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Combinatorial interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene expression in *Arabidopsis*

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Higher plants are able to integrate environmental and endogenous signals to regulate gene expression for optimal development. To define the minimal sequence requirement sufficient to integrate light and developmental signals in controlling promoter activity, we carried out a systematic analysis of the roles of four well-conserved 'light-responsive elements (LREs)' common to many nuclear-encoded photosynthetic genes. A gain-of-function assay using basal promoter–reporter fusions in stable transgenic *Arabidopsis* was employed to demonstrate that pairwise combinations of the LREs, but not the individual elements alone, can confer light-inducible expression to the reporter gene independently of the basal promoter context and the light-triggered morphological changes. The activity of the synthetic promoters with the paired LREs can be modulated at least by the phytochrome system. Further, those synthetic light-regulated promoters confer a photosynthetic cell-specific expression pattern and respond to the chloroplast development state. Our data suggest that distinct combinatorial interactions of LREs can serve as minimal autonomous promoter determinants which integrate light and developmental signals and modulate promoter activity.

Keywords: *Arabidopsis*/gene regulation/light-responsive element/promoter determinant

Introduction

Plants not only use light as an energy source, but also as an informational signal to control developmental processes for optimal growth under the prevailing light environment (Kendrick and Kronenberg, 1994; McNellis and Deng, 1995). Many of the light-controlled developments are triggered by alterations in gene expression through the regulation of transcription of specific genes in defined cell types and developmental stages (Gilmartin *et al.*, 1990; Thompson and White, 1991; Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). Some genes, such as nuclear-encoded photosynthesis-related genes for chlorophyll *a/b* binding proteins (cab) and small subunit of the ribulose 1,5-bisphosphate carboxylase (*rbcS*), are expressed at high levels upon exposure to light, whereas some others are negatively regulated by light. At least three families of photoreceptors, phytochromes, blue light receptors (also called cryptochrome) and UV light receptors (UV-B photoreceptor), have evolved to mediate light control of gene expression. Among these, phytochrome-mediated light control of gene expression has been studied most extensively and has been shown to regulate promoter activity independently of other photoreceptors and in the absence of protein synthesis or morphological changes (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). The signaling events following the light perception by photoreceptors have started to be unraveled (Bowler and Chua, 1994; Deng, 1994; Quail, 1994).

Characterization of the promoter elements involved in light regulation has been widely used as a convenient starting point for understanding the light control of gene expression (Gilmartin *et al.*, 1990; Manzara *et al.*, 1991; Anderson *et al.*, 1994; Conley *et al.*, 1994; Kehoe *et al.*, 1994; Terzaghi and Cashmore, 1995). By analyzing expression of introduced chimeric genes in stably transformed tobacco plants, minimal promoter regions (~60–200 bp, all within 500 bp of the transcription start site) required for light regulation have been defined in several promoters of light-regulated photosynthetic genes (e.g. pea *rbcS-3A*, Gilmartin *et al.*, 1990; *Arabidopsis rbcS-1A*, Donald and Cashmore, 1990; *Nicotiana plumbaginifolia cabE*, Castresana *et al.*, 1988; *Arabidopsis cab1*, Ha and An, 1988; *Arabidopsis cab2*, Anderson *et al.*, 1994; *Lemna gibba cabAB19*, Kehoe *et al.*, 1994). It is important to note that in all those cases where a minimal promoter retains proper light responsiveness, the original mode of tissue specificity and developmental regulation was largely maintained. Several consensus sequence elements, including the G motif, the GATA (or I) motif, the GT1 motif and the Z motif, are commonly found in those light-regulated minimal promoter regions and have been shown by mutagenesis studies to be neccesary for high promoter activity in the light (Kehoe *et al.*, 1994; Terzaghi and Cashmore, 1995). Those elements are commonly designated as 'light-responsive elements (LREs)'. Although similar LREs are found in many different promoters and assembled in a variety of ways, no universal element has been found in all light-regulated promoters (Terzaghi and Cashmore, 1995). In fact, some of those elements are present in promoters that are not light regulated. It remains unclear whether any of these defined elements represent the direct end points of light signaling or how the input of different signals (internal development and external light stimuli) is integrated on a light-regulated promoter.

The ability of those defined LREs to confer light-responsive expression to a non-light-regulated minimal promoter has been analyzed in some cases (Lam and Chua, 1989, 1990). Only for the GT1 motif, has it been demonstrated that insertion of a tetramer upstream of a short cauliflower mosaic virus (CaMV) 35S promoter (–90 to +8) can confer responsiveness to light in stably transformed tobacco, but this light induction depends on

a sequence motif (other than the CAAT and TATA motifs) located between –90 and –46 of the CaMV 35S promoter (Lam and Chua, 1990). Thus far, no single element has been demonstrated to confer light responsiveness to a non-light-regulated minimal promoter. Therefore, it has been speculated that it is the specific combination of promoter elements which confers the light responsiveness to a given promoter (Terzaghi and Cashmore, 1995).

To define the minimal promoter elements sufficient to mediate responses to light and developmental signals, we systematically analyzed the ability of four well characterized LREs, individually or in selected combinations, to confer light responsiveness to non-light-regulated basal promoters. This approach overcame the limitation of the previous work which often attempted to study individual LREs derived from one particular gene. Our study revealed that three distinct pairwise combinations of the motifs, but not individual motifs alone, are capable of conferring light responsiveness in the correct cell type and developmental context and independently of the basal promoters used. Further, those synthetic light-responsive promoters are sensitive to transient activation of the phytochrome photoreceptor system and are also able to respond to the developmental state of the chloroplast. The fact that multiple pairwise combinations of different LREs are able independently to mediate light responsiveness of promoters may partly account for the structural and functional diversity of the light-regulated promoters in higher plants.

Results

Experimental design

Our goal was to define which minimal promoter elements or combination of elements are sufficient for mediating light responsiveness and whether it is an intrinsic property of the same promoter elements that mediates responsiveness to other developmental signals such as tissue specificity and chloroplast development. A chimeric reporter system was constructed to test systematically the capability of previously characterized LREs for conferring light responsiveness to a non-light-regulated basal promoter (Figure 1). The basal promoter of the nopaline synthase gene (–101 to +4, designated *NOS101*), which has only the CAAT and TATA elements and was not active in transgenic plants (Mitra and An, 1989), was fused 5′ to the reporter β-glucuronidase (*GUS*) and the nopaline synthase gene (*NOS*) 3′ formation sequence (see Materials and methods). Thus the promoter activity and expression pattern can be monitored easily by assaying either GUS activity or mRNA level. When this *NOS101–GUS* fusion gene was stably introduced into *Arabidopsis*, no GUS activity in any cell type at any developmental stage was detected in all five independent single-locus transgenic lines examined (data not shown).

The four LRE motifs used (Figure 1A) are the GT1 consesus sequence derived from the pea *rbcS-3A* promoter (Lam and Chua, 1990), G and GATA consensus sequences derived from *Arabidopsis cab* and *rbcS* gene promoters (Donald and Cashmore, 1990; Schindler and Cashmore, 1990), and the putative 'Z-DNA' forming sequence (Z motif) of the *Arabidopsis cab1* promoter (Ha and An, 1988). Single or selected pairwise combinations of the

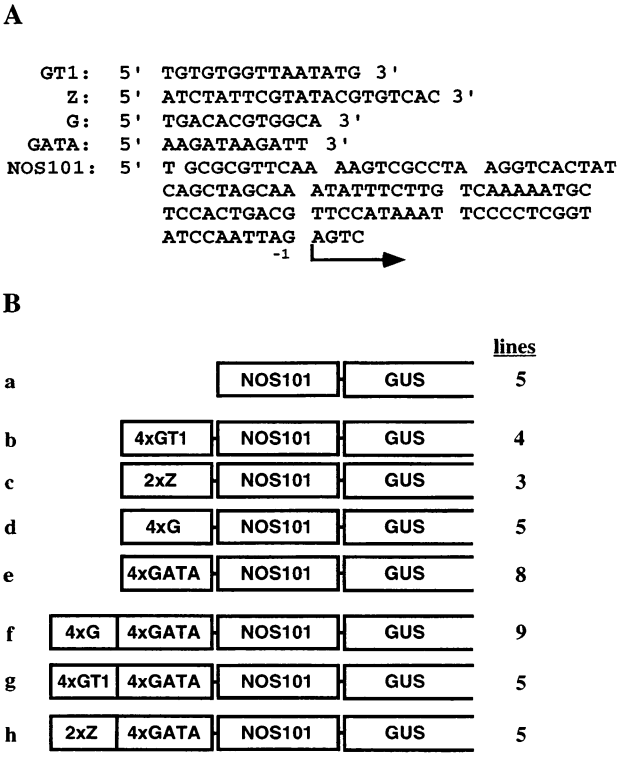

Fig. 1. Summary of the promoter elements and the *NOS101* synthetic promoter–reporter fusion genes used for this work. (**A**) The sequences of the promoter elements. The four light-responsive elements (LRE), GT1, Z, G and GATA, represent consensus sequences derived from well-characterized light-regulated promoters of photosynthetic genes (see text). The *NOS101* element is the basal promoter (–101 to +4) of the nopaline synthase gene (Mitra and An, 1989). (**B**) Diagrams of the synthetic promoter–*GUS* fusion genes used for testing the function of the four selected LREs. The number of copies (two or four) of the LRE used in the synthetic promoters is indicated. The numbers to the right of each construct indicate the total number of independent homozygous single-locus lines produced.

consensus sequences of those LRE motifs were inserted 5′ to the *NOS101–GUS* reporter construct (Figure 1B). To minimize the potential problem of inadequate spacing of the promoter elements, either tetramers (GT1, G, GATA) or dimers (Z) of the consensus sequences were used. The three element pairs used in this work all contain the GATA motif, which is commonly found in light-regulated genes (Grob and Stuber, 1987) and was shown at least in one case to be important for phytochrome-mediated light activation of a promoter (Kehoe *et al.*, 1994). The resulting constructs (Figure 1B) were introduced individually into *Arabidopsis* by stable transformation. For each construct, at least three independent transgenic lines (indicated on the left side of Figure 1B) homozygous for a single locus of the transgene were obtained and analyzed. We observed that utilization of homozygous transgenic lines carrying a single stable T-DNA locus minimized the commonly reported large variation in the transgene activity among independent transgenic lines carrying the same construct (data not shown). For a given construct, very similar or identical expression patterns were observed and thus only representative data are shown. Since each construct utilizes the same reporter and vector systems, and they only differ in the LREs used, it is expected that the expression patterns are results of transcriptional activation via the specific LRE motifs.

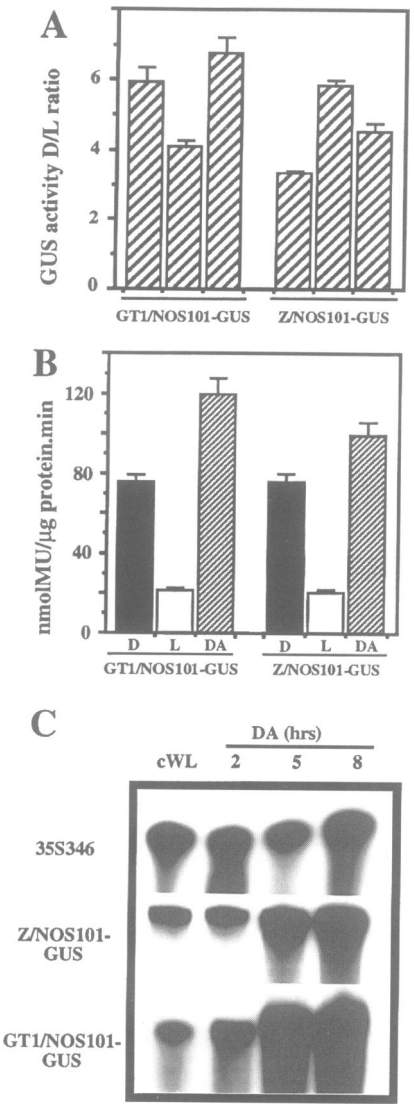

Fig. 2. Both GT1–*NOS101* and Z–*NOS101* synthetic promoters confer elevated expression of the *GUS* reporter in dark-grown seedlings and dark-adapted light-grown seedlings. (**A**) The GUS activity ratios of the dark-grown seedlings (D) versus light-grown transgenic seedlings (L). Three independent single-locus transgenic lines for each synthetic promoter–*GUS* fusion construct were analyzed. The error bars indicate standard deviations from the means of four independent assays. (**B**) Comparison of the GUS activity among the dark-grown (D), light-grown (L) and dark-adapted light-grown seedlings (DA). Only one representative line for each construct is shown. The error bars indicate standard deviations from the means of four independent assays. (**C**) Rapid accumulation of the *GUS* mRNA in transgenic seedlings during the dark adaptation (DA) process. The continuous light-grown seedlings (cWL) were transferred to complete darkness for 2, 5 and 8 h respectively. A fully active but non-light-regulated 35S promoter (–340 to +6)–*GUS* fusion transgenic line (Deng *et al.*, 1991) was used as control. Equal amounts (10 μg) were used for the RNase protection assay shown in each lane.

Individual promoter elements can confer differential expression between dark- and light-grown seedlings

As a fast and preliminary step to examine the expression patterns of the transgenes, three independent lines of each construct were chosen for quantitative fluorometric analysis (Jefferson, 1987) of GUS activity in dark- and light-grown seedlings. As shown in Figures 2 and 3, all

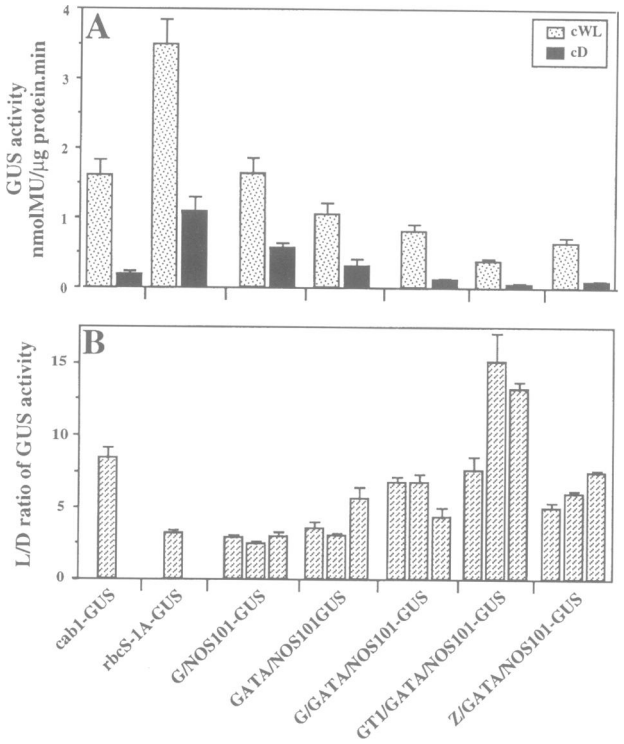

Fig. 3. Summary of the dark/light differential GUS expression levels in 6-day-old seedlings transformed with the *NOS101* series synthetic promoter–*GUS* fusion constructs. (**A**) Relative GUS activity of the 6-day-old dark-grown and light-grown transgenic seedlings. (**B**) The GUS activity ratios of the light-grown seedlings versus dark-grown transgenic seedlings. Three independent single-locus lines for each synthetic promoter–*GUS* fusion construct were analyzed. *cab1–GUS* and *rbcS-1A–GUS* transgenic seedlings are shown for comparison. The error bars indicate standard deviation from the means of four independent assays.

four individual elements conferred different levels of GUS activity between dark- and light-grown seedlings. Both GT1 and Z motifs, when located 5′ to the *NOS101–GUS* reporter, consistently exhibited ~3-to 6-fold higher GUS activity in the dark-grown seedlings than in the light-grown siblings (Figure 2A). To rule out the possibility that the differential GUS expression conferred by GT1 and Z motifs was caused indirectly simply by the drastic morphological differences between light- and dark-grown seedlings, we further examined the effect of dark adaptation of light-grown seedlings on GUS expression. While the seedling morphology was very similar between the 8-day-old light-grown seedlings and the 6-day-old light-grown seedlings subjected to 2 day dark adaptation, the dark adaptation consistently resulted in a 5- to 6-fold increase of the GUS activity based on equal amounts of total proteins (Figure 2B). Analysis of *GUS* mRNA levels indicated that significantly higher levels of *GUS* mRNA can be observed by 5 h after dark adaptation (Figure 2C). These results suggested that GT1 and Z motifs are able to confer higher reporter gene expression in the absence of light.

In contrast, both G and GATA motifs alone conferred a higher GUS activity in the light-grown seedlings than in the dark-grown siblings, as judged by the GUS activity assay (Figure 3). Among all transgenic lines examined, the G motif consistently exhibited ~2- to 3-fold higher GUS activity in the light-grown seedlings than in the

dark-grown siblings, while the GATA motif conferred ~3- to 5-fold higher GUS activity in the light (Figure 3). Under the same conditions, two native *Arabidopsis* light-regulated promoters, *cab1* and *rbcS-1A*, conferred 8- to 10- or 3-fold higher GUS activity, respectively, in the light-grown seedlings (Figure 3). The GUS activity in general correlated well with the *GUS* mRNA levels (see later). Therefore, the G and GATA motifs individually promoted a high level expression of the *GUS* reporter in the light comparable with the control *Arabidopsis* light-modulated promoters.

Paired LRE motifs result in non-additive expression patterns

Interestingly, all three pairwise combinations, G–GATA, GT1–GATA or Z–GATA, are capable of conferring a high GUS activity to the *NOS101–GUS* reporter in the light-grown seedlings (Figure 3A). The ratios of the GUS activity between light-grown and dark-grown seedlings carrying those three transgenes are more striking than those with either G or GATA motifs alone, and differ completely from those bearing GT1 and Z motifs alone. For example, G–GATA and Z–GATA combinations can confer to the *NOS101–GUS* reporter ~4- to 8-fold higher GUS activity in the light, while the GT1–GATA combination led to an 8- to 15-fold higher GUS activity in the light (Figure 3B). Remarkably, the ability of both GT1 and Z motifs to confer higher expression in dark-grown seedlings was completely suppressed when they were paired with the GATA motif. On average, those synthetic promoters are very similar or slightly better than the *Arabidopsis cab1* promoter in conferring high reporter expression in the light.

The functional role of some individual LREs, but not the paired combinations, is highly dependent on the promoter context

The four promoter elements used had been defined as required for high level expression of the *cab* or *rbcS* genes in the light in their native context (Terzaghi and Cashmore, 1995), but they nevertheless conferred drastically different effects on the basal *NOS101* promoter. This prompted us to examine the possible effect of the basal promoter context on the functional roles of the LREs tested. A set of constructs (Figure 4A), which are similar to those shown in Figure 1B except the *NOS101* basal promoter was replaced by the minimal CaMV 35S promoter (*35S90*, –90 to +6), were constructed and single-locus *Arabidopsis* transgenic lines were generated. Since the *35S90* promoter alone can confer GUS expression in roots of transgenic tobacco seedlings (Lam and Chua, 1990) as well as in *Arabidopsis* (data not shown), only the upper parts (hypocotyl and cotyledon) of seedlings were analyzed for the differential expression patterns of the transgenes (Figure 4B). Nevertheless, in the case of GT1–*35S90–GUS* and Z–*35S90–GUS*, similar results were observed when either whole seedlings or only the upper parts of seedlings were used for the GUS assay.

For individual LREs alone, both G and Z conferred similar expression patterns in the context of either *35S90* or *NOS101* promoters (Figure 4B), with G conferring a higher GUS activity in the light and Z conferring a higher GUS activity in darkness. However, in the context of the

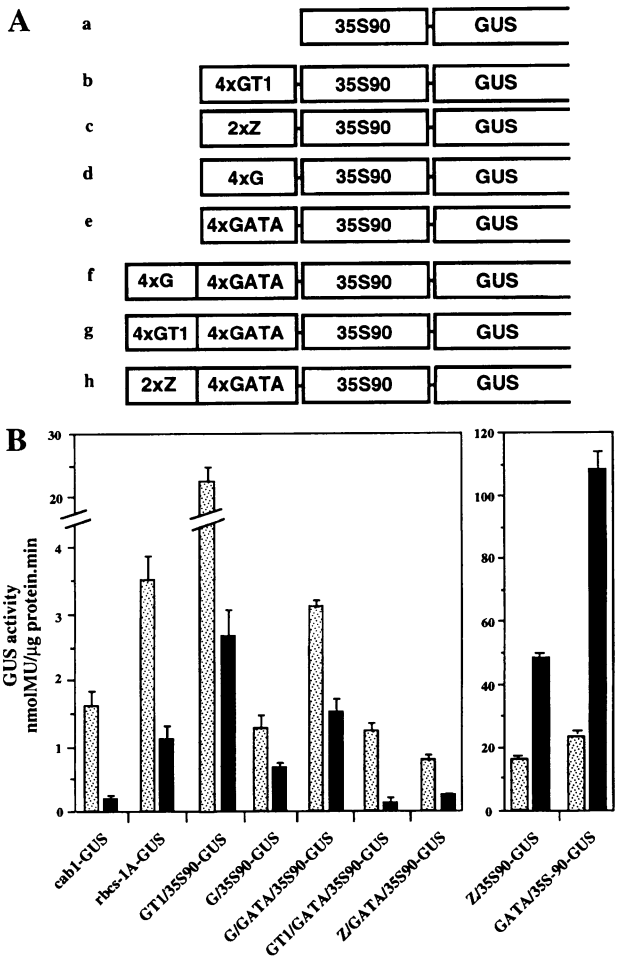

Fig. 4. Summary of the *35S90* series synthetic promoter–*GUS* fusion constructs and their dark/light differential expression patterns. (**A**) Diagrams of the eight *35S90* series synthetic promoter–*GUS* fusion genes. Those constructs are identical to those shown in Figure 1B except that the *35S90* minimal promoter replaced the basal *NOS101* promoter. (**B**) The GUS activities of the 6-day-old dark- (dark) and light-grown (dotted) seedlings from one representative line homozygous for each of the constructs shown in b–h of (A). Both *cab1–GUS* and *rbcS-1A–GUS* transgenic seedlings were analyzed at the same time as controls for comparison. The error bars indicate standard deviations from the means of four independent assays.

35S90 promoter, GT1 conferred a higher GUS activity in light-grown seedlings and GATA conferred a higher GUS activity in dark-grown seedlings (Figure 4B), in contrast to their effects in the context of the *NOS101* promoter (Figures 2 and 3). The expression pattern of the synthetic GT1–*35S90* promoter in transgenic *Arabidopsis* (Figure 4B) was similar to that reported for transgenic tobacco plants (Lam and Chua, 1990). However, the three paired elements (G–GATA, GT1–GATA, Z–GATA) behaved qualitatively similar, regardless of whether *NOS101* or *35S90* promoters were used. All LRE pairs conferred a higher GUS activity in the light-grown seedlings (Figure 4B). Therefore, although individually the LREs may confer contrasting expression patterns depending on the promoter context, the paired combinations of those elements can maintain their expression patterns when different basal promoters are used. Since the *35S90* promoter is active by itself, it is more difficult to assign a specific contribution of the individual promoter elements to the expression

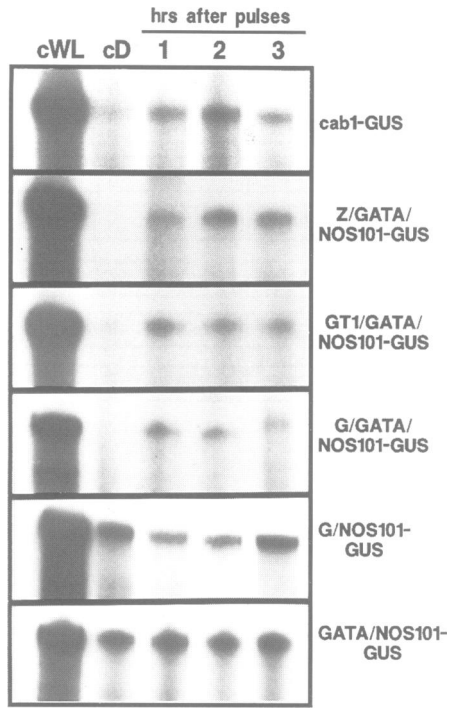

Fig. 5. The effect of short light pulses on the activity of the synthetic promoters. Dark-grown transgenic seedlings (cD) carrying each of the five *NOS101* series synthetic promoters which exhibited high expression in light-grown seedlings were subjected to three 2 min red light pulses (one pulse per hour) and then left in complete darkness for 1, 2 or 3 h before the RNA was extracted. The *GUS* mRNA levels were quantified by RNase protection assay, and equal amounts (ranging from 40 to 60 μg for the lines used) of the total RNA from light-grown (cWL), dark-grown (cD) and light-treated seedlings were used.

patterns of the *35S90* series of synthetic promoters. Therefore, we chose to focus on the *NOS101* series of synthetic promoters for further characterization.

The paired elements are capable of responding to light stimuli independently of light-induced morphological changes

We next sought to determine whether the high reporter gene expression in the light was a direct effect of the light signal, or an indirect effect of the morphogenesis triggered by light, or a combination of both. We therefore analyzed transgene expression of the 6-day-old dark-grown seedlings subjected to short light pulses that do not alter gross seedling morphology. A sensitive RNase protection assay (Zinn *et al.*, 1983) was used to quantify changes of *GUS* mRNA levels of the dark-grown transgenic seedlings after exposure to short light pulses. As shown in Figure 5, red light pulses (2 min of red light per hour for 3 h), clearly induced higher *GUS* mRNA levels in dark-grown seedlings carrying the control *cab1–GUS* transgene or the three *NOS101–GUS* chimeric genes carrying the LRE pairs. However, the *NOS101* chimeric genes which contain G or GATA elements alone did not respond to the light pulses despite the fact that they exhibited a higher GUS activity in light-grown seedlings. Since these short light pulses did not result in gross morphological changes of the *Arabidopsis* seedlings, this result suggested that the light can directly modulate the activity of the promoters containing the LRE pairs but not those with individual

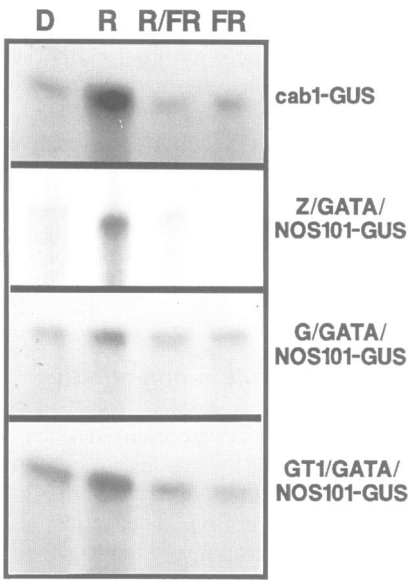

Fig. 6. The red light pulse-mediated promoter activation of the synthetic promoters can be reversed by a far red light pulse. RNase protection analysis of the *GUS* mRNA levels in three synthetic promoter–*GUS* fusions and *cab1–GUS* fusion transgenic seedlings grown in complete darkness (D), or grown in the dark for 6 days and subjected to 1 min of red light (R), 1 min of red light followed by 10 min of far red light (R/FR) or 10 min far red light alone (FR) and left in darkness for an additional 2 h before being collected for RNA isolation. An equal amount of total RNA (~60 μg) was used for each lane for individual transgenic lines.

LREs. In addition, *cab1* as well as the synthetic promoters shown in Figure 5 resulted in higher levels of *GUS* mRNA in light-grown seedling than in dark-grown seedlings, which confirmed the conclusion based on the GUS activity assay (Figure 3). In fact, the differences in the mRNA levels between light- and dark-grown seedlings are more striking than those of the GUS activities. It is evident that the short saturating light pulses were not able to induce the *GUS* mRNA level to that of the light-grown seedlings for *cab1* and the three synthetic promoters containing LRE pairs (Figure 5). This is consistent with the notion that most light-inducible promoters require light-induced morphogenesis for maximal activation (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995).

The paired elements are capable of responding to phytochrome signaling

We further tested whether the synthetic promoters are regulated by the phytochrome photoreceptor system using the classic red light/far red light reversibility assay. Since the synthetic promoters with individual LREs alone were not responsive to light pulses, only the promoters with LRE pairs were examined for the red/far red reversibility and compared with the native *cab1* promoter. As illustrated by *GUS* mRNA levels (Figure 6), a single red light pulse led to a significant increase of activity of the synthetic promoters, while a subsequent 1 min far red light pulse completely reversed this effect. The far red light pulse alone had no detectable effect on the promoter activity under our assay conditions. Those results essentially mimic that of the *cab1–GUS* transgene. Therefore, we conclude that the synthetic promoters with paired elements are responsive to the phytochrome signaling. It is worth

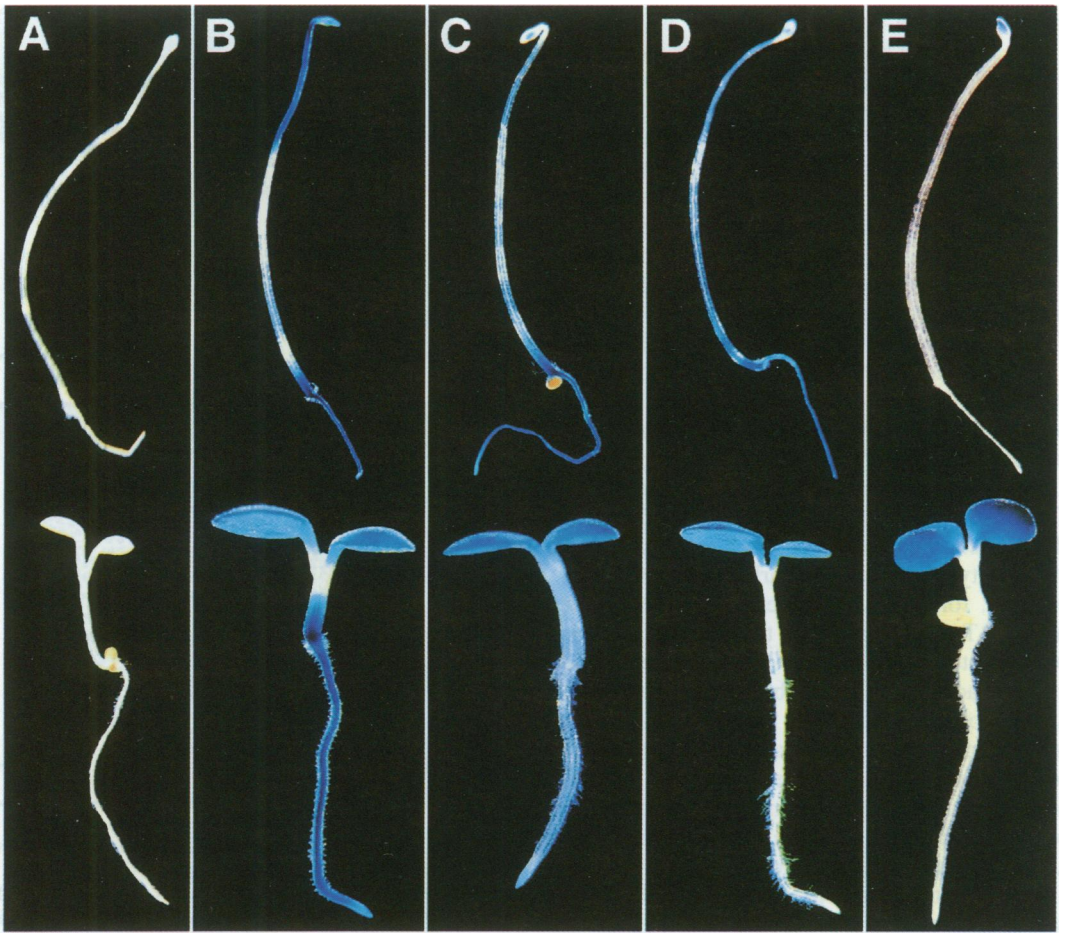

Fig. 7. Organ-specific expression patterns of the synthetic promoters in 6-day-old dark (top) and light-grown (bottom) seedlings. (**A**) The representative seedling GUS staining patterns of a *NOS101–GUS* transgenic line. (**B**) The representative seedling GUS staining patterns of a Z–*NOS101–GUS* transgenic line. The GT1–*NOS101–GUS* transgenic seedlings have identical GUS staining patterns. (**C**) The representative seedling GUS staining patterns of a GATA–*NOS101–GUS* transgenic line. The G–*NOS101–GUS* transgenic lines have essentially the same GUS staining patterns. (**D**) The representative seedling GUS staining patterns of a Z–GATA–*NOS101–GUS* transgenic line. The G–GATA–*NOS101–GUS* transgenic lines have essentially the same GUS staining patterns. The blue halo around the root hairs of the light-grown seedling (bottom) is not due to the GUS staining but is an artifact of the light reflection during photography. (**E**) The representative seedling GUS staining patterns of a GT1–GATA–*NOS101–GUS* transgenic line. The *cab1–GUS* transgenic seedlings have exactly the same GUS staining patterns. The seedlings are not shown to scale and are only intended to illustrate the relative staining pattern between different organs.

pointing out that our observed *cab1–GUS* expression after far red light differs from the reported slight increase of the endogenous *cab1* mRNA after a far red pulse (Karlin-Newmann *et al.*, 1988). This could be due to the possibility that the short *cab1* promoter used in our system did not have the ability to mediate the very low fluence response (VLFR) caused by a far red pulse.

Combinatorial interaction of promoter elements results in novel tissue expression patterns

To determine the tissue-specific expression patterns of the *NOS101* series of synthetic promoters (Figure 1B), we first examined the histological GUS staining pattern of 6-day-old dark- and light-grown transgenic seedlings carrying the synthetic promoter–reporter fusion transgenes. While there was no detectable GUS staining for all lines carrying the *NOS101–GUS* transgene (Figure 7A), four patterns were observed for transgenic lines carrying other constructs (Figure 7B–E). For a given construct, all independent single-locus transgenic lines analyzed exhibited similar or identical GUS staining patterns in either dark- or light-grown seedlings.

Addition of either GT1 or Z to the 5′ end of the *NOS101* promoter, which resulted in dark-induced expression of the *GUS* reporter, also led to the same tissue-specific GUS staining patterns (Figure 7B). In both dark- and light-grown seedlings, the roots and cotyledons showed strong GUS staining, while the hypocotyl exhibited GUS staining mostly on the two ends. Addition of either G or GATA to the 5′ end of the *NOS101* promoter, which resulted in higher expression of *GUS* reporter in light-grown seedlings, also had very similar tissue-specific GUS staining patterns (Figure 7C). Both dark- and light-grown seedlings exhibited GUS staining in cotyledons, roots and hypocotyl, although the relative staining intensity in the different organs is quite different between dark- and light-grown seedlings. In the dark, both the roots and the basal part of the hypocotyl have stronger staining than the cotyledons, while in the light the GUS staining in the cotyledons is stronger than in the roots and hypocotyls. Further, the

GUS staining of the light-grown hypocotyls was weak (sometimes hard to detect) but more uniform in the entire hypocotyl, contrasting with the localized basal staining of the dark-grown seedlings. All three synthetic promoters with LRE pairs (constructs f–h, Figure 1B) which conferred high expression of the *GUS* reporter in light-grown seedlings exhibited very similar or identical GUS staining patterns in light-grown but not in dark-grown seedlings (Figure 7D and E). In the light-grown seedlings, only the cotyledons, but not the roots and hypocotyls, showed clear GUS staining which was essentially identical to that of the *cab1–GUS* transgenic lines. For the dark-grown seedlings, a weak cotyledon staining was observed for all three constructs (f–h, Figure 1B). Interestingly, although of *cab1–GUS* and GT1–GATA–*NOS101–GUS* transgenes exhibited no root and hypocotyl GUS staining in the dark-grown seedlings (Figure 7E), the other two transgenes with G–GATA or Z–GATA LRE pairs (f and h of Figure 1B) showed GUS staining in the roots, hypocotyls, and weak staining in cotyledons of their dark-grown seedlings (Figure 7D). Thus, for the two synthetic promoters with G–GATA or Z–GATA LRE pairs, or to some degree for the synthetic promoters with G and GATA single LREs, light inhibits their activity in roots, but promotes higher expression in the cotyledons.

These results demonstrate that analogous to the light responsiveness, the tissue specificities conferred by the paired elements are novel rather than a simple additive effect of the individual LREs. Therefore, combinatorial interactions, rather than additive effects, of the *cis*-elements within a promoter define the tissue specificity, as well as light responsiveness.

Most synthetic promoters with high expression in light exhibit predominantly photosynthetic cell type-specific expression

Since all four LREs studied in this work are derived from light-inducible photosynthetic genes, we analyzed whether the synthetic promoters of the *NOS101* series actually conferred a photosynthetic cell type-specific expression. The green and photosynthetically competent cotyledons of transgenic seedlings were first stained for GUS activity and then sectioned. As anticipated, the cell type-specific GUS staining patterns in transgenic seedlings carrying all five constructs (d–h, Figure 1B) which give rise to higher GUS activity in light-grown seedlings were essentially identical as judged by GUS staining patterns, and representative results are shown in Figure 8. The GUS staining was strong in mesophyll cells and guard cells which contain photosynthetically active chloroplasts, but weak in the epidemal cells which do not have green chloroplasts (Figure 8). This cell-specific staining pattern is essentially identical to that of *cab1–GUS* transgenic lines (data not shown).

All synthetic promoters with high expression in light are responsive to the chloroplast development state

Although it is well established that the chloroplast developmental state can affect the expression of a large array of nuclear light-regulated genes encoding plastid proteins, very little is known about the promoter elements that mediate responses to chloroplast development (Simpson

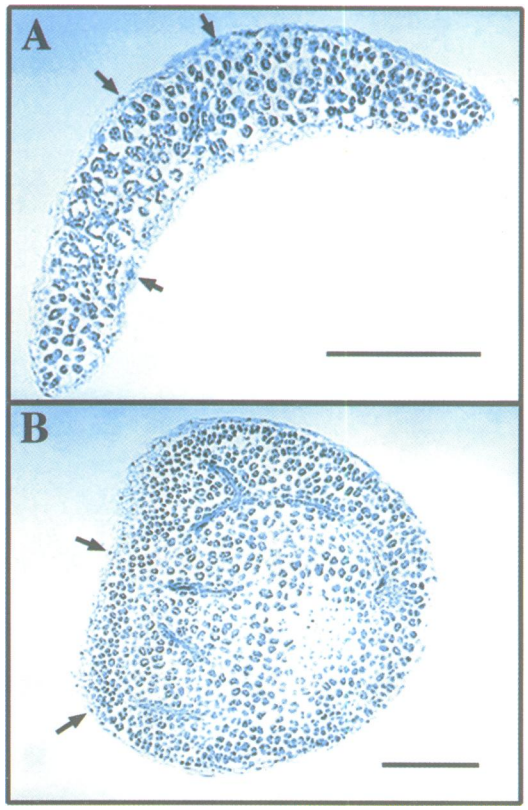

Fig. 8. Cell-specific expression of the synthetic promoters in the cotyledons of the light-grown seedlings. All five *NOS101* series synthetic promoters, G–*NOS101*, GATA–*NOS101*, Z–GATA–*NOS101*, G–GATA–*NOS101* and GT1–GATA–*NOS101*, which exhibit high expression in light-grown seedlings, have identical cell-specific GUS staining patterns in cotyledons from light-grown seedlings. (**A**) and (**B**) Examples of two different cross-sections of the light-grown G–*NOS101–GUS* seedling cotyledons, one being perpendicular (A) and one being parallel (B) to the cotyledon surface. The small arrows in both panels indicate the guard cells. The scale bars represent 0.5 mm.

et al., 1986; Bolle *et al.*, 1994). To test whether any of our synthetic promoters are also regulated by the signal reflecting chloroplast development, the activity of the synthetic promoter in response to Norflurazon-induced chloroplast photooxidative damage was examined. Norflurazon is an inhibitor of phytoene desaturase, and its presence diminishes carotenoid production and thus exposes chloroplasts to the damaging effect of free oxygen radicals generated during the photosynthetic light energy harvesting process (Chamovitz *et al.*, 1991). Since the presence of Norflurazon does not alter the normal light-grown seedling morphology, it has been used conveniently for studying the effect of chloroplast development on nuclear gene expression. In many light-regulated photosynthetic genes examined, the Norflurazon-induced chloroplast photooxidation resulted in repression of gene expression even under the light (Taylor, 1989; Tonkyn *et al.*, 1992; Susek *et al.*, 1993; Bolle *et al.*, 1994). Under our growth conditions, the presence of 100 nM Norflurazon in the growth medium resulted in pale yellow cotyledons which have abnormal plastids with a disintegrated membrane system (compare Figure 9A and B) and no chlorophyll accumulation (data not shown). As shown in Figure 9C, the expression of all synthetic promoters which have

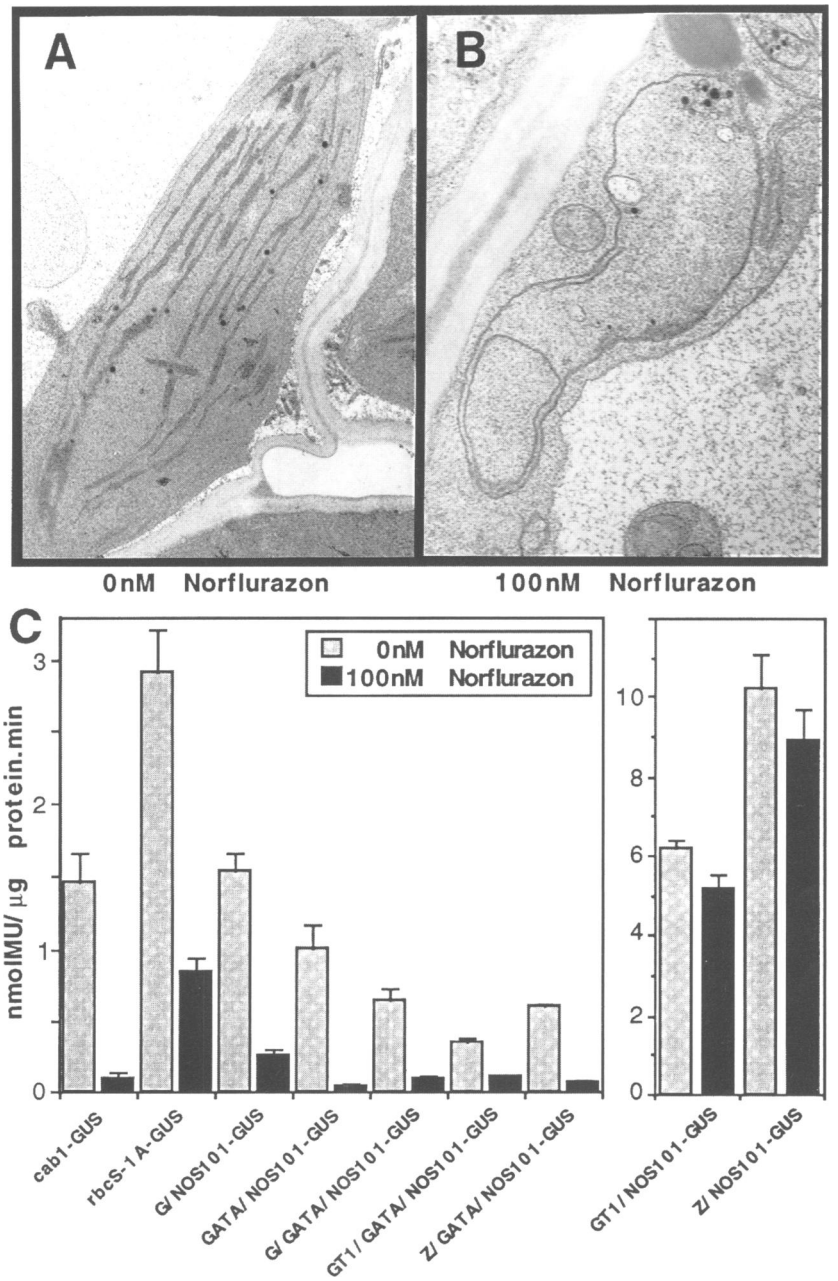

Fig. 9. The effect of Norflurazon on chloroplast development and activity of the synthetic promoters. (**A**) and (**B**) Plastid ultrastructure in cotyledons of 6-day-old light-grown seedlings in the absence (A) and presence (B) of 100 nM Norflurazon. The same magnification was used for both panels. (**C**) Effect of Norflurazon on the activity of the synthetic promoters. GUS activity of 6-day-old transgenic seedlings grown in the absence (shaded) or presence (black) of 100 nM Norflurazon under continuous white light. The results from one representative line for each synthetic promoter–*GUS* transgene and the control *cab1–GUS* and *rbcS-1A–GUS* transgenes are shown. The error bars indicate standard deviations from the means of four independent assays.

high expression in light-grown seedlings was clearly down-regulated in response to the chloroplast photooxidative damage. This response is very similar to that of the *cab1* or *rbcS-1A* promoters (Figure 9C). As anticipated, the presence of Norflurazon had a minimal effect on the two synthetic GT1–*NOS101* and Z–*NOS101* promoters which resulted in high expression in dark-grown seedlings. This result also indicates that Norflurazon treatment did not result in a general and non-specific suppression of gene expression in the light. Therefore, the five synthetic promoters which exhibit high light expression in photosynthetic cell types are also responsive to the chloroplast development state.

The synthetic light-regulated promoters have diverse expression patterns in adult plants

To reveal further the organ-specific expression patterns of the synthetic promoters, GUS staining of adult transgenic plants (~30 days old) was examined (Figure 10). With the exception of *NOS101* itself, which has no detectable activity in adult plants, all synthetic promoters result in GUS expression in green leaves but a distinct patterns in

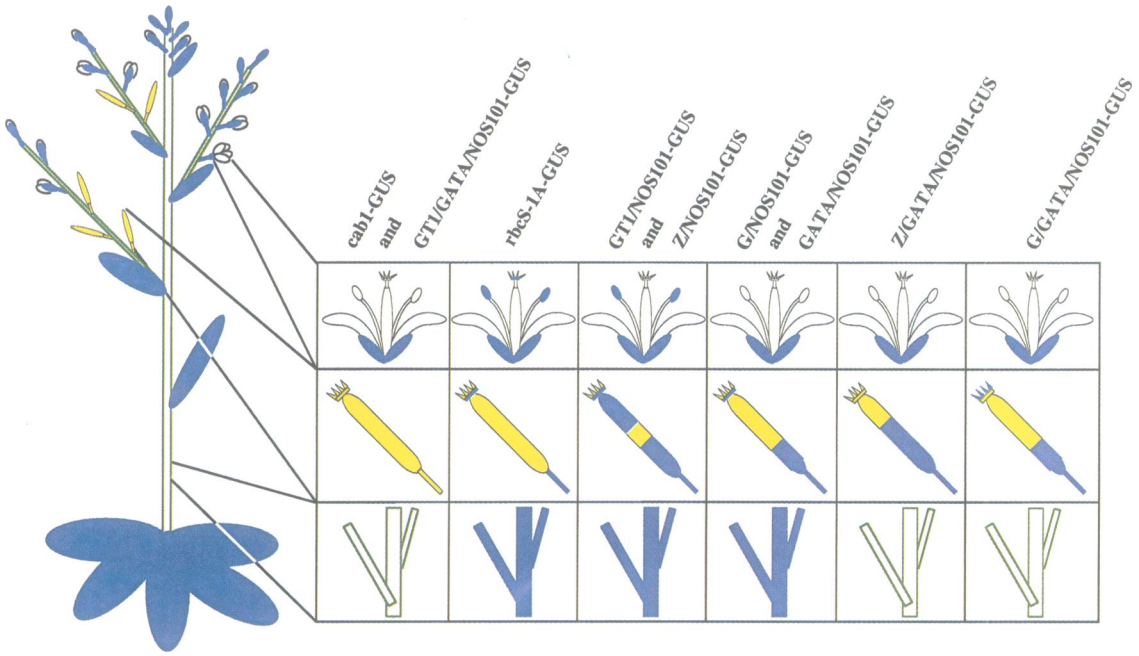

Fig. 10. Summary of expression patterns of the synthetic promoters in the adult plants. In the aerial portion of the adult plants, the GUS (blue) staining patterns of all *NOS101* series synthetic promoters and *cab1* and *rbcS-1A* control promoters are similar (left) except specific differences in the flower, fruit and stem, as indicated on the right.

flowers, siliques and mature stems. In the flower, GT1 and Z conferred to *NOS101–GUS* expression in sepals and stamens, while G, GATA and the three paired combinations conferred to *NOS101–GUS* expression only in sepals. In the siliques, GUS staining patterns are even more diverse than in the flowers (Figure 10). The individual elements conferred two distinct patterns (GT1 and Z versus G and GATA) to the *NOS101* basal promoter, while the three paired combinations all led to distinct expression patterns. In the stems, while the individual elements alone all promote GUS staining, the three paired combinations gave no detectable staining. Interestingly, *cab1* and GT1–GATA–*NOS101* promoters seemed to confer exactly the same overall staining patterns in seedlings and adult plants, while none of the synthetic promoters exactly mimic the pattern of the *rbcS-1A* promoter. These results suggested that most synthetic promoters have distinct expression patterns in both seedlings (Figure 7) and adult plants (Figure 10), and have different degrees of light responsiveness (Figures 2 and 3). The unique expression patterns conferred by different synthetic light-regulated promoters could, in part, underline the great diversity of light-regulated promoters in plants.

Discussion

Our results suggested that the pairwise interactions of multiple promoter elements, but not the individual elements alone, constitute minimal autonomous promoter determinants which dictate the light responsiveness, cell type specificity and responsiveness to chloroplast development, of a given promoter. This work represents the most comprehensive gain-of-function analysis so far of multiple promoter elements using only the single-locus stable transgenic lines in any higher eukaryotic organism. In the past, similar work in higher eukaryotes was done mostly in tissue culture cell lines for animal systems or genetically uncharacterized transgenic higher plants. In higher eukaryotes, a specific stimulus asserts its effect on gene expression through a specific and discrete regulatory DNA sequence, although that multiple such sequences may be coordinated in response to the presence of multiple stimuli at the same time (recently reviewed by Tjian and Maniatis, 1994; Hill and Treisman, 1995). Our data presented here add further to the paradigm of eukaryotic promoter activity regulation in response to development and/or extracellular stimuli and clearly suggest that multiple stimuli, both developmental and environmental, converge to unique promoter determinants comprised of pairwise combinations, but not individuals alone, of discrete DNA sequence elements.

Combinatorial LRE interactions define the minimal autonomous promoter determinants required for light responsiveness

Our data indicate that all three pairwise combinations of LREs tested can serve as minimal determinants to confer light responsiveness to the non-light-regulated basal promoters. Compared with those of the natural light-regulated promoters (*cab* and *rbcS* genes) of the photosynthetic genes from which the LREs are derived, the light responsiveness of these synthetic promoters with paired LREs meets the following three criteria. First, these synthetic promoters are responsive to the light stimulus through the phytochrome signal transduction pathway. Second, these promoters can respond to light signals even when no gross light-triggered morphogenesis has occurred. Third, the promoter activity in light-grown seedlings is significantly higher than that of the dark-grown siblings. Therefore, these promoters satisfied the strict definition of light-inducible promoters. However, none of the synthetic promoters with single LREs can meet the first two criteria.

For example, although G and GATA motifs conferred higher expression to the *NOS101–GUS* reporter in light-grown than in dark-grown seedlings, they were unable to confer measurable responsiveness to transient phytochrome activation and to light pulses which do not induce gross morphogenetic alterations. The Z and GT1 motifs alone conferred to the *NOS101–GUS* reporter lower expression in the light-grown than in dark-grown seedlings.

Regardless of whether *NOS101* or *35S90* promoters were used, the paired LRE synthetic promoters resulted in qualitatively similar expression patterns (compare Figures 3 and 4). In contrast, the expression patterns of the single LRE synthetic promoters can be qualitatively different depending on the basal promoter context (Figures 2 and 4). For example, the GATA motif gave a high expression in dark-grown seedlings when the *35S90* promoter was used instead of *NOS101*, while GT1, when fused to *35S90* instead of *NOS101*, results in high expression levels in the light-grown seedlings. The latter result confirmed the previously reported expression pattern of a similar gene construct in transgenic tobacco and it is probably the result of a combinatorial interaction of the GT1 motif and the AS-1 element within the *35S90* promoter (Lam and Chua, 1990). These results strongly indicated that, while the role of individual LREs can be quite different depending on the promoter context in which they are located, the paired LREs tested seem to behave as units, independent of the basal promoter context and different from a simple addition of the single elements alone.

Frequently, the same LRE is found in promoters with contrasting light responsiveness. As an example, the GT1 motif is found in light-inducible promoters such as *rbcS* genes (Gilmartin *et al.*, 1990; Terzaghi and Cashmore, 1995) as well as light-repressible promoters such as oat and rice phytochrome A genes (Hershey *et al.*, 1987; Kay *et al.*, 1989). Our observation that a given LRE motif, such as GT1, resulted in either light-induced or light-repressed expression patterns depending on the context of other promoter elements, provides a plausible explanation for its presence in promoters of both positively and negatively light-regulated genes. It is possible that the GT1 motif interacts with distinct promoter elements in those different promoters, which results in the opposite responses to the light stimulus. However, distinct combinatorial interactions can also potentially be achieved by distinct spacing properties of the same promoter elements involved. In these studies, we did not address the issue of spacing of the promoter elements by using multimers of the LRE elements and similar relative spacings between the LREs and basal promoter elements (Figures 1 and 4). It will be interesting to investigate the spacing requirement of the promoter elements in achieving effective combinatorial interaction.

The developmental and light signaling processes may converge to the same promoter determinants to regulate promoter activity

Most light-regulated photosynthesis-related genes are competent for responsiveness to light signals in photosynthetic cell types and are under the control of a 'plastid signal' reflecting plastid development (Fluhr *et al.*, 1986; Simpson *et al.*, 1986; Taylor, 1989; Susek *et al.*, 1993;

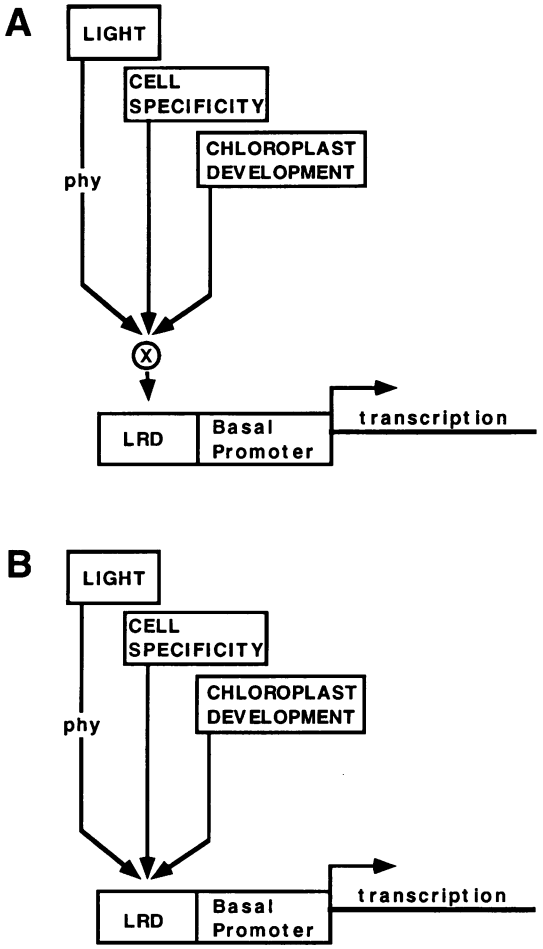

Fig. 11. A model for light and developmental control for positively light-regulated photosynthetic genes. It is hypothesized that signals representing light, cell specificity and chloroplast development state converged before (**A**) or at (**B**) the light-resposive promoter determinant(s), designated as LRD, which integrate all those signals and result in a specific expression pattern. Our results suggest that the promoter determinants are combinatorial interactions of at least two promoter motifs, rather than a single *cis*-acting element.

Bolle *et al.*, 1994). The four LREs tested were based on the consensus from two families of nuclear photosynthetic genes which encode the chlorophyll *a/b* binding proteins and the small subunit of ribulose bisphosphate carboxylase (*cab* and *rbcS*). Therefore, it is interesting to note that the three LRE pairs inserted upstream of the *NOS101* promoter not only can respond to phytochrome-mediated light signals, but also can confer correct cell specificity and responsiveness to the chloroplast developmental state in cotyledons of light-grown seedlings. Therefore, these combinatorial LRE elements, or the *trans*-factors interacting with them, may be regarded as signal integration points in the network mediating both light and developmental control of gene expression. As illustrated in Figure 11, multiple signals, such as light and endogenous developmental signals reflecting cell specificity and chloroplast development, converge either to a common factor before reaching the promoter (Model A) or directly (Model B) onto the minimal autonomous light-responsive promoter determinants (LRDs). Our data suggested that the minimal autonomous LRDs can be distinct pairwise combinations of LREs. It should be pointed out that since all three LRE

pairs analyzed contain the GATA motif, we cannot rule out the possibility that the GATA motif may play a dominant role in those combinatorial interactions. However, it is evident that light responsiveness can be conferred by promoter determinants without a GATA motif, since there are light-regulated promoters lacking the GATA motif (Terzaghi and Cashmore, 1995). Further, the synthetic GT1–*35S90* promoter does not have a typical GATA motif, but a combinatorial interaction of GT1 and AS-1 nevertheless conferred light-inducible expression to a reporter gene (Lam and Chua, 1990; also Figure 4). Further, the fact that G/GATA–*NOS101* and Z/GATA–*NOS101* exhibited significant activity in the roots and the lower part of the hypocotyl in dark-grown seedlings but this expression was suppressed in the light may suggest that correct organ-specific expression requires the presence of both light and developmental signals.

It is worth mentioning that both G–*NOS101* and GATA–*NOS101* synthetic promoters also have high expression in chloroplast-containing cells (mesophyll and guard cells) and respond to the chloroplast developmental state, similarly to the *cab1* and *rbcS-1A* promoters, while at the same time they do not respond detectably to light pulses which do not trigger gross morphological changes. At least two possible explanations could be hypothesized. First, the ability to respond to development signals is a built-in component of LRDs, but a promoter can be constructed to respond to the same developmental cues without a light-responsiveness component. Alternatively, since full activation of light-regulated promoters involves two components, one being independent of light-triggered morphogenesis and one being dependent on morphogeneic changes (Figure 5 and Results), the ability to respond to cell specificity and chloroplast development may be only associated with the morphogenesis-dependent component. In this case, both G–*NOS101* and GATA–*NOS101* promoters would be considered only capable of performing one component (morphogenesis-dependent) of the light response, while the LRE pairs are capable of both. Further study will be needed to test these models.

Materials and methods

Plant materials and growth conditions

All *Arabidopsis* lines used in this study were in No-O ecotype, except the *rbcS-1A–GUS* transgenic line (Wei and Deng, 1992) which was in Bencheim ecotype. Plant growth conditions were as described (Wei and Deng, 1992; McNellis *et al.*, 1994). For both GUS assays and mRNA analysis, *Arabidopsis* seedlings were grown in continuous white light (100 mmol/m^2/s) or continuous darkness for 6 days. For dark adaptation experiments, seedlings were grown in continuous white light for 6 days and then transferred to the dark for different time periods as indicated. The light sources for specific spectral light treatments were described previously (McNellis *et al.*, 1994).

Construction of synthetic promoters and stable transformation

All synthetic promoter–*GUS* fusion constructs were in pCIT20 (Yanofsky *et al.*, 1990) binary transformation vector. For the *NOS101* series, the basal *NOS101* promoter fragment (–101 to +4) from the nopaline synthase promoter (Mitra and An, 1989) was first synthesized by PCR with a 5′ primer containing a *Cla*I–*Xho*I linker and a 3′ primer containing a *Sal*I linker. The PCR product was digested with *Cla*I and *Sal*I and used to replace the full-length *cab1* promoter of a *cab1–GUS* fusion in the pCIT20 vector (Deng *et al.*, 1991), which resulted in the *NOS101–GUS* fusion contruct. Double-stranded DNA oligomers corresponding to dimers (Z motif) or tetramers (GATA, G and GT1 motifs) of LRE sequences (Figure 1A) were synthesized with 5′ and 3′ ends compatible with *Cla*I and *Xho*I cloning sites without restoring the *Xho*I in the resulting clones. The oligomers were ligated to the *Cla*I- and *Xho*I-digested *NOS101–GUS* fusion construct. For the GATA motif, the synthesized DNA oligomer carried a 5′ linker with *Cla*I–*Xho*I restriction sites for adding the oligomers corresponding to the Z, G and GT1 motifs mentioned above to generate three paired combinations of synthetic promoters. For the *35S90* promoter series (Figure 4), exactly the same strategy was used except that the *NOS101* sequence was replaced with the CaMV 35S promoter sequences (–90 to +8, Lam and Chua, 1990). The *cab1–GUS* fusion used as a control throughout this work contains a deletion version of the *cab1* promoter (–250 to +67, Deng *et al.*, 1991).

All chimeric promoters were confirmed by sequence analysis after construction, and were transformed subsequentely into *Arabidopsis* (No-O ecotype) through the *Agrobacterium*-mediated root transformation procedure (Valvekens *et al.*, 1988). Single-locus transgenic lines were selected by the 3:1 segregation of the T-DNA in the progeny of the primary transgenic plants and the homozygous lines were established and used for the gene expression analysis.

GUS assay

GUS enzyme activity in transgenic *Arabidopsis* seedlings was determined according to the method of Jefferson (1987). The protein concentration was determined by a Lowry assay kit according to the manufacturer's suggestion (Sigma). For histochemical analysis, the tissue was fixed in 2% paraformaldehyde in sodium phosphate buffer pH 7.0 for 10 min and then vacuum filtered for 10 min. The sample was washed in buffer twice and the staining solution (Jefferson, 1987) was added and vacuum filtered for 2 min. All procedures were performed at 4°C, except the staining reaction which was incubated overnight at 37°C. Fixation, embedding and sectioning were performed according to a published procedure (Gallagher, 1992) and the sections were examined under the light microscope.

Norflurazon treatment

The concentration of the herbicide Norflurazon (Sandoz) in the growth medium was 100 nM, which was determined empirically according to two criteria. First, under continous white light (100 mmol/m^2/s) growth conditions, this concentration of Norflurazon abolished all chlorophyll accumulation. Second, this concentration of Norflurazon was sufficient to cause maximal GUS activity reduction in the transgenic *cab1–GUS* line but at the same time had a minimal effect on the expression of a long CaMV promoter–*GUS* fusion (*35S346–GUS*, Deng *et al.*, 1991) transgenic line. The 6-day-old light-grown seedlings in the presence or absence of Norflurazon were analyzed for both GUS activity and chloroplast ultrastructure by transmission electron microscopy (Wei *et al.*, 1994).

RNA analysis

Except for the GT1–*NOS101–GUS* and Z–*NOS101–GUS* transgenic lines where the whole seedlings were used, total RNA was isolated from upper parts (hypocotyl and cotyledon) of transgenic seedlings with Trizol reagent (GIBCO BRL) following the manufacturer's instructions. The RNase protection assay was according to a standard procedure (Zinn *et al.*, 1983). The same amount of total RNA was used for the same transgenic line under different treatments, while the amount of RNA used for different transgenic lines ranged from 10 to 60 μg depending on the *GUS* mRNA levels. To generate a suitable probe, a 231 bp *Eco*RV fragment (position 855–1086 of the GUS protein coding region) from the pRTL2-GUS plasmid (Restrepo *et al.*, 1990) was subcloned into pBluescriptSK(+). The labeled RNA probe was synthesized by *in vitro* transcription with T3 RNA polymerase with radiolabeled [α-^{32}P]GTP and [α-^{32}P]CTP (Amersham, 800 Ci/mmol) and gel purified. The specific activity of the probes was 10^9 c.p.m./μg. Usually, $1–2\times10^6$ c.p.m. were used per RNase protection reaction. Protected fragments were electrophoresed on 6% polyacrylamide gels and visualized by autoradiography.

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