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Coordination of plastid and nuclear gene expression

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The coordinated expression of genes distributed between the nuclear and plastid genomes is essential for the assembly of functional chloroplasts. Although the nucleus has a pre-eminent role in controlling chloroplast biogenesis, there is considerable evidence that the expression of nuclear genes encoding photosynthesis-related proteins is regulated by signals from plastids. Perturbation of several plastid-located processes, by inhibitors or in mutants, leads to decreased transcription of a set of nuclear photosynthesis-related genes. Characterization of arabidopsis *gun* (*genomes uncoupled*) mutants, which express nuclear genes in the presence of norflurazon or lincomycin, has provided evidence for two separate signalling pathways, one involving tetrapyrrole biosynthesis intermediates and the other requiring plastid protein synthesis. In addition, perturbation of photosynthetic electron transfer produces at least two different redox signals, as part of the acclimation to altered light conditions. The recognition of multiple plastid signals requires a reconsideration of the mechanisms of regulation of transcription of nuclear genes encoding photosynthesis-related proteins.

Keywords: chloroplast biogenesis; *gun* mutants; lincomycin; norflurazon; plastid signal; redox signal

1. INTRODUCTION

It is now widely accepted that chloroplasts evolved from a free-living photosynthetic prokaryotic organism following an endosymbiotic relationship with a non-photosynthetic eukaryotic host (Douglas & Raven 2003, these proceedings). Current-day oxygenic photosynthetic organisms, such as cyanobacteria and prochlorophytes, are believed to be related to the prokaryotic endosymbiont that gave rise to chloroplasts. However, the genome sizes of the cyanobacterium *Synechocystis* PCC 6803 (*ca.* 3.57 Mbp; 3168 protein-coding genes) and chloroplasts of the higher plant *Arabidopsis thaliana* (*ca.* 154 kbp; 79 protein-coding genes) differ by more than an order of magnitude, indicating a considerable loss of genetic information during the evolution of chloroplasts. Comparison of the complete genome sequences of *Synechocystis* and *Arabidopsis* suggests that a large amount of genetic information has been transferred to the nuclear genome (Abdallah *et al.* 2000; Rujan & Martin 2001).

The transfer of genes from the prokaryotic endosymbiont to the nucleus of the host cell provided an opportunity for increased control of the endosymbiont and its biological functions by the host cell. However, this was at the expense of possible disruption of regulatory processes within the endosymbiont, for example, the loss of coordinate regulation of genes encoding subunits of a complex, or enzymes in a metabolic pathway. The organization of genes into operons is a common feature of prokaryotic genomes, enabling complex transcriptional and translational regulation. The loss of such coordinated expression of prokaryotic genes, particularly those concerned with bioenergetic processes, may have placed considerable restraints on the success of the host cell, unless the expression of the transferred nuclear gene was carefully regulated. It seems highly probable that prokaryotic genes were transferred to the nucleus, and gained appropriate regulatory features and sequences needed to target the gene product back to the endosymbiont, before the gene was lost from the endosymbiont. Very little is known about the processes by which transferred prokaryotic genes gain regulatory sequences that ensure they are functional within a eukaryotic host. It is known, however, that tracts of chloroplast DNA sequences are present in the nuclear genomes of higher plants, such as spinach and arabidopsis (Timmis & Scott 1983; The Arabidopsis Genome Initiative 2000), and this may provide material for evolutionary change in the future.

The nuclear genome now encodes the vast majority (more than 90%) of the proteins present in the chloroplasts and exerts considerable control over most of the processes that take place in the chloroplast, including the replication and expression of genes in the chloroplast genome. Indeed, it has been postulated that the nucleus has complete control over the assembly of photosynthetic complexes in chloroplasts (Ellis 1977). However, there is now a large body of evidence that 'retrograde' signals from chloroplasts regulate the expression of nuclear genes encoding photosynthesis-related proteins. Although it was originally proposed that a single 'plastid signal' or 'plastid factor' was involved (Oelmüller & Mohr 1986; Oelmüller

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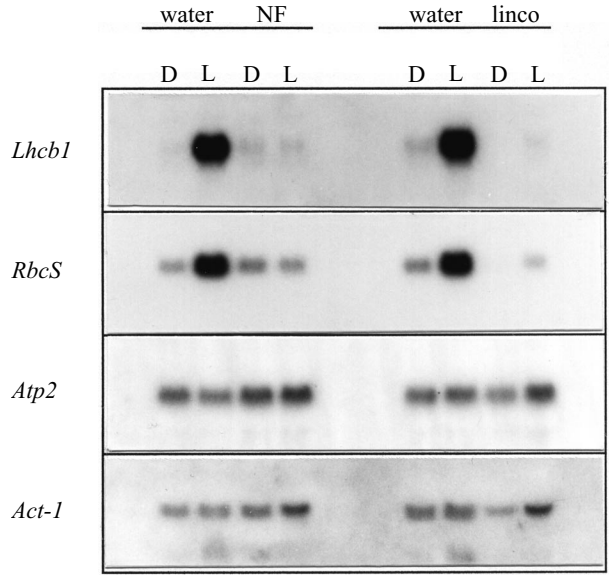

Figure 1. Northern blot analysis of transcripts in tobacco seedlings treated with norflurazon or lincomycin. Tobacco seeds were germinated and grown on water, 100 μM norflurazon (NF) or lincomycin (linco; 100 μg ml^{-1}) for 7 days in the dark (D) or for 5 days in the dark followed by 2 days in the light (L). Total RNA (10 μg) was fractionated by agarose gel electrophoresis, transferred to a GeneScreen Plus membrane and probed sequentially with ^{32}P-labelled probes for *Lhcb1*, *RbcS*, *Atp2*, *Act-1* (actin) and rRNA.

1989; Taylor 1989), it is now clear that multiple signals are produced by plastids. However, the exact nature of these signals and how they act are still to be resolved.

2. NORFLURAZON AND CAROTENOID-DEFICIENT PLANTS

Some of the earliest experiments providing evidence for plastid-to-nucleus signalling used carotenoid-deficient plants, which bleached on exposure to high light intensities. Carotenoids rapidly quench the production of triplet chlorophyll, preventing the production of free radicals and ROS. In the absence of carotenoids, either because of biosynthetic inhibitors such as norflurazon, or in mutant plants with defects in carotenoid biosynthesis, triplet chlorophyll formation results in photooxidative damage within the chloroplast. Although the damage is apparently restricted to the chloroplast (Reiss *et al.* 1983), the expression of a subset of nuclear genes encoding photosynthesis-related proteins decreases. This includes genes encoding components of the light-harvesting complexes (*Lhcb1*), the photosynthetic electron-transfer chain (*PetC*, *PetE*, *PetH*, *Fed-1*), the photosystem II oxygen-evolving complex (*PsbO*, *PsbP*) and the reductive pentose phosphate pathway (*RbcS*). Decreased accumulation of transcripts of these genes has been observed by Northern blot hybridization or by translation *in vitro* of RNA extracted from wild-type plants treated with norflurazon (Mayfield & Taylor 1984; Batschauer *et al.* 1986; Oelmüller & Mohr 1986; Burgess & Taylor 1987; Sagar *et al.* 1988). An example of the effect of norflurazon on transcripts of *Lhcb1* and *RbcS* in 7-day-old tobacco seedlings is shown in figure 1. The photobleached seedlings contain decreased amounts of transcripts of the photosynthesis-related genes, whereas transcripts of the nuclear genes encoding the β-subunit of mitochondrial ATP synthase (*Atp2*) and actin are unaffected.

Decreased accumulation of transcripts of *Lhcb1* and *RbcS* has also been observed in white tissue of carotenoid-deficient mutants of maize, barley and tomato (Harpster *et al.* 1984; Mayfield & Taylor 1984; Batschauer *et al.* 1986; Giuliano & Scolnik 1988). In addition, transcripts for several other chloroplast-located proteins have been shown to be affected by photobleaching in norflurazon-treated seedlings. These include pyruvate phosphate dikinase and NADP-malic enzyme in maize (Burgess & Taylor 1988), glutamyl-tRNA reductase (*HemA*) in arabidopsis (Kumar *et al.* 1999) and nitrite reductase in mustard (Oelmüller *et al.* 1988).

All of the gene products listed above are targeted to chloroplasts, which is the organelle damaged by photooxidation in the absence of carotenoids. However, norflurazon treatment also results in decreased expression of genes for photorespiratory enzymes, such as glycollate oxidase and hydroxypyruvate reductase, which are located in peroxisomes (Bajracharya *et al.* 1987; Schwarz *et al.* 1992; Barak *et al.* 2001). In addition, decreased transcripts for the cytosolic enzyme nitrate reductase have been observed in norflurazon-treated seedlings of mustard and squash (Oelmüller *et al.* 1988; Oelmüller & Briggs 1990). However, the expression of genes for other cytosolic proteins such as PEP carboxylase, ubiquitin and actin, is not affected (Burgess & Taylor 1988; Rapp & Mullet 1991; Schwarz *et al.* 1992).

The decreased accumulation of transcripts from these photosynthesis-related genes in photobleached carotenoid-deficient plants is due to decreased rates of transcription, as shown by nuclear run-on assays and by reporter gene expression from promoters in transgenic plants. Decreased transcription of *Lhcb1* and *RbcS* has been shown in isolated nuclei of norflurazon-treated seedlings of barley, maize, rye and pea (Batschauer *et al.* 1986; Burgess & Taylor 1988; Ernst & Schefbeck 1988; Sagar *et al.* 1988) and in isolated nuclei from white tissue of the tomato *ghost* mutant (Giuliano & Scolnik 1988).

Decreased expression of reporter genes under the control of promoter elements from an increasing number of photosynthesis-related genes has been shown in norflurazon-treated transgenic plants. This was first demonstrated for the neomycin phosphotransferase (*nptII*) reporter gene under the control of pea *Lhcb1* and *RbcS* promoters in transgenic tobacco seedlings (Simpson *et al.* 1986) and was subsequently shown for a tagged version of the potato *PsbR* gene in tobacco (Stockhaus *et al.* 1987). The promoters of arabidopsis *Lhcb1* (Susek *et al.* 1993), spinach *Lhcb1*, *PetE*, *PetH*, *PsaF* and *AtpC* (Bolle *et al.* 1994), tobacco *RbcS* and pea *PetH* (Gray *et al.* 1995) and arabidopsis *HemA* (McCormac *et al.* 2001) have all been shown to direct norflurazon-sensitive expression of the GUS reporter gene in transgenic plants. In addition, transgenic tobacco seedlings expressing the GUS reporter gene under the control of the promoter of a tobacco gene encoding peroxisomal glycollate oxidase showed decreased GUS activity in the presence of norflurazon (Barak *et al.* 2001).

Deletion analysis of several of these promoters has identified shorter regions that are able to direct similar patterns of expression in norflurazon-treated seedlings

(Bolle *et al.* 1994; Lübberstedt *et al.* 1994; Gray *et al.* 1995; Kusnetsov *et al.* 1996; Hahn & Kück 1999). However, in none of the promoters was it possible to separate the regulatory elements responding to norflurazon treatment from light-regulatory elements. This suggests that light regulation and plastid regulation of photosynthesis-related nuclear genes may operate through the same transcription factors and regulatory elements. Indeed, transgenic tobacco seedlings containing the GUS reporter gene under the control of chimeric promoters, consisting of multiple copies of known light-regulatory elements, also show decreased expression on treatment with norflurazon (Puente *et al.* 1996; Martinez-Hernandez *et al.* 2002).

These experiments provide very strong evidence for a retrograde signalling system regulating the transcription of nuclear genes encoding photosynthesis-related proteins. Unfortunately, the nature of this signalling system is still obscure. This is at least partly due to an incomplete knowledge of the effects of the photooxidative changes induced by illumination of carotenoid-deficient seedlings on plastid processes. Perturbation of tetrapyrrole biosynthesis can be inferred from the identification of mutations in genes encoding the H-subunit of Mg chelatase, haem oxygenase and phytochromobilin synthase conferring the ability on arabidopsis to express *Lhcb1* and *RbcS* in the presence of norflurazon (Susek *et al.* 1993; Mochizuki *et al.* 2001; see § 4).

Norflurazon treatment has been shown to decrease the amounts of transcripts from several chloroplast genes in mustard, barley, maize, pea and spinach (Oelmüller & Mohr 1986; Batschauer *et al.* 1986; Burgess & Taylor 1988; Sagar & Briggs 1990; Tonkyn *et al.* 1992). Decreased amounts of transcripts of *rbcL*, *psbA*, *psbEF*, *psaAB*, *atpBE*, *petA*, *rrn23* and *rrn16* have been observed by Northern blot hybridization or by translation *in vitro*. A general decrease in transcripts of virtually all plastid genes in norflurazon-treated tobacco seedlings has been observed by DNA microarray analysis (figure 2*a*). However, norflurazon treatment has different effects on transcripts of different genes, as observed previously (Tonkyn *et al.* 1992). In general, the accumulation of transcripts of plastid photosynthesis genes is affected more than the transcripts of plastid genetic system genes (figure 2*a*). Norflurazon treatment has been shown to affect transcription in spinach plastids (Tonkyn *et al.* 1992), and decreased plastid rRNA synthesis (Reiss *et al.* 1983) is at least partly responsible for the disappearance of plastid ribosomes in norflurazon-treated mustard seedlings (Frosch *et al.* 1979). This suggests that norflurazon treatment will also affect signalling dependent on plastid gene expression (see § 3).

Chlorophyll photooxidation in the absence of carotenoids will also result in the production of ROS, which have been shown to affect nuclear gene expression (Mullineaux & Karpinski 2002). ROS are also produced as an inevitable consequence of photosynthetic electron transfer, due to the direct reduction of O_2 by photosystem I in the Mehler reaction. ROS production by photosynthetic electron transfer is likely to be more prominent at high light intensities and has been suggested to provide possible signalling intermediates (Pfannschmidt *et al.* 2001*a*).

3. INHIBITORS OF PLASTID GENE EXPRESSION AND PLASTID-RIBOSOME DEFICIENT PLANTS

Analysis of Calvin cycle enzymes in the barley *albostrians* mutant provided the first evidence for the existence of plastid signals affecting the expression of nuclear genes (Bradbeer *et al.* 1979). The white tissue of the *albostrians* mutant is deficient in plastid ribosomes, and was shown to have decreased amounts of phosphoribulokinase and NADPH-glyceraldehyde-3-phosphate dehydrogenase enzyme activities (Bradbeer *et al.* 1979). Because the plants were defective in plastid protein synthesis, it was suggested that plastid RNA might provide a signal to influence nuclear gene expression (Bradbeer *et al.* 1979). Subsequent analysis using translation of extracted RNA *in vitro* and Northern blot hybridization showed that a set of photosynthesis-related genes, similar to those affected by norflurazon treatment, were downregulated in the barley *albostrians* mutant (Hess *et al.* 1991, 1994). These included genes encoding light-harvesting and Calvin cycle components, as well as enzymes of photorespiration and nitrogen assimilation (Hess *et al.* 1991, 1994). Nuclear run-on transcription assays showed that the *Lhcb1* genes were regulated at the transcriptional level (Hess *et al.* 1994).

Treatment of wild-type plants with plastid translation inhibitors, such as chloramphenicol, lincomycin, erythromycin and streptomycin, also resulted in decreased expression of nuclear genes encoding photosynthesis-related proteins (Oelmüller *et al.* 1986; Adamska 1995; Gray *et al.* 1995; Yoshida *et al.* 1998; Sullivan & Gray 1999). The effect of lincomycin on the accumulation of *Lhcb1* and *RbcS* transcripts in 7-day-old tobacco seedlings is shown in figure 1. As with norflurazon, lincomycin had no effect on transcripts of nuclear genes for mitochondrial (*Atp2*) or cytosolic (Actin) proteins. However, the inhibitors were effective in preventing nuclear gene expression only if applied within the first 2–3 days of seedling development in mustard or tobacco (Oelmüller *et al.* 1986; Bajracharya *et al.* 1987; Gray *et al.* 1995). This suggested that plastid protein synthesis during early development is required to produce a signal-generating system for use later in development. However, it has also been suggested that the inhibition of plastid protein synthesis merely prevents the plastid from reaching the necessary developmental stage where plastid-to-nucleus signalling is operational (Hess *et al.* 1994).

Plastid protein-synthesis inhibitors, such as lincomycin and erythromycin, are also able to prevent nuclear gene expression in the dark, in the pea *lip1* (*light-independent photomorphogenesis1*) and arabidopsis *cop1* (*constitutively photomorphogenic1*) mutants, which show photomorphogenic development in the dark (Sullivan & Gray 1999). This indicates that light is not an essential component of the signalling pathway affected by plastid protein-synthesis inhibitors.

The involvement of plastid transcription in signalling to the nucleus is suggested by the effect of tagetitoxin, an inhibitor of the plastid-encoded RNA polymerase (Matthews & Durbin 1990). Treatment of barley seedlings with tagetitoxin resulted in decreased transcripts of the *RbcS* and *Lhcb1* gene families, but did not affect plastid DNA replication (Rapp & Mullet 1991). Nalidixic acid, a prokarotic DNA gyrase inhibitor that affects plastid

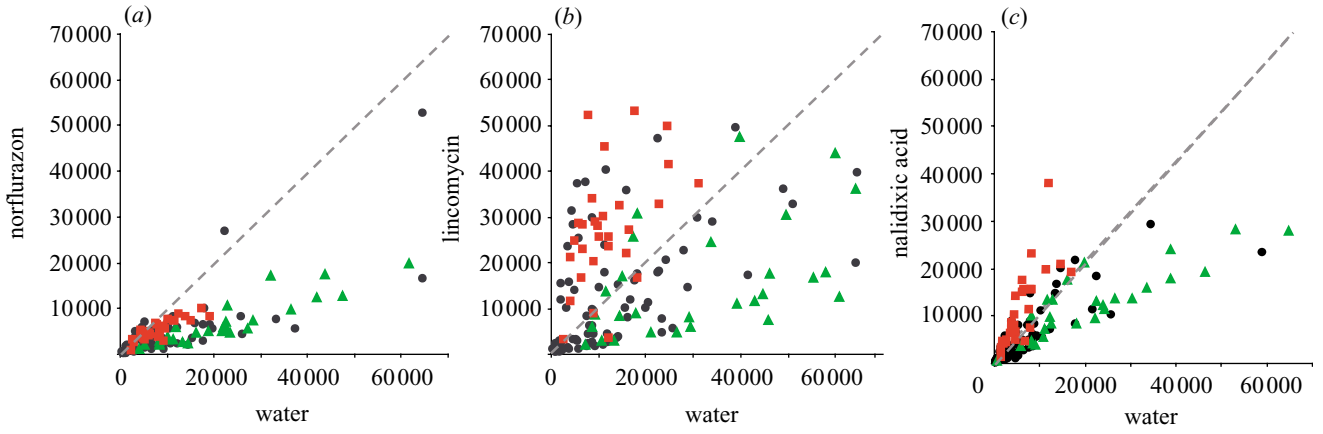

Figure 2. Plastid DNA microarray analysis of tobacco plastid transcripts. Tobacco seeds were germinated and grown on water, 100 μM norflurazon, lincomycin (250 μg ml^{-1}) or nalidixic acid (150 μg ml^{-1}) for 5 days in the dark followed by 2 days in the light. Total RNA was reverse transcribed using random primers, and Cy3- or Cy5-labelled dNTPs were incorporated into the second strand using Klenow fragment of DNA polymerase. Cy-labelled DNA produced from inhibitor-treated and control (water) samples were hybridized together to microarrays of all tobacco plastid genes, spotted either as PCR products or synthesized oligonucleotides (for transfer RNA genes). The figures on the axes are the fluorescence intensities of the individual gene spots, expressed as median pixel intensity. (*a*) Norflurazon-treated and control samples; (*b*) lincomycin-treated and control samples; (*c*) nalidixic acid-treated and control samples. Genetic system genes (*rpl*, *rps* and *rpo*) are shown as red squares; photosynthesis genes (*psa*, *psb* and *pet*) are shown as green triangles. Other genes are shown as black circles. The dashed line shows the position expected for gene transcripts that do not change due to the inhibitor treatment.

DNA replication and transcription, decreased expression of the GUS reporter gene from the *RbcS* and *PetH* promoters in transgenic tobacco seedlings (Gray *et al.* 1995). As with the plastid translation inhibitors, nalidixic acid was effective at preventing nuclear gene expression only when applied during the first 2–3 days of seedling development (Gray *et al.* 1995). The similar time-frames for the effective action of the plastid transcription and translation inhibitors suggest that plastid gene expression during early seedling development is required for signalling to the nucleus.

Plastid DNA microarrays have been used to examine changes in plastid transcripts resulting from inhibitor treatments of 7-day-old tobacco seedlings (figure 2). This shows that lincomycin and nalidixic acid have similar qualitative effects on the transcripts of most tobacco plastid genes. Transcripts from most plastid genes encoding photosynthesis-related proteins (shown as green triangles in figure 2) were decreased by both lincomycin and nalidixic acid, whereas transcripts from most plastid genes encoding genetic system components (red squares) were increased compared with untreated wild-type plants (figure 2). Increased transcription of plastid genes encoding genetic system components has previously been observed in tobacco seedlings treated with tagetitoxin (Kapoor *et al.* 1997) and in tobacco plants with deletion of the *rpo* genes encoding subunits of the plastid-encoded RNA polymerase (Allison *et al.* 1996; Hajdukiewicz *et al.* 1997; Krause *et al.* 2000). Decreased transcription by the plastid-encoded RNA polymerase appears to lead to increased transcription by the nuclear-encoded RNA polymerase, possibly due to decreased competition for template binding, or to increased availability of dNTPs. Unfortunately, these studies of plastid transcripts have not provided any clear indication of how preventing plastid gene expression results in decreased expression of nuclear genes encoding photosynthesis-related proteins.

Promoter deletion analysis of the pea *PetH* gene has shown that the effects of light, norflurazon and lincomycin on gene expression in transgenic tobacco seedlings are mediated through the same regulatory elements near the transcription start site (L. Sanders and J. C. Gray, unpublished data). This suggests that the plastid signalling pathways perturbed by norflurazon and lincomycin converge at a step before transcription initiation.

4. *GUN* (*GENOMES UNCOUPLED*) MUTANTS

A genetic approach to investigate plastid signalling was initiated by Susek *et al.* (1993), and has recently resulted in the identification of the genes affected in several *gun* (*genomes uncoupled*) mutants of arabidopsis (Mochizuki *et al.* 2001). Susek *et al.* (1993) introduced a chimeric gene construct containing the GUS reporter gene and the hygromycin-resistance *AphIV* selectable marker gene, both under the control of an arabidopsis *Lhbc1* promoter, into *Arabidopsis thaliana*, to produce plants that showed plastid-regulated GUS activity and hygromycin resistance. Seeds from these plants were mutagenized with EMS and mutants that showed GUS activity when grown in the presence of norflurazon were isolated (Susek *et al.* 1993). The mutants obtained were placed in five complementation groups. The *gun5-1* mutant was shown to result from a point mutation in the *ChlH* gene encoding the H subunit of Mg chelatase, which introduces Mg^{2+} into protoporphyrin IX as the first committed step of chlorophyll synthesis (Mochizuki *et al.* 2001). *gun2* and *gun3* were identified as alleles of the long hypocotyl photomorphogenic mutants *hy1* and *hy2*, which have mutations in the genes encoding haem oxygenase (Muramoto *et al.* 1999) and phytochromobilin synthase (Kohchi *et al.* 2001). These enzymes are required for the synthesis of the phytochrome chromophore from haem in plastids.

Genetic analysis of the *gun* mutants indicated they were all recessive and none of the mutants completely inactivated plastid signalling (Mochizuki *et al.* 2001). Analysis of *gun* double mutants suggested that *gun2*, *gun3*, *gun 4* and *gun5* all affected the same signalling pathway, whereas *gun1* appeared to affect a separate signalling pathway (Vinti *et al.* 2000; Mochizuki *et al.* 2001). *GUN2*, *GUN3* and *GUN5* all encode enzymes of tetrapyrrole metabolism, and mutations in these genes are likely to affect the relative proportions of tetrapyrrole intermediates in the plastids. However, most *gun* mutants do not show large changes in chlorophyll content or altered expression of nuclear photosynthesis-related genes when grown under normal conditions (Susek *et al.* 1993; Mochizuki *et al.* 2001); the *gun* phenotype appears to be visible only in the presence of norflurazon. This complicates the interpretation of how perturbation of tetrapyrrole metabolism provides plastid signals. Further complications were suggested by the observation that mutations in the *ChlI* gene encoding the Mg chelatase I subunit, for example in the arabidopsis *cs* and *ch42* mutants, did not produce a *gun* phenotype in the presence of norflurazon (Mochizuki *et al.* 2001). However, recent examination of tobacco lines with decreased *ChlI* expression, by expression of antisense RNA or by cosuppression (Papenbrock *et al.* 2000), indicated a *gun* phenotype (J. A. Sullivan, B. Grimm and J. C. Gray, unpublished data). The seedlings showed higher levels of *Lhcb1* and *RbcS* transcripts compared with wild-type seedlings when grown in the presence of lincomycin. The reason for the apparent difference between tobacco and arabidopsis lines is not clear.

A new collection of arabidopsis *gun* mutants has been produced following EMS mutagenesis of a line containing a reporter gene encoding endoplasmic-reticulum-targeted GFP under the control of a tobacco *RbcS* promoter (J.-H. Wang, J. A. Sullivan and J. C. Gray, unpublished data). Mutants that showed GFP fluorescence when grown in the presence of norflurazon were identified under a handheld ultraviolet lamp. Over 400 mutant lines have been selected from the M2 progeny of 35 000 mutagenized seeds, and are currently under investigation. Analysis of the first three mutant lines isolated showed they were allelic to the *gun1*, *gun2* and *gun4* mutants isolated previously (Mochizuki *et al.* 2001). These lines expressed the *gfp* reporter gene and endogenous *Lhcb1* and *RbcS* genes in 7-day-old seedlings grown in the presence of norflurazon in the light. The expression of the endogenous chalcone synthase (*CHS*) gene, which is regulated by light but not by plastid signals in wild-type plants, was not affected in the *gun* mutants. Examination of the expression of these genes in *gun* mutants grown for 7 days in the presence of lincomycin clearly distinguished two classes of *gun* mutants. The *gfp*, *Lhcb1* and *RbcS* genes were expressed in the presence of lincomycin in the *gun1* mutants, whereas these genes were sensitive to lincomycin treatment in the *gun2*, *gun4* and *gun5* mutants (J.-H. Wang, J. A. Sullivan and J. C. Gray, unpublished data). This suggests that *GUN1* encodes a component of the signalling pathway affected by inhibition of plastid protein synthesis. This provides further evidence to distinguish *gun1* from the other *gun* mutants, which all appear to be affected in tetrapyrrole biosynthesis (Mochizuki *et al.* 2001). The isolation of the *gun1* mutants by selection on norflurazon suggests that chloroplast photooxidation induced by norflurazon treatment affects signal(s) produced by the plastid protein-synthesis pathway.

5. SIGNALLING INTERMEDIATES

The identification of the arabidopsis *gun2*, *gun3* and *gun5* mutants as defective in enzymes of tetrapyrrole biosynthesis (Mochizuki *et al.* 2001) provides genetic evidence for a role for tetrapyrrole intermediates in plastid signalling. Considerable circumstantial evidence for a role for tetrapyrrole intermediates has accumulated over many years, mainly through the use of tetrapyrrole biosynthesis inhibitors or the direct application of tetrapyrrole intermediates to plants. Inhibition of tetrapyrrole biosynthesis by 2,2′-bipyridyl, an iron chelator believed to inhibit formation of the isocyclic ring of chlorophyll, resulted in the downregulation of *Lhcb* genes in *Chlamydomonas* (Johanningmeier & Howell 1984; Johanningmeier 1988). The cells treated with 2,2′-bipyridyl accumulated large amounts of Mg protoporphyrin methyl ester, leading to the suggestion that this tetrapyrrole biosynthesis intermediate acts as a negative effector of *Lhcb1* expression. Nuclear run-on experiments showed that the 2,2′-bipyridyl treatment resulted in decreased transcription of the *Lhcb1* genes (Jasper *et al.* 1991). Similar observations were made with cress seedlings treated with 2,2′-bipyridyl or 8-hydroxyquinoline (Kittsteiner *et al.* 1991). The plants showed decreased amounts of *Lhc* transcripts by Northern blot hybridization, and decreased *Lhc* transcription in nuclear run-on assays (Kittsteiner *et al.* 1991). Treatment of cress seedlings with β-thujaplicin also resulted in increased amounts of Mg protoporphyrin methyl ester and decreased transcription of *Lhcb1*, *Lhcb2* and *Lhca1* (Oster *et al.* 1996). Barley seedlings treated with amitrole, normally regarded as a carotenoid biosynthesis inhibitor, contained increased amounts of Mg protoporphyrin in the dark, and showed decreased expression of *Lhcb1* and *RbcS* when transferred to light (La Rocca *et al.* 2001). From these experiments, it appears that Mg protoporphyrin and its methyl ester may be negative signalling components for the regulation of photosynthesis-related genes. This negative regulation may be a stress response and may not have a major role in gene regulation under ambient conditions. Inhibitors of the early steps of tetrapyrrole biosynthesis do not appear to affect the expression of photosynthesis-related nuclear genes in *Chlamydomonas* or tobacco seedlings under standard growth conditions. Laevulinic acid and 4,6-dioxoheptanoic acid, competitive inhibitors of aminolaevulinate dehydratase, did not affect *Lhcb1* expression in *Chlamydomonas* (Johanningmeier & Howell 1984; Johanningmeier 1988), and gabaculine, an inhibitor of glutamate semialdehyde aminotransferase, had little effect on expression from the *RbcS* and *PetH* promoters in transgenic tobacco seedlings (Gray *et al.* 1995).

However, a number of observations indicate that conclusions on the negative regulatory effects of Mg protoporphyrin and its methyl ester are not entirely satisfactory. Decreased light induction of *Lhcb1* has been reported for the *Chlamydomonas brs-1* mutant (Johanningmeier & Howell 1984), which has recently been shown to result from a frameshift mutation in the *ChlH* gene encoding the H-subunit of Mg chelatase (Chekounova *et al.* 2001). This

mutant accumulates protoporphyrin IX, which it cannot convert to Mg protoporphyrin. The *brs-1* mutant has also been used for studies on the expression of two nuclear genes, encoding cytosolic and chloroplast hsp70 proteins, which have been reported to be induced by Mg protoporphyrin and Mg protoporphyrin dimethyl ester in *Chlamydomonas* (Kropat *et al.* 1997, 2000). The induction of the *hsp70A* gene, encoding the cytosolic hsp70 protein, by Mg protoporphyrin required the same promoter region shown to be involved in light regulation (Kropat *et al.* 1997). Light induction of *hsp70A* and *hsp70B* was not observed in the *brs-1* mutant (Kropat *et al.* 1997), suggesting that Mg protoporphyrin, or its methyl ester, is a positive regulator required for light-regulated expression of the *hsp70* genes. The light induction of an arabidopsis *hsp70* gene was delayed in a mutant affecting the ChlI subunit of Mg chelatase, but not in a mutant affecting the H-subunit (Brusslan & Peterson 2002). This adds further complexity to the role of tetrapyrrole intermediates in regulating nuclear gene expression.

Additional evidence for a possible role for tetrapyrrole intermediates in regulating nuclear gene expression may be provided by the arabidopsis *laf6* mutant, which has a reduced responsiveness to far-red light (Møller *et al.* 2001). The mutant seedlings show an approximately two-fold increase in protoporphyrin IX, and decreased accumulation of transcripts of *Lhcb1*, *PetH* and *CHS* (Møller *et al.* 2001). However, the decrease in *CHS* transcripts suggests that the mutant may be defective in a pathway separate from plastid signalling; *CHS* expression appears to be unaffected in ribosome-deficient barley (Hess *et al.* 1994) or by norflurazon and lincomycin treatments of arabidopsis seedlings (J. H. Wang, J. A. Sullivan and J. C. Gray, unpublished data). *laf6* is homologous to *ycf24*, a plastid gene in red algae, and is reported to encode an ATP-binding cassette transporter (Møller *et al.* 2001). However, more recent analysis suggests the gene is homologous to bacterial *sufB*, and is more likely to be involved in iron-sulphur centre assembly (Wilson *et al.* 2003, these proceedings).

6. PHOTOSYNTHESIS SIGNALS

Expression of nuclear genes encoding photosynthesis-related proteins is also regulated by signals derived from photosynthetic electron transfer, as part of the process of acclimation to altered light quality or intensity. Light-intensity-dependent changes in transcripts of *Lhcb1* in the green alga *Dunaliella tertiolecta* were shown to be due to changes in the plastoquinone redox state (Escoubas *et al.* 1995). Reciprocal changes in *Lhcb1* transcripts were observed following treatment of the alga with DCMU, an inhibitor of the Q_B site of photosystem II, and DBMIB, an inhibitor of the Q_O site of the cytochrome *bf* complex (Escoubas *et al.* 1995; Durnford & Falkowski 1997). Similar changes in *Lhcb1* transcripts were observed in *Dunaliella salina* following light and temperature treatments affecting the redox state of Q_A in photosystem II (Maxwell *et al.* 1995). Light treatments preferentially exciting photosystem II have also been reported to increase expression of the GUS reporter gene from the spinach *PetE*, *PsaD* and *PsaF* promoters in transgenic tobacco plants (Pfannschmidt *et al.* 2001*b*). Treatment of these plants with DCMU and DBMIB suggested that the spinach *PetE* promoter was responding to redox signals, whereas the *PsaD* and *PsaF* promoters were responding to other photosynthesis-derived signals (Pfannschmidt *et al.* 2001*b*).

The arabidopsis *PetE* promoter appears to show a rather complex set of responses to DCMU in arabidopsis cell cultures and in detached leaves (Oswald *et al.* 2001). DCMU prevented the starvation-induced increase in expression from *PetE* and *Lhcb1* promoters when added at the time of sugar removal, but DCMU had no effect when added to cell cultures or leaf discs that were actively expressing *PetE* and *Lhcb1* (Oswald *et al.* 2001). Petracek *et al.* (1997) had previously reported that DCMU had no effect on the accumulation of *Lhcb1* transcripts in illuminated dark-adapted tobacco plants. However, DCMU had a marked inhibitory effect on *Lhcb1* transcripts in light-grown tobacco plants (Sullivan & Gray 2002), and nuclear run-on assays showed that this was due largely, if not exclusively, to a decreased rate of *Lhcb1* transcription (Sullivan & Gray 2002). However, it is not known if the effects of DCMU are operating through a redox-signalling pathway, or via another separate photosynthesis-derived pathway.

Pfannschmidt *et al.* (2001*a*) have proposed that perturbation of photosynthetic electron transfer produces three different types of redox signals depending on the light intensity. They proposed that redox signalling from the plastoquinone pool would be used for fine tuning and would respond to light intensity changes at the lower end of the range. Redox signalling from thioredoxin would be used for general adaptation in moderate light conditions, whereas extreme light intensities would provoke a stress response mediated by glutathione and ROS. The identity of downstream components of these three putative redox signalling chains are not known, nor is it known if the signals converge at a single element in the promoters of responsive genes, or if they affect different regulatory steps in gene expression.

Photosynthesis signals have been shown to regulate the expression of the pea *Fed-1* and *PetE* genes in transgenic tobacco at a post-transcriptional level (Petracek *et al.* 1997; Sullivan & Gray 2002). However, in neither case has the nature of the photosynthesis signal been established. DCMU treatment caused a destabilization of *Fed-1* transcripts in 4-week-old transgenic tobacco plants, and this effect was mediated by elements in the 5′ UTR and coding regions, previously identified as light-regulatory elements (Dickey *et al.* 1992; Petracek *et al.* 1997, 1998). A requirement for sequences in the 5′ UTR and coding region for regulation by light, and by plastid signals affected by norflurazon and lincomycin, has also been shown for pea *PetE* in transgenic tobacco (Helliwell *et al.* 1997; Sullivan & Gray 2002). DCMU treatment of 4-week-old transgenic tobacco plants containing chimeric *PetE* constructs resulted in decreased stability of the *PetE* transcripts, suggesting that photosynthesis signals are used to regulate mRNA stability (Sullivan & Gray 2002).

The involvement of photosynthesis signals in the expression of nuclear genes encoding photosynthesis-related proteins does not appear to operate during early seedling development. DCMU treatment had little effect on the accumulation of *Lhcb1* or *PetE* transcripts

(Sullivan & Gray 2002), or on the expression of the GUS reporter gene from the *RbcS* and *PetH* promoters, in 7-day-old tobacco seedlings (Gray *et al.* 1995). This is perhaps not surprising; a role for photosynthesis in plastid-to-nucleus signalling during early seedling development seems unlikely, given that photosynthesis-related nuclear genes must be expressed before a functional photosynthetic apparatus can be assembled. Photosynthesis signals appear to be involved mainly in the acclimation of the photosynthetic apparatus to changes in light intensity and light quality.

7. MULTIPLE PLASTID SIGNALS REGULATING NUCLEAR GENE EXPRESSION

The currently available biochemical and genetic evidence indicates that the perturbation of a number of plastid processes, including tetrapyrrole biosynthesis, protein synthesis and photosynthesis, influences the expression of nuclear genes encoding photosynthesis-related proteins. The involvement of multiple plastid signals complicates our interpretation of experiments investigating plastid-to-nucleus signalling pathways. The original concept of a single 'plastid factor' (Oelmüller *et al.* 1986; Oelmüller 1989; Taylor 1989) must be modified considerably to explain the involvement, and probable interaction, of multiple signalling pathways. Genetic analysis of arabidopsis *gun* mutants has suggested the existence of two separate signalling pathways (Mochizuki *et al.* 2001; Vinti *et al.* 2000), one of which involves tetrapyrrole biosynthetic intermediates and the other requires plastid protein synthesis (J.-H. Wang, J. A. Sullivan and J. C. Gray, unpublished data). The requirement for plastid protein synthesis is apparently independent of tetrapyrrole signals, and independent of light (Gray *et al.* 1995; Sullivan & Gray 1999), but can be disrupted by norflurazon, as indicated by the selection of *gun1* mutants on norflurazon (Susek *et al.* 1993; J.-H. Wang, J. A. Sullivan and J. C. Gray, unpublished data). This highlights the potential difficulty of interpreting the results of inhibitor experiments when the observed effects on nuclear gene expression are secondary to the primary effect of the inhibitor. Chlorophyll photooxidation resulting from norflurazon treatment in the light may perturb several, if not all, of the plastid signalling pathways. Similarly, inhibition of plastid gene expression, with lincomycin or nalidixic acid, can be expected to have a plethora of secondary effects. It may therefore not be easily possible to define the individual signalling pathways using inhibitors with a broad range of secondary effects.

Similar difficulties in defining individual signalling pathways are apparent from studies on photosynthetic signals affecting nuclear gene expression. Although these are usually defined as redox signals (Pfannschmidt *et al.* 2001*a*), very few studies have shown that the altered redox poise of an identified electron transfer component is involved in signalling. Escoubas *et al.* (1995) showed convincingly the involvement of the plastoquinone redox state in light-intensity-dependent signalling in *D. tertiolecta* by the use of the inhibitors DCMU and DBMIB. However, most subsequent studies have used only DCMU as an inhibitor to invoke a role for redox signalling. The proposal that the redox states of thioredoxin and glutathione, and ROS, initiate signalling pathways for regulating the expression

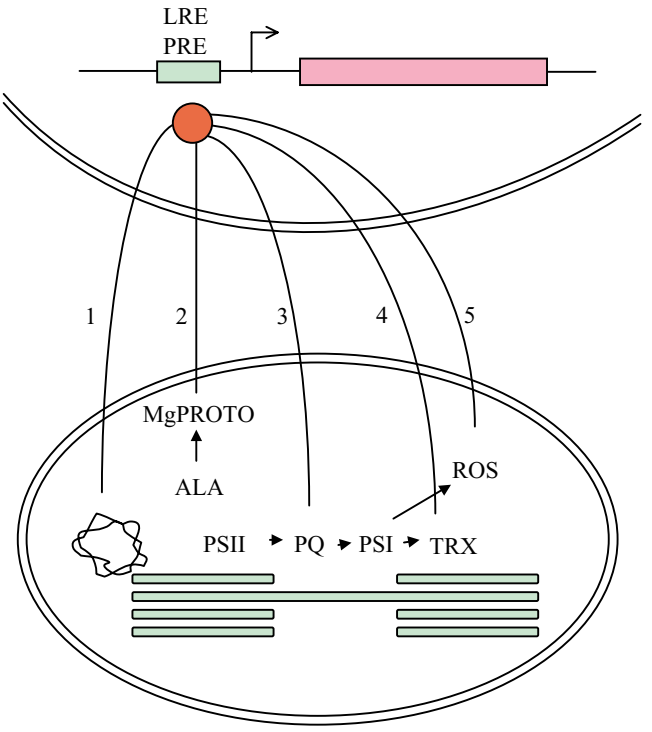

Figure 3. Model of plastid signalling pathways and their interaction with the promoter of a photosynthesis-related nuclear gene. The lower part of the figure shows a chloroplast and the processes providing information for regulating nuclear gene expression. The different signalling pathways are dependent on: (1) plastid gene expression; (2) tetrapyrrole biosynthesis; (3) plastoquinone redox state; (4) thioredoxin redox state; (5) ROS. The upper part of the figure shows a schematic nuclear gene with a common promoter element for responding to light signals (LRE) and plastid signals (PRE), and a coding region for a photosynthesis-related protein (pink box). The red circle corresponds to a common factor of the transcription machinery that integrates the signals from the various pathways. Abbreviations: ALA, aminolevulinic acid; LRE, light response element; MgPROTO, Mg protoporphyrin; PSI, photosystem I, PSII, photosystem II; PRE, plastid response element; PQ, plastoquinone; ROS, reactive oxygen species; TRX, thioredoxin.

of photosynthesis-related nuclear genes (Pfannschmidt *et al.* 2001*a*) is largely unsupported by experimental evidence. Further work is needed to define the signalling pathways responding to perturbations of the photosynthetic electron transfer chain.

Promoter analysis has indicated that the signalling pathways affected by norflurazon and lincomycin treatment appear to converge on common promoter elements that respond to light and to plastid signals (Puente *et al.* 1996; Martinez-Hernandez *et al.* 2002; L. Sanders and J. C. Gray, unpublished data). There appears to be no detailed information on whether photosynthetic signals also operate through the same promoter elements used by the other plastid signalling pathways. A model of the known and proposed plastid signalling pathways and their interaction with the promoter of a nuclear gene is presented in figure 3. This shows five separate signalling pathways initiating in the plastids. The identification of the components of these signalling pathways and an understanding of how

information from each of the pathways is integrated remain important goals for the future.

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Discussion

J. F. Allen (*Plant Biochemistry, Lund University, Lund, Sweden*). I announce that I am jealous of your experimental setup. The seven-day-old seedlings were the subjects in the microarray results, and you have not done that with four-week-old seedlings.

J. C. Gray. No, it could be done relatively easily, but we have not done this.

J. F. Allen. But you will?

J. C. Gray. It is not one of the experiments at the top of the list; there are some others.

J. F. Allen. I am in a frustrating position with microarrays. All around me where I work there are people with huge investments in microarrays and I have heard stories from medical researchers such as 'we've got all this data, and it cost an awful lot of money, with all these skilful people, and here it is, and we don't know what it means'. And the next stage of madness is to employ a whole load of computer software developers to analyse the data, to tell you what the knowledge is you will get out of it, by computer programming I have been taking shots at this systematically with the help of Adam Wilkins, for example, in BioEssays, saying 'Actually, that is not how you do science'. You begin with a hypothesis to make predictions, you do experiments to see if the real world corresponds or not with the predictions of your hypothesis, and yet there seems to be this microarray explosion of data that no one can interpret. So I say, 'Please, so why did you do that experiment in the first place?'.

J. C. Gray. That is a very good question, John, and having got the result that we got, or the results that we got, I guess we are not sure that we were asking the right questions for this particular set of experiments. The way that traditionally you do experiments is to do Northern blots on a very restricted set of genes, and try and draw conclusions. The conclusions for plastid gene expression that have been drawn are the ones that were alluded to in Reinhold Herrmann's talk, in that there are two sets of polymerases and the photosynthesis genes are transcribed by the plastid-encoded polymerase, and the housekeeping genetic system genes are encoded by the nuclear-encoded polymerase. That is on the basis of no more than 10 or 15 genes, and it seemed to us that what we really wanted to look at were the patterns of gene expression. I guess we hoped that by looking at the patterns of the whole genome, we would actually divine something. The answer is that there are some interesting features, but they have told us nothing about signalling. Now we have gone one step further in our madness; we are using whole gene arabidopsis arrays to look at the nuclear genes. The question we are asking is a very specific one, which is how many nuclear genes are under these sorts of signalling controls? We have been doing Northern blots in our experiments with five or maybe half a dozen genes and we want to know how many genes are actually under this sort of control. We think microarrays can tell us that. The problem is that if the numbers are too huge, then we are going to have to be very, very selective about what we do with particular genes. We suspect that not only will sets of gene transcripts be going down, in other words the signal is knocking gene expression down, we will find genes whose expression goes up. That would give us a clue as to what the signalling pathways might be.

D. S. Horner (*Dipartimento di Fisiologia e Biochimica Generali, University of Milan, Milan, Italy*). Do your data imply nuclear-encoded factors that are actually mediating the signalling?

J. C. Gray. I think so, yes. I mean, certainly Joanne Chory is close to isolating *GUN1*, if she has not already isolated it, and the candidate gene is reported to be a nuclear protein. In other words, this is a protein that works in the nucleus, and it is not chloroplast encoded.

C. J. Howe (*Department of Biochemistry, University of Cambridge, Cambridge, UK*). Do you see parallels between mitochondrial influence on nuclear gene expression and chloroplast influence on nuclear gene expression? I was looking at mitochondria in other organisms.

J. C. Gray. We have not done those experiments. We are aware of the work that has been done with the barley *albastrians* mutant, where you see mitochondrial effects, or chloroplast effects on mitochondria. We do see transcripts of the *Atp2* gene going up with some of our treatments, but that is the extent of any interaction with mitochondria.

C. J. Howe. Right. Really what I was getting at was the fact that, as I understand it, haem seems to be involved in mitochondrial signalling to the nucleus, and there seems to be an interesting parallel there to what you see in the chloroplast.

J. C. Gray. OK. But as far as I know that has not been shown in plants.

C. J. Howe. No, no, in other organisms.

J. C. Gray. In yeast.

C. J. Howe. So it seems interesting that that pathway seems to be implicated in plastid systems.

J. C. Gray. Well, I think that in the history of this, the reason that people were interested in tetrapyrrole signals was because of the yeast haem mitochondrial signalling. In other words, because haem was a signal from mitochondria, people thought that there might be something similar in plastids, and it is really the *gun5* mutant that is said to be possibly the answer, or one of them.

E. Lopez-Juez (*School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK*). You said that there are a variety of signals and there is this discussion about microarrays. As you know, we also think that there are a variety of signals, and in a project with you, John, and with Kevin Pyke in Nottingham, we think that there is evidence for a 'greening'-related or 'plastid function or dysfunction'-related positive signal which, if it is not a tetrapyrrole, must be produced by something else. We have also seen in parallel work that the highlight signals that John Allen was describing that control chloroplast-encoded genes also control related nuclear genes; as he mentioned, things like *psbA* goes up or down in the chloroplast, but then *PsbO* also goes up or down in the nucleus. These sorts of responses also happen later; you cannot see LHC (light-harvesting complex) regulated by high light signals early on in young arabidopsis seedlings. You can only see it in plants. And there may be other high-light-related signals that are not redox signals. My question is, are all those signals doing the same thing or separate things? We will probably not know the answer by looking at one, two or three genes, and your suggestion that one needs to find patterns—is it photosystem-related genes versus light utilization genes?—holds, to sort out a multiplicity of signals, and a multiplicity of stages or responses.

J. C. Gray. Absolutely. If you start now trying to find the promotor region through which the signals work, it all comes down to a small region that is controlling light regulation and plastid regulation. We have sort of narrowed down in the *PetH* promotor and we find that whatever norflurazon is doing, whatever lincomycin is doing and whatever light is doing, they are all operating through a region of about 30 base pairs.

R. G. Herrmann (*Department für Biologie I, Bereich Botanik, Ludwig-Maximilians-Universität, Munich, Germany*). John, you mentioned that ATP synthase genes are up– and down–regulated, and this is a system that is impaired in greening, and you refer to microarray data. Did you check the Northern blots? I am asking this because in some cases, including ATP synthase genes, we see a substantial increase factor of 10 of the signal in the array, but when we look at the Northerns everything is degraded, so this may be misleading. Do you see a clear RNA pattern?

J. C. Gray. We have not checked with Northern blots for ATP synthase genes.

R. G. Herrmann. It would be interesting to see.

C. J. Leaver (*Department of Plant Sciences, University of Oxford, Oxford, UK*). You look at levels of transcripts, what about levels of proteins, which to me are probably even more interesting?

J. C. Gray. Well, yes it is. The answer is we have not because, although we have antibodies to a range of proteins, but not all of them, these experiments are treated with lincomycin. Lincomycin is stopping protein synthesis, so even if the transcript is there, you would not expect the protein to be there—at least, not the chloroplast-encoded ones. So that experiment is not likely to give you a meaningful answer. You could look at the nuclear-encoded proteins to see whether they are present. But we are showing that the nuclear genes are being switched off, so again you would not expect to see a protein. At least with lincomycin, it is not an experiment that we think would be useful.

GLOSSARY

DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea
dNTP: deoxyribonucleoside triphosphate
EMS: ethylmethane sulphonate
GFP: green fluorescent protein
GUS: β-glucuronidase
ROS: reactive oxygen species
UTR: untranslated region