Phytochrome Control of Specific mRNA Levels in Developing Pea Buds

THE PRESENCE OF BOTH VERY LOW FLUENCE AND LOW FLUENCE RESPONSES

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ABSTRACT

We have examined phytochrome regulated changes in transcript abundance for 11 different light regulated mRNAs in developing pea buds. Fluence-response curves were measured for changes in transcript abundance in response to red light pulses in both the low and very low fluence ranges. Most transcripts show only low fluence responses, with a threshold of approximately 10 micromoles per square meter. All of the low fluence responses are reversible by far red light. One transcript shows a very low fluence response, with a threshold of approximately 10^-4 micromoles per square meter. As expected, the very low fluence response is not far red reversible and in fact can be induced by far red light.

Various fluences of red light were also used as pretreatments before transferring seedlings to continuous white light. One transcript responds to pretreatments in the very low fluence range, several respond to pretreatments in the low fluence range (including chlorophyll a/b binding protein RNA and ribulose-1,5-bisphosphate carboxylase RNA), and several show no response to the red light under these conditions. The threshold of these low fluence responses is approximately 10^2 micromoles per square meter, one order of magnitude greater than the threshold of the low fluence responses to red light alone.

The transcripts may also be grouped by their responses to white light treatment alone. Three of the clones correspond to transcripts whose abundance decreases after a 24 hour white light treatment. The remainder of the mRNAs increase between 2- and 10-fold in response to the 24 hour white light.

Excitation of phytochrome is responsible, in part, for the white light-induced changes observed in the steady state levels of several specific nuclear encoded mRNAs. This phenomenon has been reported for transcripts of the genes encoding the cab2 in barley (1), Lemma (26), pea (16, 17, 25), and mung bean (25); rbcS in Lemma (26), pea (16, 17, 25), and mung bean (25); phytochrome in oat (9); NADPH:plastide oxireductase in barley (2); and a 17 kD chloroplast polypeptide in pea (21). Recently our laboratory has demonstrated phytochrome control of steady state levels for 10 other transcripts in pea and four other transcripts in mung bean (25). In Lemma it has been shown that phytochrome affects transcription of the cab and rbcS genes in isolated nuclei (23).

Phytochrome regulation occurs over two fluence ranges of R (5, 7, 20). The LF response has a threshold of approximately 10^4 μmol m^-2 and is fully reversible by FR. This is the common phytochrome response observed in most plants. The VLF response has a threshold of approximately 10^-3 μmol m^-2. It is not reversible by FR; indeed it is induced by most FR sources (5, 7, 20).

To date, the VLF response has only been observed in a limited number of systems. These include growth rates of etiolated Avena coleoptiles and mesocotyls (3, 20), anthocyanin synthesis in mustard seedlings (4), and germination of dormant lettuce seeds (6, 7, 24). A VLF response for Chl accumulation in peas is apparent if R of varying fluence is used as a pretreatment followed by a dark period and a subsequent white light treatment (18, 22). Recently, we have also demonstrated, using etiolated pea buds, that cab transcripts have both a VLF and a LF response, whereas rbcS transcripts exhibit only a LF response (17).

The mechanism whereby phytochrome regulates transcription and/or transcript abundance is unknown. In this paper, we describe the basic characteristics of phytochrome control for 11 unidentified phytochrome regulated messages and further characterize the phytochrome responses of cab and rbcS transcripts. We have measured the fluence-dependent accumulation of these transcripts in response to single pulses of R, the ability of FR to reverse these R effects, and the ability of FR to induce transcript accumulation in the absence of prior R treatments.

We have also used a pretreatment protocol similar to that described above which demonstrates an effect of VLF R for Chl accumulation in a subsequent white light period (18, 22). For several transcripts, significant differences are apparent between responses seen in these potentiation-type experiments and responses to the R pulse alone.

MATERIALS AND METHODS

Plant Material and Light Treatments. Seeds of Pisum sativum L. cv Alaska (W. Atlee Burpee Co., Warminster, PA) were imibed for 5 h and grown on two layers of water-soaked Kimpak (Kimberly-Clarke, Roswell, GA) at 27°C, 85% RH, in absolute darkness. Plants were grown in the above conditions for 6 d, given the various light treatments, and returned to the dark for an additional 24 h after which they were either harvested or given a 24-h white light treatment. Protocols used for the various light treatments are illustrated in Figure 1. For studies of R and FR fluence dependence, groups of approximately 100 6-d-old seedlings were irradiated with single pulses of varying fluence of either R or FR and then returned to darkness for 24 h before
LOW AND VERY LOW FLUENCE RESPONSES FOR SPECIFIC mRNAs IN PEA

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Transmittance/Reflectance Scanning
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30 of total cellular RNA.

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3.

amount m–2, mol m–2, on studies elsewhere quantitated
fluorescence response
conditions and the

Densitometer. It is
addition to

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hybridized within 20%
hybridized in the

seedlings with white light (Fig. 5).

FR have been

In addition, the transcrip- tion factor is not a product of the cab gene

The irradiation protocols for the various experimental procedures are shown diagrammatically, specific details are given in the text. (A) R and FR fluence response experiments (Figs. 2 and 3) and FR reversal experiments (Fig. 4). (B) R fluence response experiment with supplemental white light (Fig. 5).

harvesting. The R irradiations were as follows: 10–7 to 10–6 μmol m–2, 0.1 s; 10–5 μmol m–2, 1 s; 10–4 μmol m–2, 10 s; 10–3 μmol m–2, 100 s; 10–2 μmol m–2, 1000 s. The FR source had an intensity of 1.25 × 107 J cm–2 s–1. FR was administered for 0, 5, 15, and 30 min. For FR reversal studies, seedlings were irradiated with 10–5 μmol m–2 of R and immediately irradiated with FR for 0, 5, 10, or 30 min. For studies on the effects of R as a pretreatment, seedlings were irradiated as for R fluence studies, returned to the dark for 24 h, and irradiated for an additional 24 h with white light (10–5 μmol m–2 s–1, cool white fluorescent) prior to harvesting. All tissue was harvested in a 4°C cold room under dim green light. Tissue was immediately frozen in liquid N2 and stored at −70°C until used for RNA extraction. The R (14), FR (15), and green safelight (14) sources have been described previously. Data points represent the average of at least two independent experiments. Each replicate experiment represents approximately 100 pea buds; these are generally within 20% (17).

Preparation and Hybridization of RNA. RNA was prepared and fixed to nitrocellulose filters (BA-85; Schleicher and Schuell, Keene NH) as previously described (25) except that a Schleicher and Schuell Mini Fold II slot blot apparatus (Schleicher and Schuell) was used. All experimental slots contained 5 μg of total cellular RNA. Hybridization conditions and the preparation and properties of the cDNA clones used as probes have been described (25). Approximately 100 ng of the cDNA clone was nick-translated and used as probe for 24 experimental slots in 10 ml of hybridization buffer. The lengths of the various cDNA clones are reported elsewhere (25).

Hybridization was quantitated by autoradiography and densitometry as described (17, 25). In addition to the RNA extracted from experimental plants, a dilution series of plasmid pBR322 DNA was present on each slot blot. Since all of the cDNA clones used are carried in a pBR322 vector, and pBR322 sequences are labeled concurrently with insert sequences during nick translation, an autoradiographic image of the pBR322 concentration series is obtained on each filter. It is possible, therefore, to construct a standard curve relating the intensity of each image with the amount of pBR322 responsible for that image. The intensity of the autoradiographic images arising from hybridization with the experimental RNA can then be quantitated relative to the pBR322 standard curve. Density of the autoradiographic images of the slots was determined using a Hoeffer GS 300 Transmittance/Reflectance Scanning Densitometer. It should be emphasized that ‘pBR322 units’ are relative units, used only to normalize slot blots hybridized on different occasions with the same probe. It should not be assumed that the numbers obtained in pBR322 units are absolute numbers.

RESULTS

Fluence Response Curves for Single Red Light Pulses. Figure 2B shows those clones corresponding to transcripts whose steady state level increases in response to R in the LF range only. They are pEA25, pEA46, pEA214, pEA215, pEA238, pEA277, pEA303, and pEA315. The LF response exhibited by those transcripts has a threshold of approximately 104 μmol m–2·R. The dark levels represent between 40 and 60% of the level achieved in response to a single R pulse with a fluence of 106 μmol m–2·R (Table I). Three of the clones, pEA25, pEA214, and pEA315 appear to reach saturation at approximately 104 μmol m–2·R. (The remainder do not reach saturation in the fluence range tested.) Saturation for the LF response would, therefore, seem to require at least two orders of magnitude more R than the threshold value. The LF response of these clones is nearly linear in the range between 104 μmol m–2 (threshold) and 105 μmol m–2, the highest fluence assayed.

Figure 2A shows the R fluence response curve for the steady state levels of the transcript corresponding to pEA170. The curve indicates a VLF response only, with a threshold at approximately 104 μmol m–2·R. Dark levels represent approximately 30% of the level resulting from a R pulse of 106 μmol m–2·R (Table I). The VLF response shown by pEA170 becomes saturated at fluences one order of magnitude above the threshold, reaching a plateau by 105 μmol m–2. Two of the cDNA clones investigated, pEA13 and pEA207, exhibit no response to single pulses of R in the fluence range examined in these experiments (Fig. 2C).

Far Red Light Reversal of the LF Responses. The ability of FR to reverse the low fluence responses exhibited by several of the transcripts is shown in Figure 3. Nearly complete reversal is obtained for all transcripts after 10 min (7.5 × 108 J cm–2) of FR.

Far Red Light Fluence Response Curves. Far red light alone is capable of inducing a VLF response, but not a LF response. Several hypotheses for this action of FR have been proposed (5, 20). Regardless of the reason, the ability of FR to induce a VLF response can be used to confirm the presence of such a response. pEA170 RNA shows a VLF response to R (see Fig. 2A). Figure 4A shows a strong FR effect for pEA170 RNA, confirming the presence of a VLF response to R. This behavior is similar to that previously observed for the VLF component of the cab RNA response (17).

Figure 4B shows fluence response curves to FR for the rest of the cDNA clones examined. These clones recognize RNAs which either show no response to R (pEA13 and pEA207) or which exhibit only a LF response (pEA25, pEA46, pEA214, pEA215, pEA238, pEA277, pEA303, and pEA315) (Fig. 2, B and C). The response curves to FR indicate no significant increase in transcript abundance in response to increasing fluence of FR. The lack of any response by these transcripts confirms the absence of any major VLF responses.

The transcript represented by pEA238 does show a small increase in response to the FR alone. In addition, it also seems to require more FR to reverse the response to low fluence R (Fig. 3) than do the other transcripts. It is possible that the transcript represented by pEA238 does have a small VLF response, although neither the red fluence response curve (Fig. 2B) nor the ability of the FR to fully reverse the R response (Fig. 3) indicate any VLF FR activity.

Red Light Potentiation Effects. It is possible that steady state transcript levels might respond differently to excitation of phytochrome (by either VLF or LF red light) under conditions other than those tested. For example, it is reasonable to suppose that stabilization and accumulation of specific transcripts might be
Fig. 2. Red light fluence response curves without supplemental white light treatments. Groups of 6-d-old, dark grown, pea seedlings were irradiated with single pulses of R at the indicated fluences. The seedlings were returned to darkness for 24 h before the buds were harvested (Fig. 1). RNA was isolated and fixed to nitrocellulose filters. Separate filters were probed by hybridization with 32P-labeled DNA from each of the cDNA clones indicated. The relative abundance of the RNA corresponding to each cDNA was determined by comparing the density of the autoradiographic images of the slots with a standard curve constructed from pBR322 DNA. Abundances have been normalized to the values obtained with 10^6 μmol m^-2. (A), cDNA clones corresponding to transcripts showing LF responses only; (B), cDNA clones corresponding to transcripts showing LF responses only; (C), cDNA clones corresponding to transcripts showing no response to R in the range of fluences tested.

Table 1. Transcript Levels under Various Light Treatments

<table>
<thead>
<tr>
<th>Clone</th>
<th>Dark</th>
<th>Red</th>
<th>White</th>
<th>R/D</th>
<th>W/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAB96</td>
<td>0.14</td>
<td>0.70</td>
<td>0.78</td>
<td>5.0</td>
<td>5.6</td>
</tr>
<tr>
<td>pSS15</td>
<td>0.06</td>
<td>0.31</td>
<td>0.58</td>
<td>5.2</td>
<td>9.7</td>
</tr>
<tr>
<td>pEA13</td>
<td>0.08</td>
<td>0.08</td>
<td>0.15</td>
<td>1.0</td>
<td>1.9</td>
</tr>
<tr>
<td>pEA25</td>
<td>0.03</td>
<td>0.08</td>
<td>0.16</td>
<td>2.6</td>
<td>5.3</td>
</tr>
<tr>
<td>pEA46</td>
<td>0.10</td>
<td>0.18</td>
<td>1.00</td>
<td>1.8</td>
<td>10.0</td>
</tr>
<tr>
<td>pEA170</td>
<td>0.13</td>
<td>0.42</td>
<td>0.06</td>
<td>3.2</td>
<td>0.5</td>
</tr>
<tr>
<td>pEA207</td>
<td>1.20</td>
<td>1.30</td>
<td>0.60</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>pEA214</td>
<td>0.14</td>
<td>0.24</td>
<td>0.52</td>
<td>1.7</td>
<td>3.6</td>
</tr>
<tr>
<td>pEA215</td>
<td>0.08</td>
<td>0.13</td>
<td>0.02</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>pEA238</td>
<td>0.17</td>
<td>0.25</td>
<td>1.46</td>
<td>1.5</td>
<td>8.6</td>
</tr>
<tr>
<td>pEA277</td>
<td>0.03</td>
<td>0.07</td>
<td>0.16</td>
<td>2.2</td>
<td>5.3</td>
</tr>
<tr>
<td>pEA303</td>
<td>0.19</td>
<td>0.32</td>
<td>0.53</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td>pEA315</td>
<td>0.07</td>
<td>0.12</td>
<td>0.26</td>
<td>1.7</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Fig. 3. Far red light reversal of the LF response to red light. Seedlings, grown as for Fig. 2, were irradiated with a single pulse of R with a fluence of 10^6 μmol m^-2. The seedlings were then irradiated with FR (1.25 × 10^-4 J cm^-2 s^-1) for the amount of time indicated on the figure, returned to the dark for 24 h, and harvested (Fig. 1). Hybridization and quantitation were as for Fig. 2.
shows no change (Fig. 2). The remainder of the transcripts increase their steady state levels between 2- and 10-fold in response to the 24 h white light treatment. This is in contrast to the 1.5- to 5-fold in response to the single pulses of R (Table I).

When R pulses of varying fluence are used as a pretreatment, followed by a 24-h dark period and a subsequent 24-h white light illumination period, pAB96, pSS15, pEA25, pEA170, pEA238, pEA303, and pEA315 RNAs show only LF responses. These responses have thresholds of approximately $10^3 \mu$mol m$^{-2}$ of R (Fig. 5A), one order of magnitude higher than the threshold for LF response to R alone (Fig. 2B). Except for pEA170 and pAB96, these transcripts show only low fluence responses to R alone. pEA170 shows only a VLF to R alone (Fig. 2A) and thus its response in the pretreatment protocol is quite different. pAB96, which exhibits both a VLF and a LF response to R alone (17), exhibits only a LF response to pretreatment before a white light treatment is given. The two remaining clones (pEA303 and pEA315) do not reach a plateau within the range of fluences examined and the protocol used.

pEA215, which shows only a LF response to R alone, shows both a VLF and a LP response to R in plants treated with supplemental white light (Fig. 5A). The thresholds, at approximately $10^{-3}$ and $10^2 \mu$mol m$^{-2}$, respectively, and the fluorescence response curves in general are similar to that measured for cab RNA in response to R alone (17).

The remaining clones, pEA13, pEA46, pEA207, pEA214, and pEA277, exhibit neither a VLF nor a LF response under these conditions (Fig. 5C). Although pEA13 and pEA207 do not respond to R alone (Fig. 2C), the remainder of this group of transcripts do show LF responses for R alone (Fig. 2B).

**DISCUSSION**

We have used a variety of light treatments to further describe the characteristics of the previously reported (25) phytochrome responses exhibited by 11 different transcripts in etiolated pea buds. Fluence-response curves using single, short, R pulses show that LF and/or VLF responses can be observed for different transcripts. FR reversal studies and studies of the fluorescence-dependent accumulation to FR pulses have confirmed the VLF and LF responses observed. The cDNA clones corresponding to the transcripts comprising these categories are listed in Table II.

When only a single R pulse is given, a majority of the transcripts we have studied show only a LF response. These include the transcripts encoded by the rbcS genes (17) and eight other transcripts reported here. One transcript, pEA170, shows a VLF response only. Aside from the transcripts encoded by the rbcS genes (17), no transcripts show both a VLF and a LF response to R alone.

The use of R pulses of varying fluence as a pretreatment has allowed us to group the transcripts using a different protocol than the simple R fluence response curves. This pretreatment protocol has been used to demonstrate the ability of a pulse of VLF red light to potentiate subsequent Chl accumulation in developing pea buds (18, 22). Several of the transcripts exhibit LF responses to the R pretreatment, indicating the ability of a pulse of LF R to potentiate the accumulation of these transcripts. At least one transcript, pEA215, also exhibits a VLF response, indicating a similar potentiation effect of R in the VLF range. The two transcripts showing no response to the R treatment alone, pEA13 and pEA207, also show no response to the R pretreatment.

Several transcripts show a VLF and/or LF response to R when given alone, but not when the R is given as a pretreatment before white light (Table II). There are several possible reasons for this observation. First, it is possible that the phytochrome signal resulting in the response to R alone is not capable of potentiating a white light response under these conditions. It is also possible of the white light alone. By comparing the abundance of transcripts in buds of dark-grown plants with the abundance in buds of plants receiving 24 h of white light, it is possible to determine a white light induction ratio for each of the cDNA clones examined (Fig. 1 for protocol). These white light induction ratios are listed in Table I. It is noteworthy that three of the clones (pEA170, pEA207, and pEA215) correspond to transcripts whose abundance decreases in response to the white light irradiation. This has been previously reported for pEA207 (25). Both pEA170 and pEA215 increase in response to red light alone, approximately 1.5- and 3-fold, respectively (Table I), while pEA207
Table II. Type of Fluence Response Displayed by the Various Transcripts

<table>
<thead>
<tr>
<th>Clone</th>
<th>No</th>
<th>LF Only</th>
<th>VLF Only</th>
<th>VLF and LF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R*</td>
<td>RW</td>
<td>R</td>
<td>RW</td>
</tr>
<tr>
<td>pAB96</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>pSS15</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA13</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA25</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>pEA46</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>pEA170</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>pEA207</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA214</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA215</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA238</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>pEA277</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA303</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pEA315</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*(R), Response to R alone. (RW), Response to R as a pretreatment to a subsequent white light irradiation.

that the phytochrome signal regulating these transcripts does not interact with the continuous white light signal. The two responses remain distinct and the increase in transcript level as a result of the white light is large enough to make the phytochrome response undetectable. (Recall that, except for pEA170, the white light response for these transcripts is greater in magnitude than the respective responses to the single R pulses [Table I].)

This latter possibility could easily occur in the case of a multigene family, wherein one copy of the gene is regulated by a VLF R pulse, but not by continuous white light, and a second copy is regulated by continuous white light, but not a VLF R pulse. Accumulation of the transcript from the white light regulated copy may make the accumulation from the VLF R sensitive copy undetectable. This may be the case for the cab RNA where a VLF response is observed for R alone (17) but not to R used as a pretreatment.

Many of the transcripts which show a LF response to R alone are also sensitive to LF pulses of R used as a pretreatment. Although it is possible that the same signal transduction mechanism regulates the two different responses, we feel this is probably not the case. If both responses are mediated by the same mechanism, white light would not be expected to alter the threshold of the R fluence-response curve. However, the threshold for the LF response to R alone is different from that seen when the R is used as a pretreatment (compare Figs. 2B with 3B). Thus, we believe that the signal transduction mechanisms for the LF responses seen in the two protocols are probably different.

The transcripts may also be grouped on the basis of changes in abundance in response to white light alone (i.e. white light induction ratios). Three of the clones, pEA170, pEA207, and pEA215, show a decrease in response to 24 h of white light. Both pEA215, and pEA170 increase in response to a single pulse of R in the LF range, even if continuous white light follows that pulse. The mechanism by which the R pretreatment lessens the decrease in response to white light should prove very interesting. Furthermore, both pEA170 and pEA215 show VLF responses, with and without supplemental white light respectively. In contrast, pEA207 does not respond to the single pulses of R. However, the abundance of pEA207 decreases in response to three R pulses showing both LF and VLF responses; (B), cDNA clones corresponding to transcripts showing LF responses only. (C), cDNA clones corresponding to transcripts showing no response to R in the range of fluences tested.
on three successive days (25). This decrease in abundance is reversible if each R pulse is followed by FR.

Apel (2), working with barley, has demonstrated that the transcript encoding NADPH:photolide oxidoreductase decreases in abundance upon R or white light illumination. By analogy, it seems possible that pEA207, pEA215, or pEA170 might represent the mRNA for the enzyme in pea, although we have no direct evidence addressing this point. The increase of pEA170 and pEA215 RNA in response to VLF levels of R and their decrease in response to 24 h of white light, may suggest that their encoded products are transient and may have a regulatory role.

The data presented in this paper do not address the question of whether the observed changes in transcript abundance are due to changes in transcription, degradation, or both. Silverthorne and Tobin (23), working with *Lemma*, have demonstrated that phytochrome regulation of *cab* and *rbcS* can occur at the level of transcription. Gallagher and Ellis (13), using pea, have demonstrated white light regulation of *cab* and *rbcS* at the level of transcription. It would seem, therefore, that at least a portion of the observed changes in transcript abundance for these two gene families is probably a consequence of phytochrome control of transcription. However, the possibility that phytochrome does control other steps in mRNA accumulation, such as processing or turnover, has not yet been addressed.

Southern hybridization experiments show that several of the sequences represented by our cDNA clones are multigene families (8, 10–12, 19). Dunsmuir et al. (11, 12), using petunia, suggested that both the *cab* and *rbcS* gene families consist of subfamilies, distinguishable by the sequence in the untranslated region 3' to the coding sequence of the mRNA. It seems reasonable to suppose that different gene copies (or perhaps whole subfamilies) might be differently expressed under different light conditions (i.e. LF R, VLF R, etc.).

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