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ORIGINAL PAPER

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Differential regulation of genes transcribed by nucleus-encoded plastid RNA polymerase, and DNA amplification, within ribosome-deficient plastids in stable phenocopies of cereal albino mutants

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Abstract We isolated stable albino plants of barley and maize by inhibiting plastid protein synthesis with streptomycin and propagating bleached seedlings in the absence of antibiotics in vitro. Albino plants are deficient in plastid translation products and plastid ribosomal RNAs, and are stable phenocopies of the barley *albostrians* and maize *iojap* mutants, which contain ribosome-free plastids. Once plastid ribosomes are lost they cannot be re-synthesized because about one-third of plastid ribosomal proteins are themselves plastid encoded. The group II/subgroup IIA intron in plastid *rpl2* transcripts was not processed in albinos, providing strong evidence for the absence of plastid translation. Photosynthesis-related plastid mRNAs and plastid tRNAs were down-regulated in albino leaves. A differential influence of plastid ribosome deficiency on mRNA levels allowed us to divide genes transcribed by nucleus-encoded plastid RNA polymerase into two groups. Northern analysis revealed increases in the levels of *clpP*, *rpl2*, *rpl23*, *rps15* and *rpoB* mRNAs in total RNA from albino leaves relative to those in green leaves. In contrast, albinism did not increase the band intensities of *rps2* and *rps4* messages. Plastid ribosome-associated factor(s) or plastid-encoded product(s) play a role in the initiation, termination, processing or stability of transcripts containing *trnG*(UCC) and *rps4*. Excision and 100-fold amplification of a 5.2-kb region of plastid DNA encompassing the *trnG*(UCC) and *trnE*(UUC) genes was observed in one of four albino barley plants. Gene amplification was correlated with the accumulation of abundant novel transcripts derived from regions flanking the *trnG*(UCC) gene.

Keywords *albostrians* · Chloroplast DNA · *iojap* · RNA polymerases · Streptomycin

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Introduction

Plastids contain their own genetic system comprised of a circular double-stranded DNA genome and a prokaryotic-like expression apparatus. The cereal plastid genome is typically 135–140 kb in size and encodes around 70 polypeptides, a complete set of 30 tRNAs, and the structural RNAs of plastid ribosomes (Hiratsuka et al. 1989; Maier et al. 1995). Expression of plastid genes is essential for the development of differentiated plastids, such as chloroplasts, from proplastids (Baumgartner et al. 1993). An *Escherichia coli*-like, plastid-encoded plastid RNA polymerase (PEP) is responsible for the synthesis of abundant mRNAs from photosynthesis-related plastid genes in chloroplasts (Hess et al. 1993; Allison et al. 1996). The core subunits of PEP are encoded by plastid DNA (ptDNA), whilst the sigma subunits are encoded in the nucleus (Allison 2000). A T7/T3 phage-like nucleus-encoded plastid RNA polymerase (NEP) appears to be largely responsible for transcribing non-photosynthetic plastid genes in chloroplasts and non-green plastids (Hess et al. 1993; Allison et al. 1996; Hedtke et al. 1997). NEP and PEP transcribe distinct sets of plastid genes (Hajdukiewicz et al. 1997). Variations in the levels of plastid transcripts during development result from differential usage of NEP and PEP promoters (Hajdukiewicz et al. 1997; Hubschmann and Börner 1998) and changes in RNA stability (Baumgartner et al. 1993; Krause et al. 2000).

The involvement of NEP in plastid gene expression has been studied in mutant plants that are deficient in PEP (Allison et al. 1996; De Santis-Maciossek et al. 1999). This elegant reverse genetics approach is limited to those few species in which methods for plastid

transformation have been established. The roles of nucleus-encoded factors in plastid gene expression can also be studied in albino plants lacking plastid ribosomes. Ribosome-deficient plastids are unable to translate plastid mRNAs and contain nucleus-coded proteins. Analyses of ribosome-deficient plastids in the *albostrians* (Hess et al. 1993) and *iojap* (Walbot and Coe 1979) mutants in barley and maize, respectively, have identified NEP transcribed genes (Hess et al. 1993; Silhavy and Maliga 1998), and nucleus-encoded editing and splicing factors in plastids (Zeltz et al. 1993; Hess et al. 1994; Karcher and Bock 1998; Vogel et al. 1999). Studies on plants with ribosome-deficient plastids have also revealed a requirement for functional plastids in specifying cell type (Pyke et al. 2000) and in inter-organellar signalling (Hedtke et al. 1999). Despite these advances, the limited abundance of experimental material, which is restricted to a few genotypes, has hindered the widespread use of ribosome-deficient plastids to study the plastid genetic system in cereals.

We recently described conditions in which inhibition of plastid protein synthesis with spectinomycin leads to irreversible loss of plastid ribosomes and results in stable albinism in Brassicaceae (Zubko and Day 1998). However, the binding site for spectinomycin is not present in 16S ribosomal RNA (rRNA) from cereals (Harris et al. 1994), making them resistant to this antibiotic. The target site for streptomycin is conserved in cereal 16S rRNA (Harris et al. 1994), and streptomycin-induced bleaching has been demonstrated in barley (Kirk and Juniper 1963) and rice (Sarma and Patnaik 1982; Yoshida et al. 1998). However, removal of the antibiotic resulted in re-greening (discussed in Kirk and Tilney-Bassett 1967), which prevented the isolation of stable albino plants. Here we report the successful isolation of stable albino plants following exposure of barley and maize seeds to streptomycin. These albino plants are stable phenocopies of the *albostrians* and *iojap* mutants. We characterized protein and RNA levels in phenocopies by Western and Northern analyses. Our results indicate a role for plastid protein synthesis in the differential accumulation of RNA from NEP transcribed genes. In addition, we found that loss of plastid ribosomes can lead to excision and amplification of the *trnG*(UCC)-*trnE*(UUC) region of ptDNA and the production of novel and abundant transcripts.

Materials and methods

Isolation of chlorophyll-deficient plants

Seeds of *Hordeum vulgare* L. (cv Maris Otter) and *Zea mays* L. (hybrid F1 Dickson) (Sutton Seeds) were sterilized in 10% sodium hypochlorite (BDH, approximately 12% w/v available chlorine) for 30 min and subsequently washed five times with sterile water. Sterilized seeds of barley and maize were germinated on MS salts medium (Murashige and Skoog 1962) supplemented with: 100 mg/l *myo*-inositol, 1 mg/l thiamine hydrochloride, 2 mg/l pyridoxine hydrochloride, 1 mg/l 6-benzylaminopurine (Sigma), 80 μg/l α-napthylene acetic acid (Duchefa), 30 g/l sucrose, 0.5 g/l 2-(N-morpholino)-ethanesulfonic acid (Duchefa) and 0.5–2 g/l streptomycin sulphate (Sigma). In some cases, barley seeds were first germinated in sterile water containing 0.5 g/l streptomycin for 1 week before being transferred to solid medium containing streptomycin sulphate. Bleached shoots were cut after 2–5 weeks and transferred to streptomycin-free medium. Shoots were transferred to fresh media every 3 weeks, resulting in the isolation of variegated and albino plants. Bleached maize seedlings were obtained by soaking seeds in 3 g/l or 10 g/l streptomycin for 48 h before planting them in soil. Unlike albinism, which is stable and requires the propagation of albino plants in vitro, bleaching can result in re-greening if the plants survive.

Isolation and Western analysis of total plant proteins

Protein extracts were prepared from 150 mg of frozen tissue (green and chlorophyll-deficient lines), which was homogenized in 300 μl of buffer [80 mM TRIS-HCl pH 7.5, 9% (v/v) glycerol, 8% (w/v) SDS, 0.35% (v/v) β-mercaptoethanol], boiled for 3 min and clarified by centrifugation. A 15-μl aliquot of each extract was fractionated by SDS/PAGE (10% gels). Protein bands were visualized by staining with silver salts (Sambrook et al. 1989). For immunoblot analysis, proteins were electroblotted to nitrocellulose (Hybond-ECL, Amersham) and incubated with polyclonal anti-D1 antibodies or monoclonal antibodies raised against α-tubulin (TAT antibody; Woods et al. 1989) and Rubisco LS (Theobald et al. 1998). Bound antibodies were detected by chemiluminescence (Amersham ECL Western blotting detection kit).

Nucleic acid analyses

Total RNA (3 μg) was fractionated on 1% (w/v) agarose-formaldehyde gels, and blotted (10×SSC) onto Gene Screen nylon membranes (NEN). Aliquots of total DNA (1 μg) were digested with restriction enzymes, fractionated on TRIS-acetate agarose gels (0.8%) and alkali-blotted to Gene Screen membranes. Gel-purified probes were prepared from restriction enzyme fragments and PCR products generated from previously cloned barley ptDNA fragments (Day and Ellis 1985) and labelled by random priming (High Prime Kit, Roche). The positions of the probes used are given with reference to the 134,525-bp rice plastome (Hiratsuka et al. 1989): *psbA*, 1-kb *Pvu*II fragment (134198–687); *rps15*, 0.8-kb *Xho*I-*Apa*I fragment (100509–101278); *rbcL*, 1.1-kb *Pst*I-*Hin*dIII fragment (54263–55369); *clpP*, 0.64-kb *Pst*I-*Hin*dIII fragment (67731–68373); plastid rDNA, 8.4-kb *Pst*I fragment (88398–96795). The PT14 probe, a *Pst*I-*Sac*I fragment (11265 to ~14800; the *Sac*I site is not conserved in rice), spans from *trnS*(UGA) to *trnG*(UCC). Probes for tRNA-Glu(UUC) and *atpB* mRNA were made by annealing *trnE*-F (5′-CTCTCTTTCAAGGAGGCAGCGGGGATTCGACTT-3′) with *trnE*-R (5′-TGGTACCCCCAGGGGAAGTCGAAT-3′) (15678–15722), and *atpB*-F (5′-CTTGTGARGTACARCAATTATTAGGAAATAATCG-3′) with *atpB*-R (5′-GCACTCATAGCTACAGCTCTAACTCGATTATTT-3′) (53069–53126), and filling-in 5′ overhangs with [α-^{32}P]dCTP, dATP, dGTP, dTTP and Klenow enzyme. Gene-specific probes for *atpF*, *rpoB*, *rps2*, *rps4*, *rpl2*, *rpl23*, *trnG*(UCC) and *ycf9* were prepared from cloned barley ptDNA (Day and Ellis 1985) sequences by PCR amplification using the following primer pairs: (1) for *atpF*, 5′-ATGAAAATGTAACCCATC-3′ and 5′-GCACGAATCGTACGGAAATG-3′; (2) *rpoB*, 5′-ATGCTCCGGAATGGAAACGAGGGAA-3′ and 5′-TCAAACTTCCTCCCTATTAACC-TGG-3′; (3) *rps2*, 5′-ATGACAAGAAGATATTGGAACATCA-3′ and 5′-TCAACGATTTCTTATATAGAGAGAA-3′; (4) *rps4*, 5′-TC AAGTCTGACGAGAGTAATATTCT-3′ and 5′-GAGTCTTCATGTCCCGTTATCGAGG-3′; (5) *rpl2*, 5′-AACACCTATCCCGAGCACAC-3′ and 5′-TTACTTACGGCGACGAAGA-3′; (6) *rpl23*, 5′-TTACTTACGGCGACGAAGAATAAAA-3′ and 5′-ATGGATGGAATCAAATACGCAGTAT-3′; (7) *trnG*(UCC), 5′-GGTTATAGTCGACGTTGGTTGATTA-3′ and 5′-GTGTGATTCGTTCTATTAATAACTG-3′; and (8) *ycf9*, 5′-AGGAGAGAGAG-

GGATTCGAACCCTC-3′ and 5′-CTACGGTATATATTTTAT-AGCGTAT-3′.

The 7-kb insert from pBG35 (Goldsbrough and Cullis 1981) was used as a probe for nuclear rDNA. After DNA hybridization blots were washed in 0.1×SSC, 0.1% (w/v) SDS at 50–60°C. Blots were incubated in 50 μg/ml RNase A in 1×SSC at room temperature for 8 h to remove filter-bound RNA. Membrane-bound ^{32}P was quantified by phosphorimaging with a Fuji BAS2000 Bioimager and AIDA (v 2.0) Image analysis software.

Results

Treatment with streptomycin induces stable albinism in barley and maize

Seeds were germinated in vitro on medium containing streptomycin. The resulting bleached seedlings were then transferred to medium lacking streptomycin. Transfer of barley seedlings to antibiotic-free medium gave rise to albino (Fig. 1A), green and variegated plants. In contrast, bleached maize plants remained uniformly white on medium lacking streptomycin (Fig. 1B). Variegated barley plants segregated green, or more rarely albino, shoots. No green sectors were detected on maize and barley albino plants cultivated on antibiotic-free medium for over 10 months. Variegation was maintained in a number of barley plants after their transfer to soil (Fig. 1C) and this variegation persisted in the spike (Fig. 1D). The spikes in variegated regions of these plants resembled those found in variegated barley mutants (Döring et al. 1999). One hundred and sixty seeds collected from these spikes were incubated in vitro. Only 22% of these seeds germinated and all of these produced green seedlings. Genetic analysis of albino plants was not possible since they did not flower.

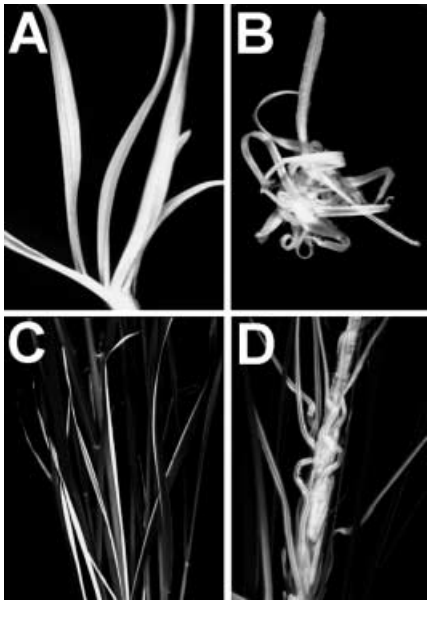

Fig. 1A–D Streptomycin-induced albinism in cereals. **A** Stable albino barley plant. **B** Stable albino maize plant. **C** Streptomycin-induced variegated barley plant growing in soil. **D** Spike from a tiller of a variegated barley plant

Analyses of plastid translation products in albino barley plants

Streptomycin-induced albinism can be explained by permanent loss of plastid ribosomes (Zubko and Day 1998). The levels of plastid translation products were investigated by SDS-PAGE and Western analyses. The top panel in Fig. 2 shows total soluble proteins from green (G) and albino (A) plants derived from separate seeds. No differences were found between the four green barley lines. The large subunit (LS) of ribulose bisphosphate carboxylase (Rubisco) and the D1 protein of photosystem II are two abundant, plastid-encoded, proteins normally present in green leaves. When total soluble proteins were compared, the most prominent difference between green and albino plants was the absence of a major LS-Rubisco band (Fig. 2) in the extracts from albinos. The absence of LS-Rubisco in albino plants was confirmed by Western analysis using a monoclonal antibody specific for the LS (Fig. 2). The D1 protein was also not detectable in samples from albino leaves on Western blots. In contrast, the nucleus-encoded protein α-tubulin accumulates to similar levels in green and albino plants (Fig. 2). The absence of normally abundant plastid translation products is consistent with a deficiency of plastid ribosomes in albino plants.

Reduced accumulation of plastid rRNAs, mRNAs for photosynthesis-related proteins, and tRNAs in albino barley plants

Samples of total RNA from the green and albino plants used for Western analysis were fractionated by electrophoresis on denaturing agarose gels, and analyzed on Northern blots. Ethidium bromide staining (upper

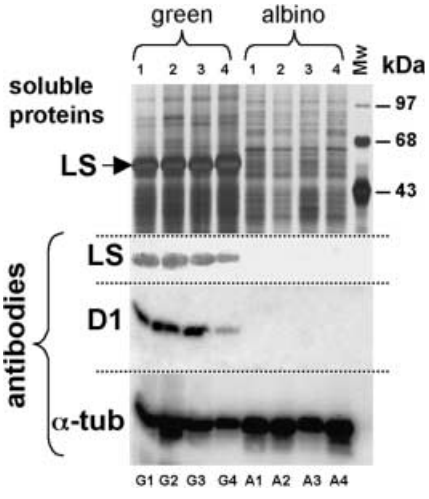

Fig. 2 Plastid translation products in green and albino barley plants. *Upper panel* Total soluble proteins extracted from green (G) and albino (A) plants, fractionated by SDS/PAGE and stained with silver salts. LS is the large subunit of Rubisco. *Lower panels* Western analysis of soluble proteins using the indicated antibodies. D1 is the 32-kDa core protein of photosystem II; α-tub is α-tubulin

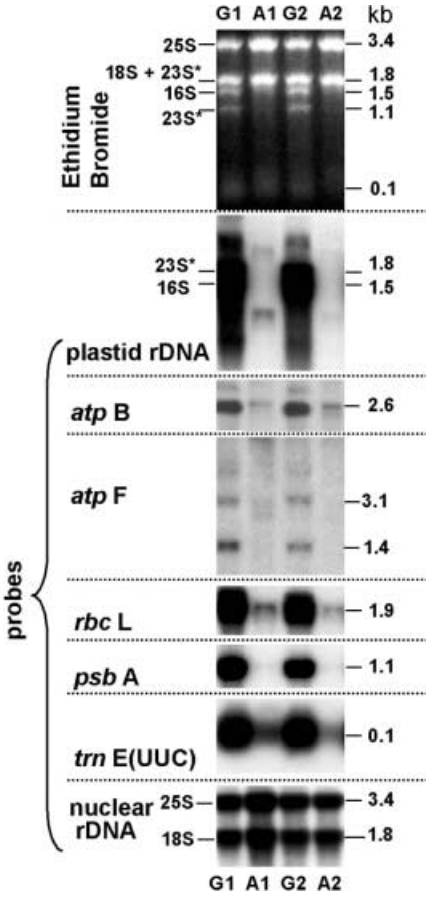

Fig. 3 Down-regulation of plastid transcripts in albino barley plants, as revealed by Northern analysis of total RNA. *Top panel* Ethidium bromide-stained formaldehyde gel. Nuclear rRNAs (25S, 18S) and plastid rRNAs (23S, 16S) are marked. Most of the 23S rRNA migrates as two bands of 1.8 and 1.1 kb (23S*). *Lower panels* Blots were hybridized with the indicated probes

panel, Fig. 3) revealed similar amounts of total RNA per lane, as judged by the intensities of the bands representing 25S and 18S rRNAs. This was confirmed by probing blots with a fragment of nuclear rDNA (lower panel, Fig. 3). Intact 23S (2.9 kb) rRNA is a minor species and most 23S rRNA migrates as 1.8 and 1.1 kb bands as a result of breakage (Poulsen 1983). These 23S derivatives are denoted as 23S*. The 1.8-kb 23S* rRNA band co-migrates with 18S cytosolic rRNA. The plastid 23S (1.1 kb) and 16S (1.5 kb) rRNA bands accumulate to high levels in green plants (G), but are not detectable in albino (A) lanes. Northern analysis with a probe for the 1.8-kb 23S* and 1.5-kb 16S rRNA bands revealed faint rRNA signals in albino lanes, which do not co-migrate with the major plastid rRNA species present in green plants. Image analysis showed that these minor, albino-specific, bands represent less than 1% of the rRNA band intensities in lanes G1 and G2. Similar minor rRNA plastid transcripts with altered sizes have also been detected in barley plants derived from albino pollen (Dunford and Walden 1991). These results suggest that plastid ribosome assembly, which is blocked in albino leaves due to lack of plastid-encoded ribosomal proteins, is involved in the maturation and stabilization of plastid rRNA.

The plastid genes *atpB*, *atpF*, *rbcL* and *psbA* encode components of ATPase (beta and I subunits), LS-Rubisco and the D1 protein, respectively. The *atpB*, *atpF*, *rbcL* and *psbA* mRNAs are clearly detectable by Northern blot analysis in green plants. Albino plants contain dramatically reduced levels of these mRNA species. Phosphor image analysis showed that the *rbcL* and *psbA* mRNAs in albino leaves were present at 9% and 2%, respectively, of the levels found in green leaves. These genes are predominantly transcribed by PEP, which cannot be synthesized in plastids lacking functional ribosomes (Hess et al. 1993; Allison et al. 1996). Low levels of *atpB* and *rbcL* mRNAs were also detected in albino RNA on gels where albino and green lanes were well separated (not shown). The product of the *trnE* gene is tRNA-Glu(UUC), which has a dual function in protein and chlorophyll synthesis (Schon et al. 1986). Although *trnE*(UUC) is transcribed by a NEP (Hess et al. 1992a; Chiba et al. 1996), the levels of its product are reduced by from 10% (clone A2) to 18% (clone A1) in albino leaves compared to green leaves. Transcripts of *trnE*(UUC) are also reduced in the *albostrians* mutant (Hess et al. 1992b). The *atpF* probe hybridizes to two RNA species of 1.4 and 3.1 kb in samples from green leaves. The 1.4-kb species is not detected in albino leaves, and appears to be replaced by a band of higher molecular weight that migrates just below the 3.1-kb *atpF* species (Fig. 3, lane A1). The *atpF* gene contains a group II/subgroup IIA intron (Bonen and Vogel 2001), which requires plastid translation for splicing (Hess et al. 1994; Vogel et al. 1999). Because *atpF* RNAs are down-regulated in ribosome-deficient plastids – making it difficult to visualize unprocessed transcripts – analysis of intron-containing messages such as *rpl2* that are up regulated in albino plants provides a better demonstration of plastid splicing defects in albinos (see below).

Accumulation of plastid transcripts of non-photosynthetic genes in albino barley plants

NEP transcribes plastid genes whose products are not related to photosynthesis. These include the genes for the proteolytic subunit (*clpP*) of the ATP-dependent protease Clp, the beta subunit of PEP (*rpoB*) and components of the small (*rps2*, *rps4*, *rps15*) and large (*rpl2*, *rpl23*) subunits of plastid ribosomes (Hess et al. 1993; Hubschmann and Börner 1998; Silhavy and Maliga 1998). Only transcripts synthesized by the NEP will accumulate in streptomycin-induced albino plants, since these lack a functional translation apparatus and cannot make PEP. Figure 4 shows that transcripts containing the *rps15*, *clpP*, *rpl23*, *rpl2*, *rps2*, *rps4* and *rpoB* sequences indeed accumulate in albino leaves. A

comparison of band intensities between lanes loaded with albino-leaf RNA versus green-leaf RNA distinguishes two broad classes of genes transcribed by NEP. In the first class, illustrated by *rps2* and *rps4*, similar band intensities are observed in green and albino lanes. In the second class, exemplified by *rps15*, *clpP*, *rpl23*, *rpl2* and *rpoB*, band intensities are higher in albino than in green lanes. Part of this increase is due to the absence of plastid rRNAs (which make up ~30–40% of total green-leaf RNA) in albino RNA. Because equal amounts of green- and albino-leaf RNA were loaded per lane, the hybridization intensity of a constitutively expressed RNA species will be enhanced by 1.4- to 1.7-fold in albino RNA samples, which lack plastid rRNA. The *rps15*, *rpl2*, *rps2*, *rps4* and *rpoB* probes hybridize to multiple transcripts, which are mainly polycistronic. The adjacent *rpl2* and *rpl23* genes are transcribed together (Kanno and Hirai 1993), and the multiple faint bands above the strong 2.6-kb *rpl2* band in the albino lanes (Fig. 4) were also detected with the *rpl23* probe on other blots (not shown). The *rpl2* gene contains a group II/subgroup IIa intron, which is not removed from *rpl2* transcripts in ribosome-deficient mutants (Hess et al. 1994). The unprocessed 2.6-kb *rpl2* message accumulates to high levels in albino leaves (Fig. 4). A very faint band corresponding to processed *rpl2* mRNA (1.9 kb) in albino lanes was too close to background levels of hybridization, within albino lanes, to allow its accurate quantification. This faint 1.9-kb *rpl2* band in albino lanes represents substantially less than 5% of the hybridization intensity of the unprocessed 2.6-kb *rpl2* band. The processed *rpl2* message, lacking an intron, accumulates to low but detectable levels in green leaves. This processed *rpl2* message is more easily seen in leaf RNA from streptomycin-bleached maize seedlings grown in soil (see below), which contain detectable levels of plastid rRNA. A 2.6-kb *rps4* band in green-leaf RNA is replaced by a *rps4* band of higher molecular weight in albino-leaf RNA (Fig. 4). This could reflect differences in RNA processing, stability, initiation or termination.

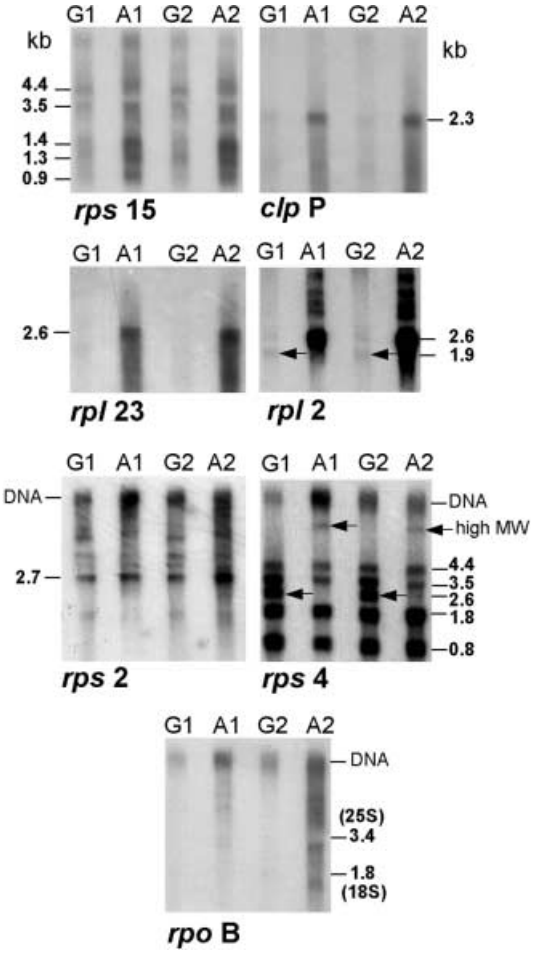

Fig. 4 Constitutive and up-regulated NEP transcripts in albino barley plants detected by Northern analysis of total RNA. Hybridization probes and band sizes are indicated

Excision and amplification of the *trnG*(UCC)-*trnE*(UUC) region of ptDNA

The integrity of the plastid genome in albino plants was studied by Southern analysis of total DNA. The hybridization probes used covered approximately 30% of the plastid genome. No differences in the intensities or sizes of hybridizing bands were found between DNA from green plants and barley albino clones 1, 3 and 4 (not shown). Although base changes and small rearrangements cannot be ruled out, this result is consistent with the presence of an intact plastid genome in three barley albino plants.

Uncleaved DNA samples from green leaves (green plant 2) and albino clone 2 were fractionated by agarose gel electrophoresis. Ethidium bromide staining located most of the DNA in the high-molecular-weight region of the gel (Fig. 5A). The A2 DNA contained a 10.4-kb band below the high-molecular-weight smear of DNA. Blot analysis revealed that this 10.4-kb DNA molecule hybridizes to pHvcPT14 and is part of a multimeric series; the dimer (20.8 kb) and trimer (31 kb) are clearly visible (lane 2 in Fig. 5B). Comparisons of band intensities by phosphor imaging show that this multimeric series is amplified by approximately 100-fold relative to the levels of wild-type ptDNA in green and albino leaves. In this series the phosphor image intensities of the dimer, trimer and tetramer (compared to the monomer) are 75%, 30% and 13% , respectively. The probe pHvcPT14 corresponds to the T10 region of ptDNA (Day and Ellis 1985) and contains the genes *trnG*(GCC) and *trnG*(UCC) (for map see Fig. 6C). Small ptDNA molecules arising from this region have also been found in albino plants obtained from cereal anther culture (Day and Ellis 1985; Ellis and Day 1986; Harada et al. 1992) and long-term rice cultures (Kawata et al. 1997). These linear DNA molecules have been studied in detail and are double-stranded with hairpin ends (Ellis and Day 1986; Harada et al. 1992; Kawata et al. 1997).

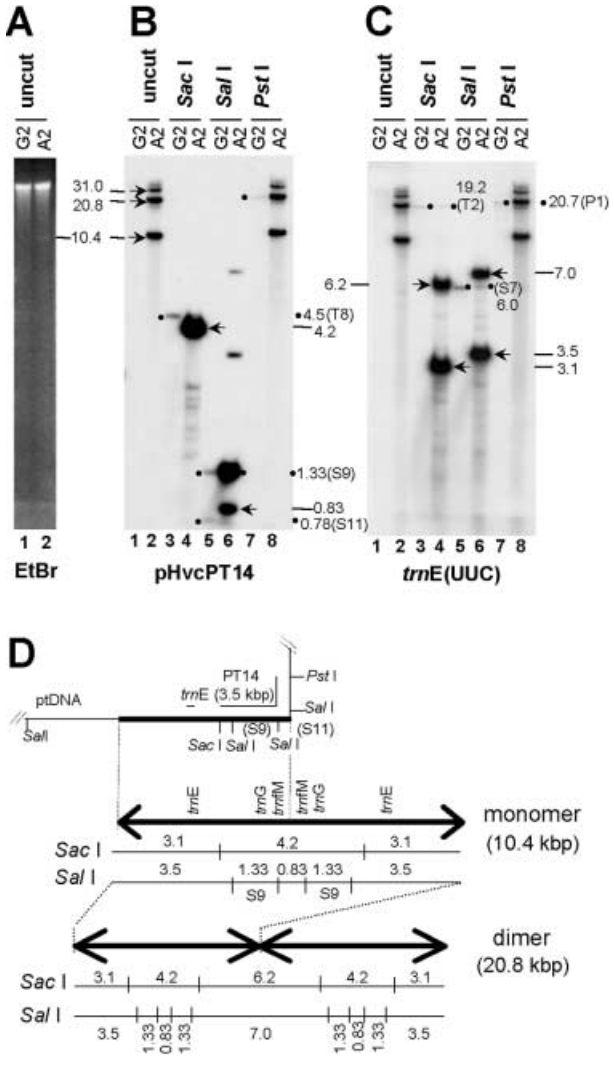

Fig. 5A–D Amplification of ptDNA in barley albino plant 2. **A** Ethidium bromide (EtBr)-stained gel of undigested total DNA from albino and green leaves. A 10.4-kb band is *arrowed* in lane 2. **B, C** Southern blots probed with pHvcPT14 and *trnE*(UUC). Wild-type ptDNA fragments are indicated by *dots*. Fragments unique to A2 are *arrowed*. **D** Amplified ptDNA in barley A2 is composed of a multimeric series based on a 10.4-kb linear monomer. The monomer is an inverted repeat of the *trnfM*(CAU)–*trnE*(UUC) region of plastid DNA with the centre of symmetry in *Sal*I fragment S11. The 20.8-kb dimer and larger multimers produce the 6.2-kb *Sac*I and 7.0-kb *Sal*I fragments

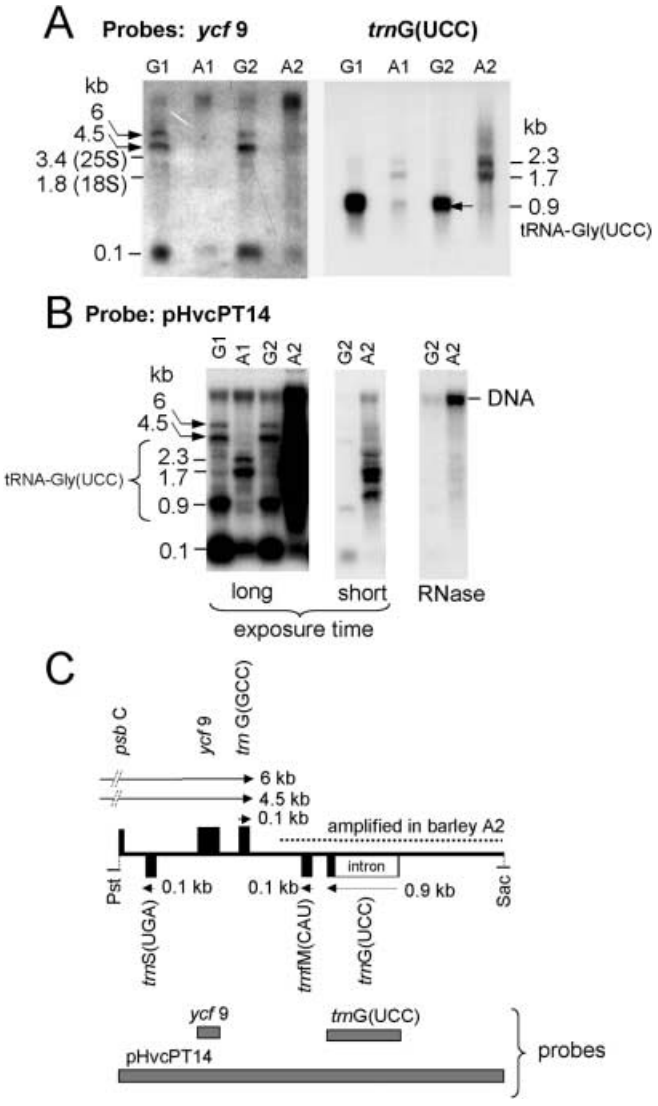

Fig. 6A–C Plastid DNA amplification is associated with the production of abundant NEP-dependent transcripts. **A, B** Northern blots of total RNA from green and albino plants hybridized with the indicated probes. Panel **B** includes a short autoradiographic exposure and shows the effects of RNase A treatment on lanes G2 and A2. **C** Map locations of transcripts detected in green leaves by the 3.5-kb barley ptDNA insert in pHvcPT14

The structure of the 10.4-kb molecule was determined by digesting A2 DNA with *Sac*I, *Sal*I and *Pst*I. Probing of digests on blots with pHvcPT14 and *trnE*(UUC) probes produced the hybridization patterns shown in Fig. 5B and C. The molecule does not contain *Pst*I sites. It contains the *Sal*-9 (S9) fragment (Day and Ellis 1985) of ptDNA. The sizes of all the other major *Sal*I and *Sac*I bands in A2 lanes do not correspond to those found in intact ptDNA. The structure of the small DNA molecule in A2 is shown in Fig. 5D. It is an inverted repeat centred in the intergenic region between *trnG*(GCC) and *trnfM*(CAU) (see Fig. 6C). Multimers contain additional copies of the 10.4-kb inverted repeat. The structure of the dimer is also shown (Fig. 5D). Whilst all linear inverted repeat ptDNA molecules previously characterized contain *trnE*(UUC), the centres of these molecules map to different locations. Linear ptDNAs resembling those found in A2, with a symmetrical centre between *trnG*(GCC) and *trnfM*(CAU), have been described before (Ellis and Day 1986; Harada et al. 1991; Chiba et al. 1996). Hybridization probes located outside the amplified region detect normal levels of intact ptDNA restriction fragments in A2 (not shown). This is consistent with maintenance of an intact plastid genome in A2, which gives rise to the minor T2 (lane 4) and S7 (lane 6) bands seen in Fig. 5C.

Amplification of ptDNA in albino clone 2 is associated with the appearance of novel transcripts

The 3.5-kb *Sac*I-*Pst*I (PT14) fragment of barley ptDNA in pHvcPT14 (Day and Ellis 1985) encodes a number of tRNA genes, including tRNA-Ser(UGA), tRNA-Gly(GCC), tRNA-fMet(CAU) and tRNA-Gly(UCC) (Oliver and Poulsen 1984). Chloroplast transcripts from this region (Fig. 6C) have been mapped (Oliver and Poulsen 1984; Kanno and Hirai 1993). Transcripts in albino clone A1 were compared with those present in A2, which contains the amplified ptDNA molecule shown in Fig. 5D. A *ycf*9 probe hybridizes to bands of ~0.1, 4.5 and 6 kb in green-leaf RNA (Fig. 6A). The large bands are polycistronic transcripts, which encompass the photosynthetic genes *psbD* and *psbC*. These bands are not readily detectable in A1 and A2 lanes. A *trnG*(UCC) probe detects an abundant transcript of approximately 0.9 kb in green-leaf RNA of barley. This corresponds in size to the unprocessed form of tRNA-Gly(UCC), which contains a 680-nt group II/subgroup IIB intron (Vogel et al. 1999). About 90% of the *trnG*(UCC) probe is composed of intron sequences, and the mature tRNA-Gly(UCC), which does accumulate in green and to a lower extent in white *albostrians* leaves (Vogel et al. 1999), is not readily visualised in Fig. 6A. The *trnG*(UCC) probe also hybridizes to 1.7- and 2.3-kb bands in albino-leaf RNA, which could reflect differences in RNA processing, stability, initiation or termination between albino and green leaves.

The *ycf*9 and *trnG*(UCC) probes are located within pHvcPT14. Figure 6B shows that pHvcPT14 detects the RNA bands hybridizing to *ycf*9 and *trnG*(UCC) in green-leaf RNA and albino clone A1 (Fig. 6A). An abundant ~0.1-kb band in green-leaf RNA corresponds to the combined hybridization of tRNA-Gly(GCC), tRNA-Ser(UGA) and tRNA-fMet(CAU). This ~0.1-kb band is reduced in intensity in albino lanes. The pHvcPT14 probe hybridizes intensely to A2 RNA. A short autoradiographic exposure revealed multiple hybridizing bands. These A2-specific RNA bands represent abundant novel transcripts synthesized by a NEP, which are not detectable in albino clone A1. The intense bands observed in A2 RNA are not DNA contaminants since RNase A removes them (Fig. 6B). RNase A does not remove DNA, which migrates at the top of the gel and is a minor high-molecular-weight contaminant in our RNA extracts.

Plastid translation and transcription products in stable albino maize plants

Western and Northern analyses were used to compare plastid gene products in stable albino maize plants (A) and streptomycin-bleached seedlings (Bl.) grown in soil (Fig. 7). In rice, low doses of streptomycin (10–100 μg/ml) reduce the expression of PEP-transcribed genes in soil-grown plants (Yoshida et al. 1998). The LS of

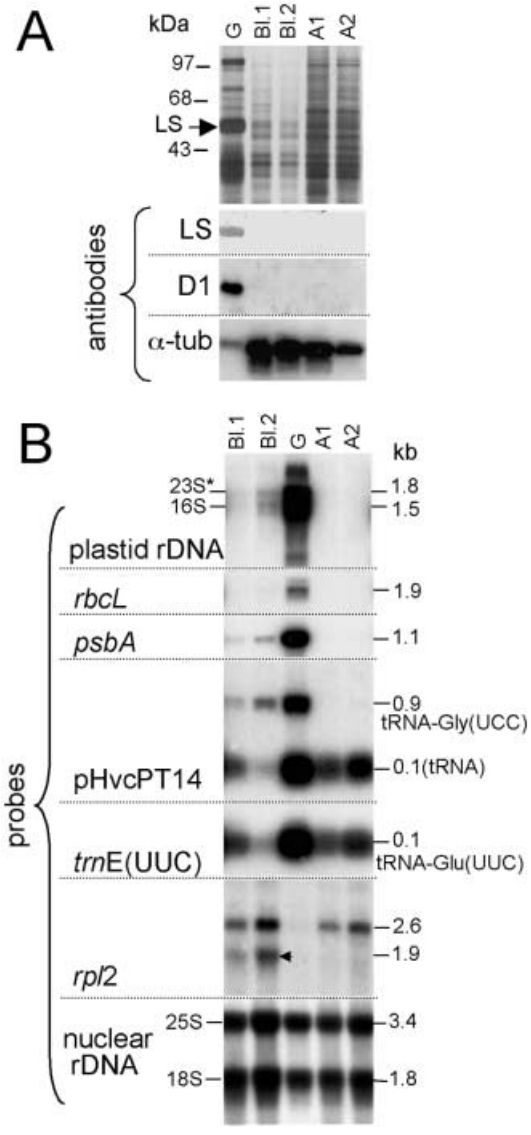

Fig. 7A, B Plastid gene expression in green (G), bleached (Bl.) and stable albino (A) maize leaves. **A** Western analyses of total soluble proteins. *Top panel* Total soluble proteins extracted from green (G) and albino (A) plants fractionated by SDS/PAGE and stained with silver salts. *Lower panels* Western blots incubated with the indicated antibodies. **B** Northern analysis of total RNA. Hybridization probes and band sizes are indicated

Rubisco was not detected in gel-fractionated protein extracts of albino and bleached maize stained with silver or probed with LS-specific antibodies (Fig. 7A). Western analyses revealed that albino and bleached material was also depleted in the D1 protein. An anti-α-tubulin antibody was used to verify that all lanes contained similar amounts of protein. The weak α-tubulin signal in the G lane is probably due to competition with LS-Rubisco for filter binding sites during blotting. Maize α-tubulin and LS-Rubisco co-migrate on gels.

A comparison between albino and bleached maize leaves illustrates the differences in transcript accumulation between ribosome-free plastids (albino) and ribosome-depleted plastids (bleached). Propagation of

bleached material (still containing plastid ribosomes) in vitro allows the cell and plastid divisions needed to isolate stable albino shoots totally lacking plastid ribosomes. Plastid rRNA was not detected in maize albino plants by Northern analysis (Fig. 7B). Low amounts of plastid rRNA (10% of green-leaf levels) were detected in soil-grown bleached seedlings, which indicates incomplete depletion of plastid ribosomes. Ribosomal RNA bands in bleached material co-migrate with those present in green leaves. RNA blots were also hybridized with *rbcL*, *psbA*, pHvcPT14 (Fig. 6C) and *trnE*(UUC) probes. The levels of all trancripts hybridizing to these probes were reduced in albino and bleached material compared to green leaves. The *rbcL* and *psbA* messages in bleached leaves accumulate to 10% and 4% of the levels in green leaves, respectively, whilst the values for albino leaves were 2% and 1%, respectively. The 0.9-kb tRNA-Gly(UCC) band accumulates to higher levels in bleached leaves than in albino leaves (pHvcPT14 probe). The pHvcPT14 probe also detects the combined hybridization signals from tRNA-Gly(GCC), tRNA-Ser (UGA) and tRNA-fMet(CAU). These tRNAs and tRNA-Glu(UUC) accumulate to varying levels in bleached and albino leaves. The hybridization intensities of tRNA-Glu(UUC) in Bl.1, Bl.2, A1 and A2 leaves were 20%, 8%, 18% and 26% of green-leaf levels, respectively. Transcripts containing *rpl2* were up-regulated in albino and bleached leaves. Both processed (1.9 kb) and unprocessed (2.6 kb) messages were present in bleached leaves. The *rpl2* probe, containing the intron (45% of the probe), hybridized twice as intensely to the 2.6-kb intron-containing band (Hess et al. 1994) as to the 1.9-kb processed band in RNA from bleached leaves. Only the unprocessed 2.6-kb *rpl2* message was detected in RNA from albino maize plants (Fig. 7). A nuclear rDNA probe verified that all lanes had been loaded with similar amounts of maize cytosolic rRNAs.

Discussion

Exposure of barley and maize seeds to streptomycin prior to germination has allowed us to isolate stable albino plants. Propagation of bleached seedlings in vitro, in the absence of antibiotic, is an essential component of our procedure. The provision of a carbon source allows the cell and plastid divisions (Zubko and Day 1998) in bleached shoots necessary for cytoplasmic sorting and the isolation of stable albino shoots from variegated and re-greened shoots. Albino plants were deficient in plastid translation products and plastid rRNAs. The stability of albinism is consistent with an irreversible loss of plastid ribosomes. Once plastid ribosomes are lost they cannot be reconstituted (Zubko and Day 1998). Twenty-one of the approximately sixty proteins found in chloroplast ribosomes (Subramanian 1993) are encoded by the plastid genome (Hiratsuka et al. 1989; Maier et al. 1995) and if lost they cannot be re-made without functional plastid ribosomes. Lack of splicing of the group II/subgroup IIA intron (Vogel et al. 1999; Bonen and Vogel 2001) present in plastid *rpl2* transcripts (Hess et al. 1994) provided a rigorous proof of the absence of plastid translation in our barley and maize albino plants. The barley and maize albino plants that we isolated are phenocopies of the *albostrians* and *iojap* mutants, which contain ribosome-deficient plastids. Isolation of these phenocopies is consistent with the suggestion that mutations in ptDNA are not necessarily responsible for albinism in *albostrians* and *iojap* mutants (Walbot and Coe 1979; Börner and Sears 1986).

Early experiments on the isolation of streptomycin-resistant mutants in *Chlamydomonas* led to the suggestion that streptomycin is a mutagen of non-chromosomal genes (Sager 1962). This suggestion was not prompted by the frequency of extra-chromosomal streptomycin-resistant mutations recovered (1.4×10^{-7} mutations per cell division), which was lower than the frequency of spontaneous nuclear streptomycin-resistant mutants ($0.8–1.1\times10^{-6}$ mutations per cell division). The idea that streptomycin might be a specific mutagen of extra-chromosomal genes was based on distinguishing between induced mutation and selection (Sager 1962). This is a difficult problem that is not easy to address experimentally. Since streptomycin is not a known mutagen and its precise target within the 30S ribosomal subunit has been determined (Moazed and Noller 1987; Carter et al. 2000), the effects of streptomycin may be indirect. For example, streptomycin may facilitate the isolation of plastid mutants by perhaps reducing the copy number of plastid DNA (see Harris 1989). The extremely high frequencies (100% in some cases) of streptomycin-induced albinism in cereals and spectinomycin-induced albinism in the Brassicaceae (Zubko and Day 1998), as compared to the low frequencies of *Chlamydomonas* plastid mutants selected with streptomycin, are consistent with irreversible loss of plastid ribosomes (as discussed in this paper), rather than induction of plastome or nuclear mutations (Sarma and Patnaik 1982), as the causal basis of streptomycin-induced albinism in cereals.

The stable maintenance of variegation in soil-grown barley plants contrasts with the instability of variegation induced by spectinomycin in *Brassica napus* (Zubko and Day 1998). This probably reflects developmental differences between monocotyledonous and dicotyledonous plants. The maintenance of variegation in soil-grown plants should allow genetic analysis of streptomycin-induced albinism. Variegation was not sexually transmitted at a detectable frequency in the barley variety used here. This indicates that dysfunctional plastids might increase embryo lethality (Coe et al. 1988), or the absence of white plastids in the cell lineages giving rise to eggs. Use of alternative varieties might increase the frequency of sexual transmission. In maize, specific alleles of the *R* gene or at a closely linked locus can enhance sexual transmission of white plastids in *iojap* crosses (Coe et al. 1982). In rice, the small fraction of green and variegated (*viridis* type) plants emerging from

streptomycin-treated seeds gave rise to 0.1–0.6% white seedlings amongst their progeny (Sarma and Patnaik 1982).

Streptomycin-induced albinism provides a rapid method for isolating plants with pure populations of ribosome-deficient plastids in a range of genotypes. The study of phenocopies overcomes a potential problem of mutant analysis where mutation effects unrelated to plastid ribosome deficiency might contribute to an observed phenotype. The precise mechanisms that induce ribosome loss in mutants such as *iojap* and *albostrians* remain unclear. Mutations in nuclear genes of known function that reduce plastid ribosome number (Tokuhisa et al. 1998; Schultes et al. 2000) have not led to the isolation of plants with ribosome-free plastids.

Ribosome-deficient plastids are unable to produce transcripts normally synthesized by PEP, allowing the identification of mRNAs synthesized by NEP (Hess et al. 1993). The analysis of PEP transcript accumulation in phenocopy plants is in agreement with similar studies on *albostrians* (Hess et al. 1993) and *iojap* mutants (Silhavy and Maliga 1998). The pattern of transcript accumulation in stable albino plants lacking plastid 23S and 16S rRNAs is clearly different from that observed in bleached maize seedlings that retain low but detectable levels of correctly processed plastid ribosomal RNAs. Importantly, these results demonstrate that streptomycin-induced bleaching per se, which renders the plastid-encoded Rubisco large subunit and D1 protein undetectable on Western blots, is not an indicator of ribosome-free plastids. Bleaching is only indicative of a reduction and not a total loss of plastid ribosomes. Stable albino cell lines and shoots lacking plastid ribosomes can only be isolated following further growth ofbleached cells, involving cell and plastid divisions (Zubko and Day 1998). The levels of plastid tRNAs appear to be regulated by the demand for these molecules in plastid protein synthesis. When protein synthesis is abolished in phenocopies, tRNA levels are also reduced. Analyses of the relative levels of transcript accumulation in albino, bleached and green leaves revealed, however, that tRNA levels are not tightly coupled to the levels of plastid rRNAs, *rbcL* or *psbA* mRNAs. For example, tRNAs accumulate to relatively high levels in albino maize plants, in contrast to plastid rRNAs, *rbcL* and *psbA* mRNAs (Fig. 7).

Comparisons between albino and green leaves revealed differences in the expression of genes transcribed by NEP. Plastid RNA levels are regulated at the transcriptional and post-transcriptional levels (Baumgartner et al. 1993). Two broad classes of genes transcribed by NEP were observed. In the first class, illustrated by *clpP*, *rps15*, *rpl2*, *rpl23* and *rpoB*, the hybridization intensities of transcripts in total RNA extracts from albino leaves were increased relative to those in green leaves. In contrast, the hybridization intensities of transcripts containing *rps2* and *rps4* were similar in green- and albino-leaf RNA. Although, NEP transcribes *rps2* and *rps4* in ribosome-deficient plastids, we cannot rule out an involvement of PEP in the expression of these genes in chloroplasts. Our data indicate an influence of plastid protein synthesis on the differential accumulation of RNA from NEP transcribed genes. This could be mediated by ribosome-associated activities and/or plastid-encoded products. Alternatively, the effect might be more indirect, and reflect regulation of transcript accumulation dependent on the developmental state of plastids.

Differences in the sizes of *rps4* and *trn*G(UCC) transcripts in albino and green plants could be due to changes in initiation/termination sites, in the stability of precursor transcripts, or in RNA processing. Transcript maturation could conceivably require association with ribosomes or a plastid-encoded protein. Only a subset of chloroplast introns, including the *rpl2* intron, is not spliced normally in *albostrians* plants (Hess et al. 1994; Vogel et al. 1999). Incomplete processing of the *rpl2* intron was observed in our barley and maize albino phenocopies.

Recent data from tobacco plants lacking PEP indicates regulation of NEP-dependent transcript accumulation at the post-transcriptional level (Krause et al. 2000). Such tobacco plants appear to contain functional ribosomes (De Santis-Maciossek et al. 1999). In contrast to our data on cereal albino plants, tobacco plants lacking PEP RNA polymerase accumulate relatively high levels of correctly processed plastid rRNAs (Allison et al. 1996; De Santis-Maciossek et al. 1999) and *atpB* mRNA (Krause et al. 2000). This could either reflect differences in the regulation of tobacco and cereal plastid genes or the effects of plastid-encoded components, such as ribosomal proteins, on plastid RNA accumulation. The isolation of stable albino tobacco plants lacking plastid ribosomes will be required to distinguish between these possibilities. This is not possible at present since tobacco is recalcitrant to the induction of plastid ribosome deficiency by inhibitors of plastid protein synthesis (Zubko and Day 1998). This could indicate that plastid protein synthesis is essential for cell viability in tobacco (Drescher et al. 2000).

The *trnG*(UCC) to *trnE*(UUC) region of barley ptDNA was amplified as a multimeric series of linear DNA molecules based on a 10.4-kb monomer in barley plant A2, which also contains an intact plastid genome. The formation of these linear DNA molecules can be distinguished from the recombination events that produce deleted circular plastid genomes in plants derived from albino pollen (Day and Ellis 1984, 1985) and cultured rice cells (Cuzzoni et al. 1995). Amplified linear ptDNA molecules have been observed in anther culture-derived callus and plants (Day and Ellis 1985; Ellis and Day 1986; Harada et al. 1992; Kawata et al. 1997), and long-term cell lines of rice derived from scutella (Kawata et al. 1997). Strand switching at the replication fork provides a simple model for the origin of linear inverted repeat DNAs (Ellis and Day 1986; Qin and Cohen 2000). A common theme, such as loss of functional plastid ribosomes, probably explains their presence

in albino plants from diverse sources. Plastid DNA amplification appears to be a secondary consequence of plastid ribosome deficiency, since only one of four albino barley plants contained amplified DNA. Amplification might provide a mechanism to enhance the levels of specific plastid gene products, which are down-regulated in ribosome-deficient plastids. Alternatively, since most plastid genes cannot be expressed without ribosomes, the plastid genome becomes dispensable and the accumulation of variant plastid replicons might reflect a relaxation of the mechanisms that normally maintain the integrity of the plastid genome. Common mechanisms might be responsible for the linear amplicons in albino plants and the rare hairpin molecules found in green leaves (Collin and Ellis 1991).

A common feature of linear ptDNA molecules is the retention of the *trnE*(UUC) gene (Harada et al. 1992). The *trnE*(UUC) gene product tRNA(Glu) is required for plastid protein synthesis and is also a precursor for heme, which is essential and is utilized by both mitochondria and plastids. The requirement for tRNA(Glu) in heme synthesis has been suggested to account for the retention of its coding sequence in deleted plastid genomes (Howe and Smith 1991; Harada et al. 1992). In ribosome-deficient plastids, the demand for tRNA(Glu) in protein synthesis is reduced and this may down-regulate its synthesis to an extent that impedes heme synthesis. Indeed, tRNA(Glu) levels are reduced in *albostrians* and *iojap* phenocopies (this work) and the *albostrians* mutant (Hess et al. 1992b). This is in contrast to the situation in etioplasts, where tRNA(Glu) levels remain high in the dark (Berry-Lowe 1987). Paradoxically, we found that amplification of *trnE*(UUC) did not increase the steady-state levels of tRNA(Glu) in albino leaves. Whilst this might suggest that demand for the *trnE*(UUC) gene product is not responsible for gene amplification, it remains possible that amplification at an earlier developmental stage did raise tRNA(Glu) levels. Once linear amplicons are formed their levels need not correlate with the dosage of encoded RNA species. RNA levels will not only be determined by gene dosage but by rates of RNA synthesis, processing and decay – which could vary with cell type. Amplification of the *trnG*(UCC) region of ptDNA was associated with abundant novel transcripts in albino plants, which must reflect the activity of a NEP. Whether these transcripts play a role in the maintenance of linear plastid amplicons remains to be determined. The promoter(s) responsible for these abundant transcripts are suitable for constructing plastid transformation vectors because they are likely to be active in the non-green target organs used for DNA-mediated transformation of cereals (Barcelo et al. 1994).

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