mutants. Furthermore, since the absence of individual subunits often indicates coordinate loss of the entire complex (Barkan et al. 1986), it can be assumed that the Cyt *f*/*b*6 complex and the chloroplast ATPase fail

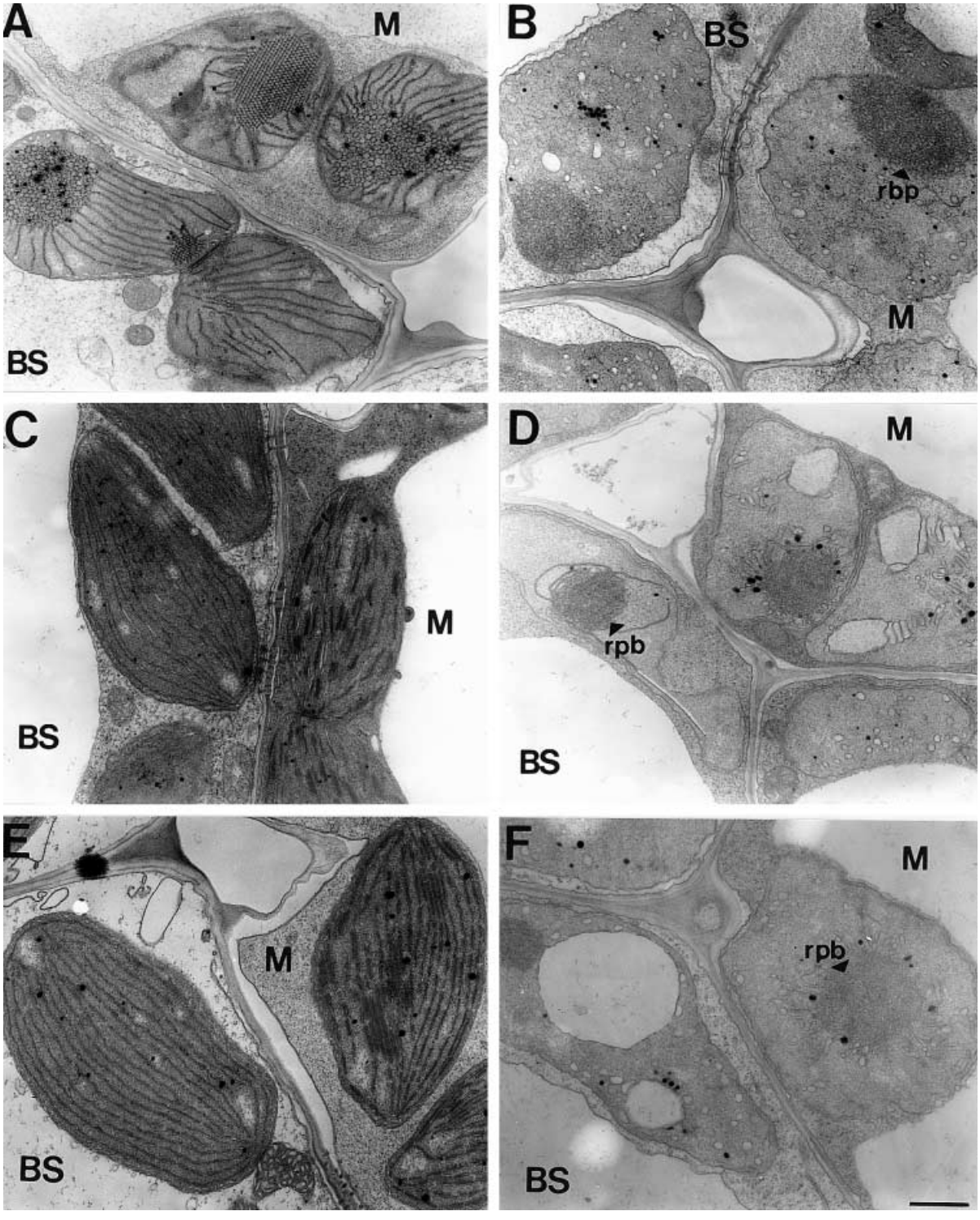

Fig. 2 Transmission electron microscopy of etiolated and light-shifted leaves of wild-type (**A, C, E**) and *pun* mutant (**B, D, F**) seedlings of maize. **A, B** Etiolated leaves; **C, D** greening leaves after a 24-h shift to moderate light (100 μmol m^{-2} s^{-1}); **E, F** greening leaves after a 24-h shift to low light (10 μmol m^{-2} s^{-1}). *BS* bundle sheath, *M* mesophyll, *rpb* rudimentary prolamellar body. Bar = 1 μm

to accumulate in *pun* mutants. Thus, chloroplasts in *pun* mutant plants are not photosynthetically competent.

Rubisco Lsu protein does not accumulate in *pun* mutant leaves

The observations described above suggest that proteins encoded by the chloroplast genome do not accumulate

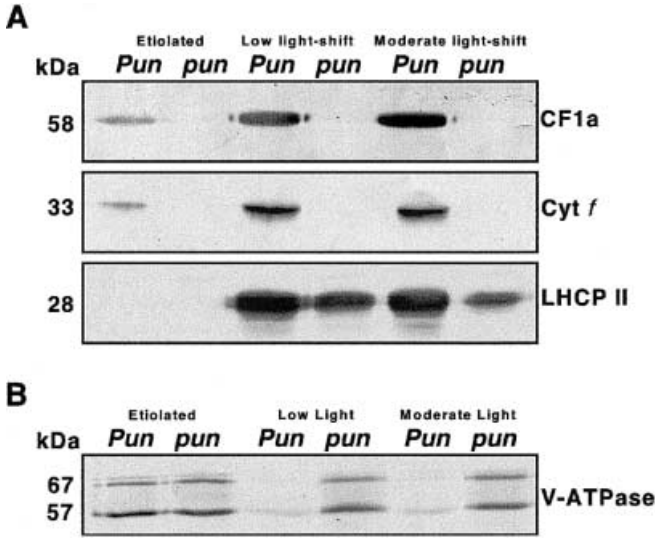

Fig. 3A, B Immunoblot analysis of insoluble proteins extracted from wild-type (*Pun*) and mutant (*pun*) maize seedlings. Seedlings were either grown totally in the dark, or grown in the dark and then shifted for 24 h to low (10 μmol m^{-2} s^{-1}) or moderate (100 μmol m^{-2} s^{-1}) light. **A** Immunoreaction with antibodies raised against the chloroplast proteins CF1a, Cyt *f* and LHCPII. **B** Immunoreaction with antibodies raised against subunits of the vacuolar ATPase. Protein was isolated using a standard weight of tissue per volume of extraction buffer. Equal sample volumes were loaded in each lane. Respective molecular masses (kDa) are indicated on the *left*

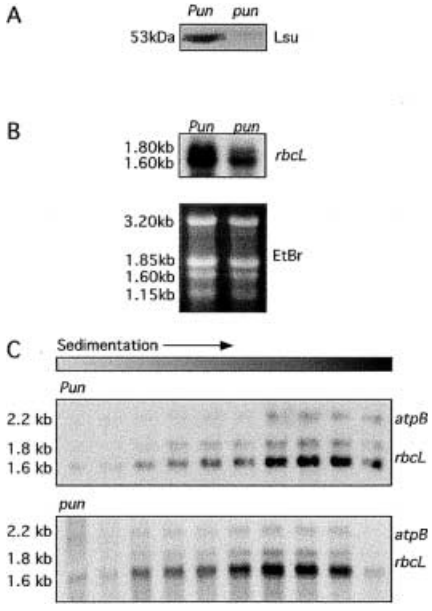

Fig. 4A–C Analysis of Rubisco Lsu accumulation patterns in wild-type (*Pun*) and mutant (*pun*) third leaf blades of maize seedlings grown in low light (10 μmol m^{-2} s^{-1}). **A** Immunoblot of total leaf protein reacted with antibody raised against Rubisco Lsu protein. **B** Northern blot of total leaf RNA hybridised with *rbcL*. Equal amounts of RNA were loaded in each lane as judged by ethidium bromide (*EtBr*) staining of ribosomal RNA. Transcript sizes are indicated. **C** Northern blot of sucrose-gradient-fractionated RNA. Blots were hybridised with a fragment that recognises both *rbcL* and *atpB* sequences. Transcript sizes are indicated

in *pun* mutant plants. To determine whether this effect is limited to membrane-associated proteins or instead represents a more general defect in chloroplast gene expression mechanisms, we assayed the accumulation pattern of the stromal protein Rubisco Lsu in the wild type and in *pun* mutant seedlings. Plants were grown in low light (10 μmol m^{-2} s^{-1}) to avoid possible secondary perturbations due to photooxidative damage. Figure 4A shows that Lsu protein was virtually undetectable in *pun* mutant leaves. This observation supports the idea that the *pun* mutation may condition a general defect that perturbs the accumulation of all proteins encoded by the chloroplast genome.

To determine the level at which *rbcL* gene expression is perturbed in *pun* mutants, we investigated the extent to which *rbcL* transcripts accumulated and are translated. Northern blot analysis showed that *rbcL* transcripts accumulated to approx. 50% of normal levels (as determined by densitometry) in *pun* mutant leaves grown in low light (10 μmol m^{-2} s^{-1}) (Fig. 4B). Thus, *rbcL* transcription or transcript stabilisation processes are at least partially defective in *pun* mutants. Relative to the wildtype, however, Lsu protein levels were decreased to a greater extent than *rbcL* transcript levels. To investigate this further, we examined the association of *rbcL* transcripts with polysomes. The extent to which transcripts are associated with polysomes, provides a means to examine the efficiency of translation initiation and elongation (Barkan 1993; Berry et al.1988). RNA was extracted from wild-type and *pun* mutant tissue, fractionated on 15% to 45% sucrose gradients to separate polysome-associated transcripts from monosomes and free RNA, and then hybridised to *atpB* and *rbcL*. As shown in Fig. 4C, *atpB* and *rbcL* transcripts from wild-type and mutant plants sedimented at similar rates. Furthermore, the primary 1.8-kb *rbcL* transcript was processed correctly into a 1.6-kb transcript and both transcripts associated with polysomes in *pun* mutants. Since translation initiation and the early steps of elongation proceed normally in *pun* mutants, the failure to accumulate Lsu protein must therefore be due to a post-translational defect.

Nuclear photosynthetic gene expression is essentially normal in light-grown *pun* mutant leaves

Previous studies of carotenoid-deficient seedlings suggested that plastid integrity is required for the production of plastid factor(s) which induce transcription of the nuclear genes *Cab* and *RbcS* (reviewed in Oelmuller 1989). We have established that plastid integrity is severely perturbed in *pun* mutants. A number of chloroplast-encoded proteins fail to accumulate (Figs. 3, 4), chloroplast gene expression is perturbed at more than one level (Fig. 4) and chloroplast morphology is aberrant throughout development (Figs. 1 , 2). To examine the extent to which these plastid aberrations influence

nuclear gene expression, *Cab* and *RbcS* transcript levels were compared in wild-type and *pun* mutant seedlings that were either grown in total darkness, in low light (10 μmol m^{-2} s^{-1}) or in moderate light (100 μmol m^{-2} s^{-1}). Figure 5A shows that *Cab* and *RbcS* transcript levels were slightly reduced in light-grown *pun* mutant plants, most noticeably in plants grown in moderate light. This observation suggests that if plastid factors are required for *Cab* and *RbcS* gene expression, those factors are at least partially functional in *pun* mutants and that the decreased transcript levels in plants grown in moderate light may result from photooxidative damage. Notably, however, *RbcS* transcript levels were also reduced in etiolated *pun* plants where photooxidative damage cannot occur. *rbcL* transcript levels were similarly perturbed in both dark- and light-grown plants (Fig. 5B), supporting the idea that PUN plays a light-independent role in chloroplast biogenesis.

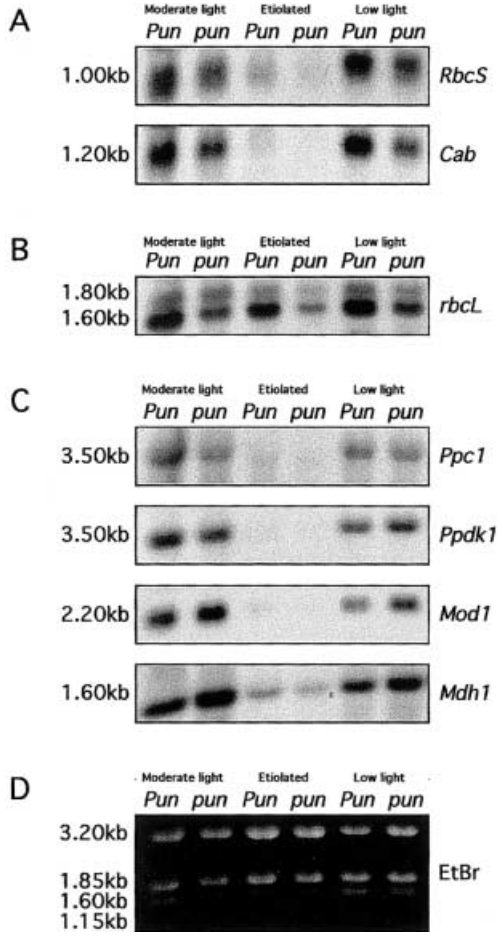

Fig. 5A–D Gel blot analysis of total RNA isolated from wild-type (*Pun*) and mutant (*pun*) third leaf blades of maize. Seedlings were either grown in the dark, grown in low light (10 μmol m^{-2} s^{-1}) or grown in moderate light (100 μmol m^{-2} s^{-1}). **A** Hybridisation of *RbcS* and *Cab* transcripts. **B** Hybridisation of *rbcL* transcripts. **C** Hybridisation of the C_4 transcripts *PPdK1*, *Ppc1*, *Mod1* and *Mdh1*. **D** Equal amounts of total RNA were loaded in each lane as determined by ethidium bromide (*EtBr*) staining of ribosomal RNA. Respective transcript sizes are indicated on the *left* of each panel

To determine the effect of aberrant plastidogenesis on other nuclear photosynthetic genes, we analysed accumulation patterns of transcripts encoding the C_4 carbon-shuttle enzymes. Figure 5C shows that levels of transcripts encoding PEPCase, PPDK, ME and MDH were normal in etiolated, low-light-grown and moderate-light-grown *pun* mutant tissue. Notably, even growth at moderate light, where some degree of photooxidative damage may occur (see above) did not lead to a decrease in transcript levels. Thus, aberrant plastid biogenesis conditioned by the *pun* mutation does not influence C_4 gene expression patterns.

C_4 carbon-shuttle and Calvin cycle enzymes accumulate in *pun* mutants

To determine whether nuclear-encoded photosynthetic proteins are synthesised and accumulate in *pun* mutants, leaf proteins were extracted from seedlings that were grown in the dark and then shifted to low light (10 μmol m^{-2} s^{-1}) or moderate light (100 μmol m^{-2} s^{-1}). Immunoblots were reacted with antibodies raised against cytosolically localised (PEPCase) and chloroplast-targeted (NADP-ME, PPDK, NADP-MDH) carbon-shuttle enzymes and against representative Calvin cycle enzymes [sedoheptulose bisphosphatase (SBPase) and Rubisco Ssu and Lsu]. Figure 6A shows that all of the carbon-shuttle enzymes accumulated to essentially normal levels in *pun* mutant leaves. Similarly, SBPase accumulated to normal levels (Fig. 6B), suggesting that there is no general decrease in Calvin cycle proteins. The only nuclear-encoded photosynthetic protein that failed to accumulate to normal levels in *pun* mutant leaves was Rubisco Ssu. However, since *RbcS* transcripts accumulated (Fig. 5), it is likely that Ssu protein levels are compromised due to aberrant accumulation of the Lsu protein (Rodermel et al. 1988; Avni et al. 1989; Jiang and Rodermel 1995) rather than as a direct result of either the *pun* mutation or aberrant plastid biogenesis.

Spatial and temporal patterns of *Rbcs* and *rbcL* transcript accumulation are normal in *pun* mutants

To determine whether reduced Rubisco levels in *pun* mutants are coupled to aberrant spatial and/or temporal patterns of gene expression, *rbcL* and *RbcS* transcripts were examined in situ in wild-type and mutant P1–5 leaf primordia and seedling leaves grown in moderate light (100 μmol m^{-2} s^{-1}), i.e. in tissue exhibiting the plastid phenotypes shown in Fig. 1. In wild-type leaves, *rbcL* mRNAs accumulate specifically in bundle sheath progenitor cells between P4 (surrounding major vein sites) and P5 (surrounding intermediate vein sites) (Langdale et al. 1988a). As shown in Fig. 7A, although the levels of *rbcL* transcripts were reduced, the timing of

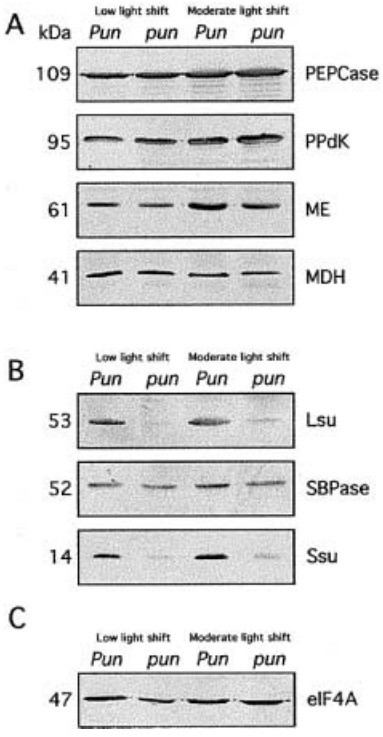

Fig. 6A–C Immunoblot analysis of soluble proteins extracted from wild-type (*Pun*) and mutant (*pun*) maize seedlings. Seedlings were grown in the dark and then shifted for 24 h to low (10 μmol m^{-2} s^{-1}) or moderate (100 μmol m^{-2} s^{-1}) light. **A** Immunoreaction with antibodies raised against the carbon-shuttle enzymes PEPCase, PPDK and NADP-ME, NADP-MDH. **B** Immunoreaction with antibodies raised against the Calvin cycle proteins SBPase and Rubisco (*Lsu* and *Ssu*). **C** Loading control: immunoreaction with antibody raised against the translation factor eIF4A. Protein was isolated using a standard weight of tissue per volume of extraction buffer, using the same tissue as used for the insoluble proteins shown in Fig. 3. Equal sample volumes were loaded in each lane. Respective molecular masses (kDa) are indicated on the *left*

rbcL transcript accumulation in *pun* mutant leaf primordia appeared to be the same as in the wild-type. Furthermore, in both leaf primordia and third leaf blades, *rbcL* transcripts were localised specifically in bundle sheath cells (Fig. 7A). Similarly, spatial and temporal patterns of *RbcS* transcript accumulation were unperturbed (Fig. 7B). These results suggest that the spatial regulation of Rubisco gene expression does not depend on the presence of fully functional chloroplasts or on a functional *Pun* gene product.

Discussion

Mutations that disrupt pigment accumulation are very common in higher plants owing to the large number of nuclear- and chloroplast-encoded gene products that are essential for photosynthesis. Pigment-deficient mutants in maize have therefore been divided into two groups to distinguish those with perturbed pigment biosynthetic pathways from those with developmental defects (Coe et al. 1988). Class-I mutants, such as *lemon white* and *white3* are blocked in the carotenoid biosynthetic pathway. These mutants are partially or fully pigmented in dim light but are completely bleached by moderate light as a result of photooxidative damage (Robertson 1975). Similarly, chloroplast morphology is more severely disrupted in moderate-light-grown as compared to dim-light-grown plants (reviewed in Robertson 1975). In contrast, yellow and pale-green class II mutants represent a group of mutations that disrupt chloroplast biogenesis or chloroplast gene expression in a light-independent manner (Han et al. 1993 , 1995). Although some photooxidative damage is observed in *pun* mutants, particularly with respect to pigment concentrations and LHCPII accumulation, plastid morphology is perturbed in a light-independent manner. Based on these observations, the *pun* mutant falls within class II and is therefore unlikely to be defective in the carotenoid biosynthetic pathway. We also believe that the *Pun* gene does not have a direct role in the chlorophyll biosynthetic pathway, primarily because chloroplast structure in *pun* mutants is disrupted as early as P5 yet chlorophyll biosynthetic mutations characteristically cause chloroplast biogenesis to be arrested during relatively late stages of chloroplast development (Mascia and Robertson 1978; Hudson et al. 1993; Cowan 1995; Runge et al. 1995).

The morphological features of the *pun* mutant phenotype suggest that the *Pun* gene product facilitates normal chloroplast biogenesis in both the light and the dark. In the absence of PUN, chloroplast biogenesis is perturbed very early in development (at least as early as P5). Chloroplasts fail to develop normal membrane structures and there is a concomitant decrease in accumulation of thylakoid-associated photosynthetic complexes. Notably, chloroplast-encoded thylakoid proteins are more severely affected than the nuclear-encoded LHCPII protein. This observation suggests that the *pun* mutation may condition a general defect that affects the accumulation of chloroplast-encoded proteins. In support of this idea, chloroplast-encoded *rbcL* transcript and protein levels are also reduced in mutant plants. Since Lsu protein levels are more severely perturbed than *rbcL* transcript levels and *rbcL* transcripts load onto polysomes, the *pun* mutation probably does not condition a general defect in the chloroplast transcriptional machinery or in translation initiation events. It is therefore likely that PUN plays either a specific or widespread role in the post-translational regulation of chloroplast-localised complexes. A number of observations support the suggestion that PUN has a general role in these post-translational processes. For example, characterisation of the maize mutant *bundle sheath defective 2* (*bsd2*) has shown that specific loss of Rubisco protein is not accompanied by developmental defects in plastid biogenesis programs (Roth et al. 1996; Brutnell et al. 1999). Similarly, characterisation of the maize *psa*, *psb* and *pet* mutants has shown that individual chloroplast membrane complexes can be perturbed without affecting other chloroplast components (Barkan et al.

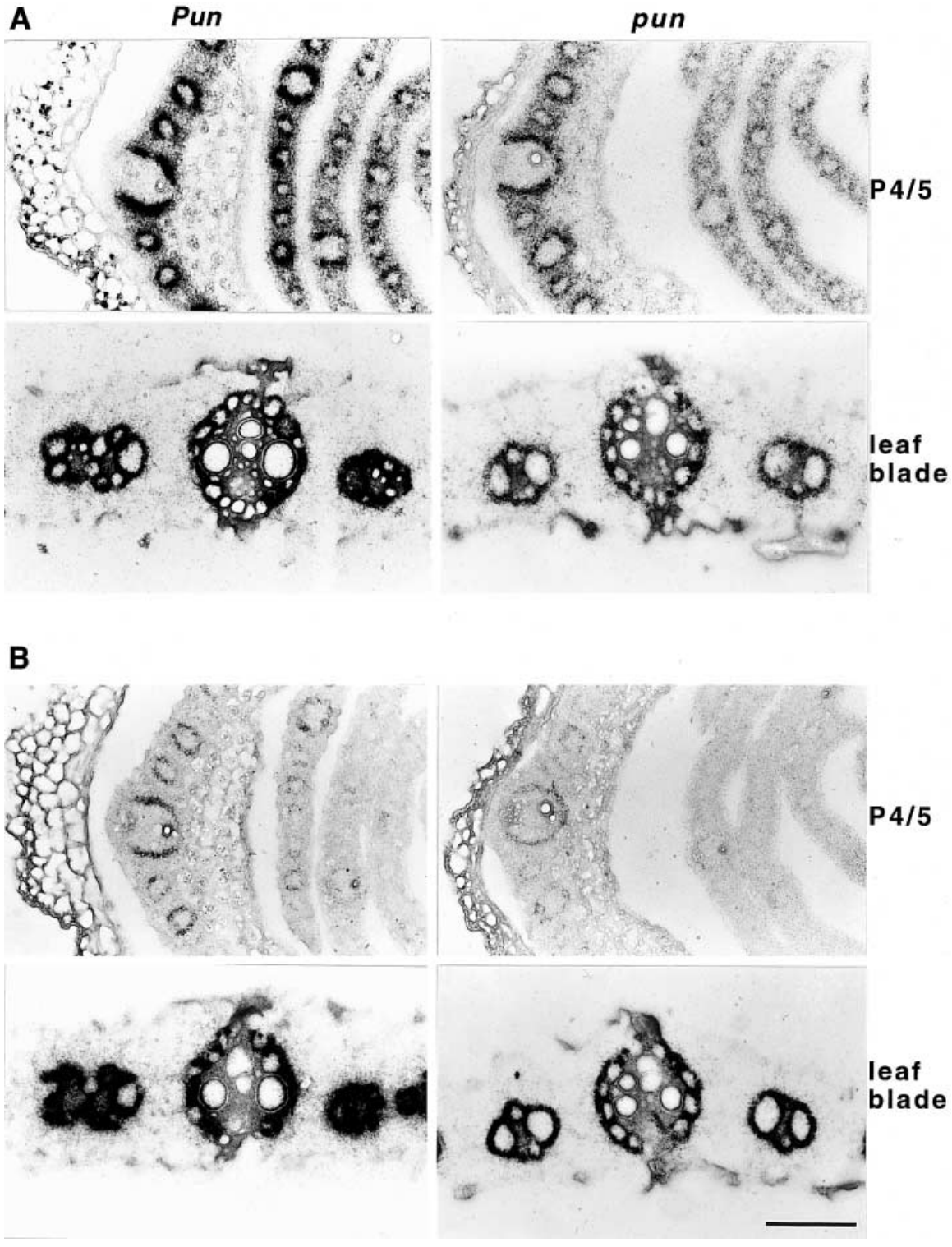

Fig. 7 In situ detection of *rbcL* (**A**) and *RbcS* (**B**) transcripts in plastochron 4/5 (P4/5) leaf primordia and third leaf blades of wild-type (*Pun*) and mutant (*pun*) maize siblings grown in moderate light (100 μmol m^{-2} s^{-1}). Bar = 50 μm

1995). Thus, it is unlikely that the *pun* mutant phenotype results from the specific misregulation of any individual chloroplast component.

Previous work has suggested that plastid signals are required for the accumulation of nuclear-encoded *RbcS* and *Cab* transcripts (reviewed in Oelmuller 1989; Taylor 1989). In *pun* and other maize yellow mutants (Mayfield and Taylor 1984), *RbcS* and *Cab* transcript levels are essentially normal. Thus, plastid signals are intact in these mutants despite aberrant plastid morphology. The accumulation of both carbon-shuttle and Calvin cycle

enzymes in *pun* mutants suggests that neither the *Pun* gene product nor chloroplast function is required to establish C_4 photosynthetic gene expression patterns. It has previously been proposed that during photomorphogenesis in maize, light-enhanced signals emanate from the leaf veins to pattern bundle sheath and mesophyll cells (Langdale et al. 1988b). Cells perceive and respond to these signals on the basis of their distance from the vein. Since spatial and temporal patterns of gene expression are unperturbed in *pun* mutants, bundle sheath and mesophyll cells adopt their appropriate fate. Thus, the proposed light-enhanced positional signals that emanate from the vein to establish the spatial patterning of photosynthetic gene expression are not dependent on chloroplast development or function.

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References

Avni A, Edelman M, Rachailovich I, Aviv D, Fluhr R (1989) A point mutation in the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase affects holoenzyme assembly in *Nicotiana tabacum*. EMBO J 8:1915–1918

Barkan A (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell 5:389–402

Barkan A, Miles D, Taylor WC (1986) Chloroplast gene expression in nuclear photosynthetic mutants of maize. EMBO J 5:1421–1427

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

Batschauer A, Mosinger E, Kreuz K, Dorr I, Apel K (1986) The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light harvesting chlorophyll *a*/*b* protein. Eur J Biochem 154:625–634

Berry JO, Carr JP, Klessig DF (1988) mRNAs encoding ribulose-1,5-bisphosphate carboxylase remain bound to polysomes but are not translated in amaranth seedlings transferred to darkness. Proc Natl Acad Sci 85:4190–4194

Bradbeer JW (1981) Development of photosynthetic function during chloroplast biogenesis. In: Stumpf PK, Conn EE (eds) Biochemistry of plants, vol 8. Academic Press, New York, pp 424–472

Brown WV (1975) Variations in anatomy, associations, and origins of Kranz tissue. Am J Bot 62:395–402

Brutnell TP, Sawers RJH, Mant A, Langdale JA (1999) BUNDLE SHEATH DEFECTIVE2, a novel protein required for post-translational regulation of the *rbcL* gene of maize. Plant Cell 11:849–864

Coe EH, Neuffer MG, Hoisington DA (1988) The genetics of corn. In: Sprague GF, Dudley JW (eds) Corn and corn improvement. American Society of Agronomy, Madison, pp 111–224

Cowan AK, Botha CEJ, Hartley BJ, Cross RHM (1995) Ultrastructural changes in ageing leaves of a light-grown achlorophyllous mutant of barley. Physiol Plant 94:391–398

Edwards GE, Walker DA (1983) C3, C4: mechanisms and cellular and environmental regulation of photosynthesis. Blackwell, Oxford

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

Han C, Derby RJ, Schnable PS, Martienssen RA (1995) Characterization of the plastids affected by Class II albino mutations of maize at the morphological and transcript levels. Maydica 40:13–22

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

Jiang C, Rodermel SR (1995) Regulation of photosynthesis during leaf development in *RbcS* antisense DNA mutants of tobacco. Plant Physiol 107:215–224

Kirchanski SJ (1975) The ultrastructural development of the dimorphic plastids of *Zea mays* L. Am J Bot 62:695–705

Klaff P, Gruissem W (1991) Changes in chloroplast mRNA stability during leaf development. Plant Cell 3:517–529

Langdale JA, Kidner CA (1994) *bundle sheath defective*, a mutation that disrupts cellular differentiation in maize leaves. Development 120:673–681

Langdale JA, Metzler MC, Nelson T (1987) The *argentia* mutation delays normal development of photosynthetic cell-types in *Zea mays*. Dev Biol 122:243–255

Langdale JA, Rothermel BA, Nelson T (1988a) Cellular patterns of photosynthetic gene expression in developing maize leaves. Genes Dev 2:106–115

Langdale JA, Zelitch I, Miller E, Nelson T (1988b) Cell position and light influence C4 versus C3 patterns of photosynthetic gene expression in maize. EMBO J 7:3643–3651

Langdale, JA, Hall LN, Roth R (1995) Control of cellular differentiation in maize leaves. Philos Trans R Soc Lond Ser B 350:53–57

Lichtenthaler HK, Wellburn AR (1983) Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochem Soc Trans 11:591–592

Mascia PN, Robertson DS (1978) Studies in chloroplast development in four maize mutants defective in chlorophyll biosynthesis. Planta 143:207–211

Mayfield SP, Taylor WC (1984) Carotenoid-deficient maize seedlings fail to accumulate light-harvesting chloroplhyll *a*/*b* binding protein. Eur J Biochem 144:79–84

Mayfield SP, Taylor WC (1987) Chloroplast photooxidation inhibits the expression of a set of nuclear genes. Mol Gen Genet 208:309–314

Meierhoff K, Westhoff P (1993) Differential biogenesis of photosystem II in mesophyll and bundle sheath cells of monocotyledonous NADP-malic enzyme-type C4 plants: the non-stoichiometric abundance of the subunits of photosystem II in the bundle sheath chloroplasts and the translational activity of the plastome-encoded genes. Planta 191:23–33

Nelson T, Langdale JA (1992) Developmental genetics of C4 photosynthesis. Annu Rev Plant Phys Plant Mol Biol 43:25–47

Oelmuller R (1989) Photooxidative destruction of chloroplasts and its effect on nuclear gene expression. Photochem Photobiol 49:229–239

Robertson DS (1975) Survey of the albino and white endosperm mutants of maize: their phenotypes and gene symbols. J Hered 66:67–74

Rodermel SR, Abbott MS, Bogorad L (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell 55:673–681

Roth R, Hall LN, Brutnell TB, Langdale JA (1996) *bundle sheath defective2*, a mutation that disrupts the coordinated development of bundle sheath and mesophyll cells in maize. Plant Cell 8:915–927

Runge S, van Cleve B. Lebedev N, Armstrong G, Apel K (1995) Isolation and classification of chlorophyll-deficient *xantha* mutants of *Arabidopsis thaliana*. Planta 197:490–500

Sharman BC (1942) Developmental anatomy of the shoot of *Zea mays* L. Ann Bot 6:245–284

Sylvester AW, Cande WZ, Freeling M (1990) Division and differentiation during normal and *liguleless-1* maize leaf development. Development 110:985–1000

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu Rev Plant Phys Plant Mol Biol 40:211–233

Wrischer M (1989) Ultrastructural localization of photosynthetic activity in thylakoids during chloroplast development in maize. Planta 177:18–23