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Localization of a Phytochrome-Responsive Element within the Upstream Region of Pea *rbcS-3A*

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The pea *rbcS-3A* promoter with a 5′ deletion to −166 (−166 *rbcS-3A*) contains two GT-1-binding sites. Mutational analyses demonstrated that a decrease in affinity for GT-1 correlates with reduced promoter activity. Transcription of −166 *rbcS-3A* in transgenic etiolated seedlings is induced by red light and suppressed by far-red light, indicating that it contains a phytochrome-responsive element.

Pea *rbcS-3A* (5) has been the focus of our studies to identify *cis* elements and *trans*-acting factors for light-responsive transcription (18–20, 22). Several *trans*-acting factors that bind conserved sequence motifs present within the upstream region of *rbcS-3A* have been identified (2, 12–14). The *rbcS-3A* promoter with a 5′ deletion to −166 (−166 *rbcS-3A*) contains two discrete sequence elements, boxes II and III (20). Both elements are essential for transcriptional activity (18) and are bound in vitro by the same nuclear factor, GT-1 (14, 15). This is reminiscent of observations of HAP1 in yeast cells (27), EBP20 (16), and OBP100 (1) in animals in which degenerate elements can act as specific binding sites for the same factor.

We investigated the significance of sequence differences between boxes II and III by comparing their abilities to activate transcription in vivo and to bind GT-1 in vitro. Box replacements (10) were introduced into −166 *rbcS-3A* (Fig. 1A) to substitute the wild-type box II-III configuration (Fig. 1B) with either a box II-II (Fig. 1C) or a box III-III configuration (Fig. 1D). Constructs were verified by sequence analysis and subcloned into the test gene site of pMON200, placing −166 *rbcS-3A* adjacent to the nopaline synthase (*nos*) promoter at −150 (19). Constructs were transferred into *Agrobacterium tumefaciens* and *Nicotiana tabacum* (var. Xanthi), as described elsewhere (7).

Transgene expression levels were assayed by S1 nuclease analyses (6) of total RNA isolated from mature leaves of light-grown plants and also from the same plants following 3 days of dark adaptation (18). RNA samples from 7 to 10 primary transformants expressing *rbcS-3A* were pooled for further analysis to minimize variation due to position effects (3, 17, 26). The −166 II-II *rbcS-3A* promoter conferred wild-type levels of activity (Fig. 2A). In contrast, transcript levels from the −166 III-III *rbcS-3A* promoter were lower than those from the wild type. RNA titration analyses showed a 20-fold reduction in promoter activity (data not shown). Both mutant promoters remained light responsive, suggesting that boxes II and III may play similar roles in regulation yet differ in their quantitative ability to do so.

To correlate in vivo activity with in vitro affinity for GT-1, gel shift competition studies were performed by using pea nuclear extracts (14) with the wild-type II-III promoter and the corresponding fragments from the II-III, II-II, and III-III promoter elements (Fig. 1) as competitor DNA. The affinity of the −166 II-II promoter for GT-1 is indistinguishable at this level of resolution from that of the wild-type −166 II-III promoter. In contrast, the affinity of the −166 III-III promoter for GT-1 is severely reduced.

The −166 *rbcS-3A* promoter (Fig. 3A, construct 1) can confer light responsiveness on the −150 *nos* promoter (11). As observed for *rbcS-3A*, the II-II element (Fig. 1C) confers wild-type levels of expression while the III-III element (Fig. 1D) confers lower levels of expression on −150 *nos* (Fig. 3B). Therefore, this activity is dependent on the GT-1-binding site composition of the −166 *rbcS-3A* promoter. These data parallel observations of *rbcS-3A* expression and demonstrate a correlation between reduced affinity for GT-1 in vitro (Fig. 2B) and a decrease in transcriptional activity in vivo (Fig. 2A). Previous studies demonstrated that loss of GT-1 binding abolished transcriptional activity (18, 23). Therefore, differences in the contribution of boxes II and III to the activity of the light-responsive element are likely due to their disparate abilities to interact with GT-1.

Two independent light-responsive elements are located downstream of −166 (22). The −166 to −50 region can confer light responsiveness on the light-insensitive cauliflower mosaic virus 35S TATA region. Furthermore, the *rbcS-3A* TATA region is independently light responsive. We have assayed the ability of the −166 *rbcS-3A* promoter, following replacement of the TATA region with that of the cauliflower mosaic virus 35S TATA box (Fig. 3A, construct 2) (22), to confer light responsiveness on −150 *nos*. Analysis of *nos* transcripts present in pooled RNA samples from mature leaves of plants expressing either construct 1 or construct 2 (Fig. 3A) demonstrates that the observed *nos* activity is independent of the light-responsive *rbcS-3A* TATA element and is due to the −166 to −50 region of *rbcS-3A*. Therefore, the GT-1-binding sites are critical for the light-responsive expression of both *rbcS-3A* and *nos*.

Expression studies of *rbcS-3A* deletion and chimeric constructs have primarily used white light as the inductive cue (5, 18, 22, 23). However, expression mediated by the −330 to −50 region of *rbcS-3A* is in response to a combination of both phytochrome and a blue light photoreceptor (4). The *cis* regulatory elements that elicit this response were not further delineated. Definition of the *cis*-acting elements through which the transcriptional phytochrome response (8, 29) is mediated is an important step towards identifying interme-

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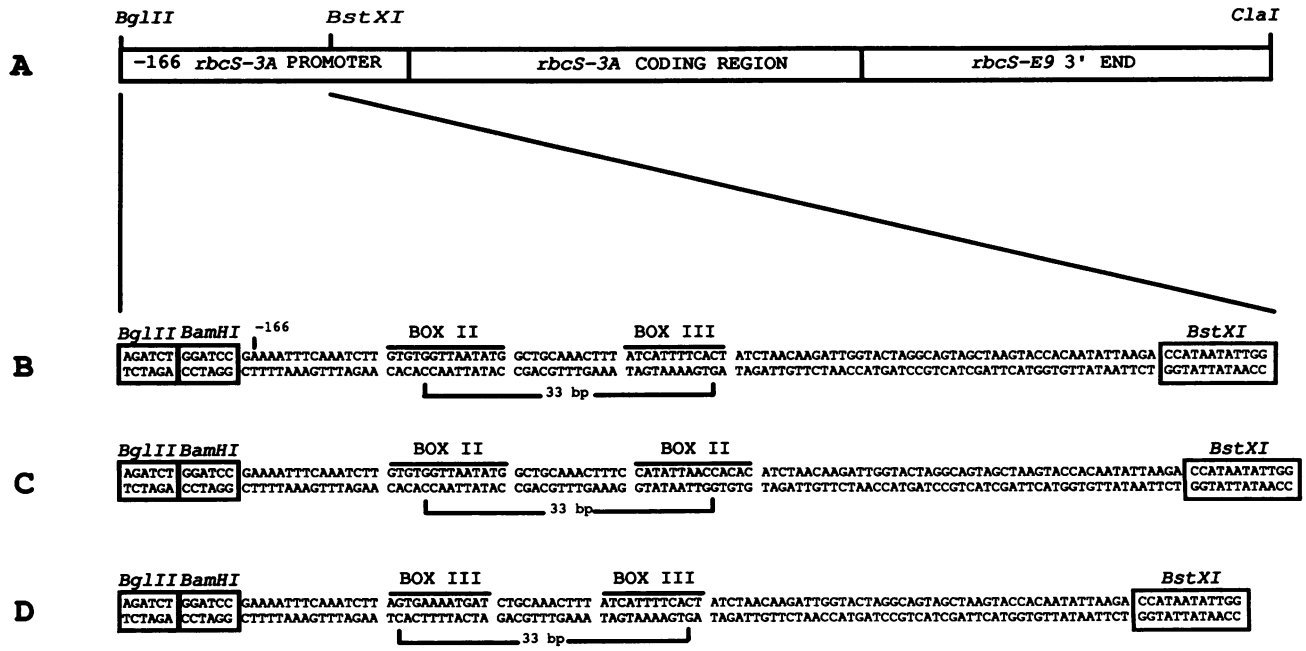

FIG. 1. Organization of GT-1-binding sites within the *rbcS-3A* promoter. (A) Schematic representation of −166 *rbcS-3A*. The *Bgl*II and *Cla*I restriction sites are located at the 5′ and 3′ ends of the gene, respectively. The *Bst*XI site within the promoter is at −55. The 3′ polyadenylation sequence is derived from *rbcS-E9* (19). (B) Nucleotide sequence of the *rbcS-3A* promoter between the *Bgl*II and *Bst*XI sites. Boxes II ($^{-151}$GTGTGGTTAATATG^{-138}) and III ($^{-125}$ATCATTTTCACT^{-114}) are overlined. The distance between G^{-147} (box II) and G^{-113} (box III) (14) is indicated at 33 base pairs (bp) (11). (C) Nucleotide sequence of the *rbcS-3A* promoter following replacement of box III with box II. (D) Nucleotide sequence of the *rbcS-3A* promoter following replacement of box II with box III. The distance between the critical G residues (14) is maintained at 33 base pairs (11) in the box replacement promoters.

diary components of the signal transduction pathway linking photoperception to a transcriptional response. Therefore, we wanted to define the wavelength component of white light which mediates −166 *rbcS-3A* and −150 *nos* transcription through boxes II and III.

Seeds from representative transgenic plants expressing −166 *rbcS-3A* (Fig. 1B) and −166 II-II *rbcS-3A* (Fig. 1C) were germinated in complete darkness for 10 days, and the etiolated seedlings were treated with various light regimes (Fig. 4). Following 16 h of constant white light, −166 *rbcS-3A* transcript levels significantly increased over transcript levels in the dark (Fig. 4A). A similar induction is elicited following 3 min of red light and is partly reversible by 10 min of far-red light. This red light induction and subsequent reversal by far-red light are characteristic of a phytochrome-mediated response (21, 25, 30). The −166 II-II *rbcS-3A* promoter also mediates the same photoresponse (Fig. 4B). The −166 III-III *rbcS-3A* promoter is not sufficiently active to assay in seedlings. The light-responsive expression of *nos* conferred by the II-III (Fig. 4C) and II-II *rbcS-3A* promoters (Fig. 4D) is also mediated via phytochrome. The induction of *nos* in response to both white and red light appears to be lower than that for *rbcS-3A* because of a higher background of *nos* transcript levels in etiolated seedlings. This most likely reflects the presence of additional regulatory elements within the −150 *nos* promoter absent from the −166 *rbcS-3A* promoter, for example *nos-1* (24). These observations localized a phytochrome response element within the −166 *rbcS-3A* promoter.

Our studies demonstrate a functional significance for sequence differences between boxes II and III. In addition, they define a role for the red light component of white light in the expression mediated by the −166 *rbcS-3A* promoter

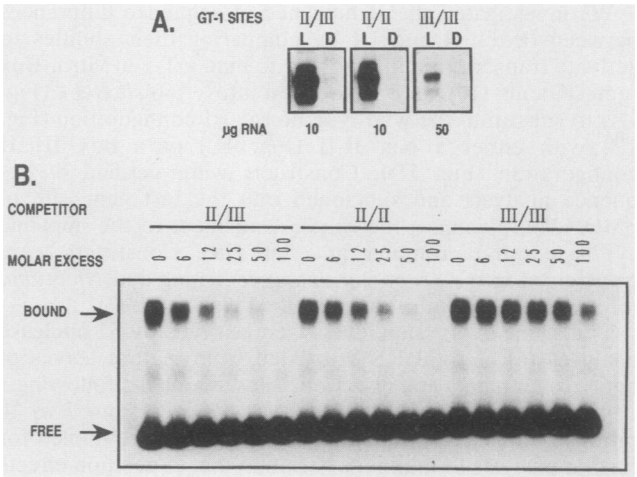

FIG. 2. Effects of GT-1-binding site substitutions on *rbcS-3A* promoter activity. (A) S1 nuclease analysis of pooled RNA samples from transgenic plants expressing *rbcS-3A* from the −166 promoter and the mutant derivatives. The following combinations of GT-1-binding sites within the promoter are indicated: the wild-type box II plus box III promoter, II-III (Fig. 1B); paired box II promoter, II-II (Fig. 1C); and paired box III promoter, III-III (Fig. 1D). RNA samples derived from light-grown (L) and dark-adapted (D) plants were pooled from 18 II-III, 7 II-II, and 7 III-III independent primary transformants. The amount of RNA used is indicated. (B) The *Bgl*II (−166)-to-*Bst*XI (−55) restriction fragment of *rbcS-3A* (Fig. 1B) was used as a gel shift probe with pea nuclear extract. Specific competitor fragments were derived from either wild-type (II-III [Fig. 1B]), paired box II (II-II [Fig. 1C]), or paired box III (III-III [Fig. 1D]) promoter elements digested with *Bgl*II and *Bst*XI and were added in the indicated molar excesses.

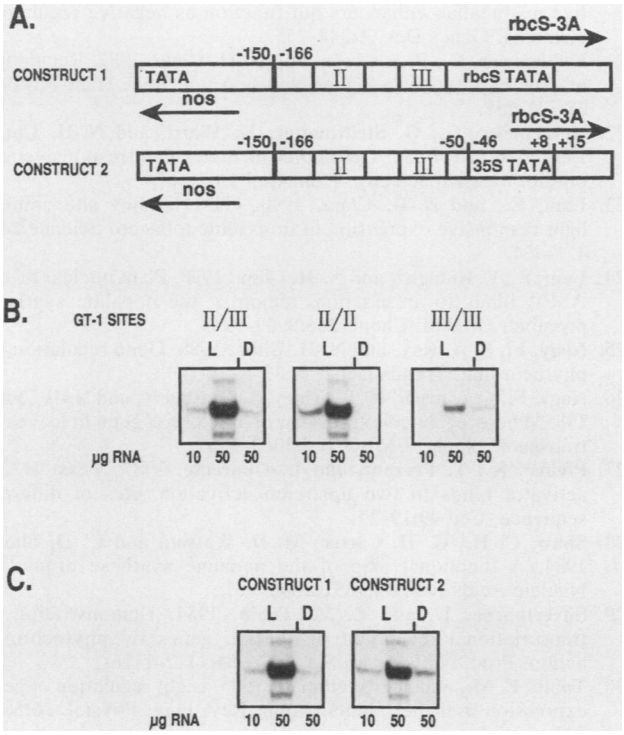

FIG. 3. Effects of *rbcS-3A* promoter substitutions on *nos* promoter activity. (A) Construct 1. Schematic representation of −166 *rbcS-3A* adjacent to −150 *nos* within pMON200. The direction of transcription of each gene and the 5′ endpoints are indicated. Construct 2. Like construct 1, except the light-responsive *rbcS* TATA element (−50 to +15) was replaced by the light-insensitive cauliflower mosaic virus 35S TATA element (−46 to +8). (B) 5′ RNAse protection analysis (9) of *nos* transcripts within pooled RNA samples from light-grown (L) and dark-adapted (D) plants. The *Eco*RI-*Cla*I fragment of the *nos* gene (28) deleted to −131 was used as a probe. The configuration of GT-1-binding sites within the −166 *rbcS-3A* promoter (construct 1) and the number of plants assayed are as described in the legend to Fig. 2. (C) 5′ RNAse protection analysis of *nos* transcripts in pooled RNA samples from plants carrying either construct 1 or construct 2. RNA samples were pooled from 18 and 7 independent light-grown (L) or dark-adapted (D) transgenic plants for constructs 1 and 2, respectively. The amounts of pooled RNA in panels B and C are as indicated.

and therefore link a specific photoreceptor (phytochrome) to a transcriptional response through a characterized *trans*-acting factor (GT-1) and its cognate *cis*-acting elements (boxes II and III). Furthermore, they demonstrate that additional *cis*-acting elements present within −330 to −50 of *rbcS-3A* (GBF [13], GAF-1 [12], and AT-1 [2]) are not essential for this phytochrome-mediated response.

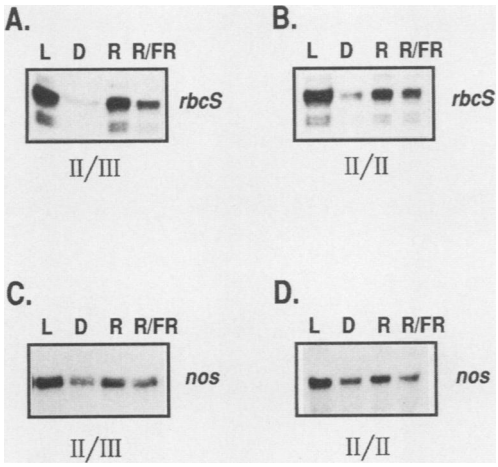

FIG. 4. Paired GT-1-binding sites constitute part of a phytochrome-responsive element. RNA (20 µg) was isolated from 10-day-old etiolated seedlings from representative plants carrying either the wild-type −166 *rbcS-3A* (II-III [Fig. 1B]) promoter or the paired box II promoter (II-II [Fig. 1C]). Light treatments were as follows: 16 h of white light illumination (L), 16 h of darkness (D), 3 min of red light followed by 16 h of darkness (R), or 3 min of red light followed by 10 min of far-red light followed by 16 h of darkness (R/FR). RNA samples were analyzed for *rbcS-3A* transcripts by 3′ S1 nuclease protection analysis with a *rbcS-E9* probe (20) (panels A and B) or by 5′ RNAse protection for *nos* transcripts (panels C and D).

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