Phytochrome B in the Mesophyll Delays Flowering by Suppressing FLOWERING LOCUS T Expression in Arabidopsis Vascular Bundles

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Light is one of the most important environmental factors that determine the timing of a plant’s transition from the vegetative to reproductive, or flowering, phase. Not only daylength but also the spectrum of light greatly affect flowering. The shade of nearby vegetation reduces the ratio of red to far-red light and can trigger shade avoidance responses, including stem elongation and the acceleration of flowering. Phytochrome B (phyB) acts as a photoreceptor for this response. Physiological studies have suggested that leaves can perceive and respond to shade. However, little is known about the mechanisms involved in the processing of light signals within leaves. In this study, we used an enhancer-trap system to establish Arabidopsis thaliana transgenic lines that express phyB–green fluorescent protein (GFP) fusion protein in tissue-specific manners. The analysis of these lines demonstrated that phyB-GFP in mesophyll cells affected flowering, whereas phyB-GFP in vascular bundles did not. Furthermore, mesophyll phyB-GFP suppressed the expression of a key flowering regulator, FLOWERING LOCUS T, in the vascular bundles of cotyledons. Hence, a novel intertissue signaling from mesophyll to vascular bundles is revealed as a critical step for the regulation of flowering by phyB.

INTRODUCTION

Plants can perceive and respond to various environmental stimuli. One of the most important factors influencing plant growth and reproduction is light quantity and quality. Plants use light signals for regulating various developmental processes, such as seed germination, deetiolation, hypocotyl elongation, and floral induction. Plants have evolved several different photoreceptors for light perception, including the red/far-red light photoreceptor phytochrome (Sullivan and Deng, 2003) and the blue light photoreceptors, cryptochrome and phototropin (Briggs and Huala, 1999; Briggs and Christie, 2002).

Phytochrome is a well-characterized photoreceptor in plants because it is encoded by a small gene family. In Arabidopsis thaliana, the phytochrome family consists of five members (phytochrome A [phyA] to phyE) (Mathews and Sharrock, 1997). Phytochrome can exist in two spectrally distinct forms (i.e., Pr and Pfr). Red light activates phytochrome by converting it from the Pr form to Pfr form. Conversely, far-red light inactivates phytochrome by converting Pfr to Pr. This photoreversible nature of phytochrome enables plants to monitor the ratio of red to far-red light, which is greatly reduced in the shade. The shade of nearby vegetation can trigger shade avoidance responses, including stem elongation and acceleration of flowering. Among photoreceptors, phyB acts as a major photoreceptor for these responses. For example, phyB mutants exhibit constitutive shade avoidance responses (Halliday et al., 1994; Smith and Whitelam, 1997).

Timing of flowering is greatly affected by environmental light conditions. Not only daylength but also spectral quality of light can affect flowering. In the shade, flowering is accelerated (Smith and Whitelam, 1997). CONSTANS (CO), FLOWERING LOCUS T (FT), and PHYTOCHROME AND FLOWERING TIME 1 (PFT1) are flowering regulators that act downstream of phyB (Cerdan and Chory, 2003; Halliday et al., 2003; Valverde et al., 2004). phyB regulates the abundance of CO protein posttranslationally (Valverde et al., 2004). In the phyB mutant, CO is maintained at a high level, which leads to the promotion of FT expression (Samach et al., 2000). The analysis of pft1 mutants suggests that PFT1 also acts downstream of phyB for regulating FT expression (Cerdan and Chory, 2003). However, the relationship between CO and PFT1 remains unclear.

Spectral and immunochemical analyses suggest that phytochrome is present in various organs throughout the life cycle of plants (Pratt, 1994). Expression analyses of the phytochrome promoter and β-glucuronidase (GUS) gene fusion further support this view (Adam et al., 1994, 1996; Somers and Quail, 1995; Goosby et al., 1997). These observations indicate that most phytochrome responses are cell-autonomous. However, irradiation of restricted parts of plants and grafting experiments have revealed that a complex communication exists between different organs. It is well established that leaves perceive the photoperiodic stimulus, send signals to the shoot apex, and regulate flowering (Knot, 1934; Chailakhyan, 1936; Zeevaart, 1976). The state of phytochrome in leaves is more important than that in the...
stem in determining the rate of stem elongation (Casal and Smith, 1988a, 1988b).

Recent molecular tools can be used to study light perception and signal transduction in different plant tissues. Green fluorescent protein (GFP) has proved to be an excellent fluorescent tag (Chalfie et al., 1994). In Arabidopsis, phyB-GFP fusion proteins have successfully been used to examine the intracellular distribution of phyB (Kircher et al., 1999; Yamaguchi et al., 1999). The enhancer/promoter-trap system uses a reporter gene construct, which can respond to cis-acting transcriptional signals when integrated into chromosomal DNA. It has been demonstrated that this technique is an efficient means of expressing reporter proteins in various patterns (Brand and Perrimon, 1993). Using this system, Kiegle et al. (2000) expressed the calcium reporting protein, aequorin, fused to a fluorescent protein to demonstrate the importance of the endodermis and pericycle in Ca²⁺-mediated stress signal transduction in roots.

In this study, we applied a Cauliflower mosaic virus (CaMV) 35S minimal promoter-based enhancer-trap system to express phyB-GFP in the phyB-deficient mutant of Arabidopsis. The resultant lines, named phyB-GFP tagged (PBT), exhibited various patterns of phyB-GFP expression. In some of these lines, recovery from the phyB-deficient phenotype was observed. Phenotypic and expression analyses of these plants revealed that phyB-GFP in mesophyll suppressed the expression of a key flowering regulator, FT, in the vascular bundles, whereas phyB-GFP in the vascular bundles did not. Hence, an intertissue signaling from mesophyll to vascular bundles is revealed as a critical step in the regulation of flowering by phyB.

RESULTS

Expression of phyB-GFP in PBT Lines

To examine tissue-specific functions of phyB, we expressed a phyB-GFP fusion protein using a CaMV 35S minimal promoter-based enhancer-trap system. We constructed a vector containing the PHYB-GFP fusion gene and the CaMV 35S minimal promoter (Figure 1A), which is thought to be expressed only when it is inserted near cis-acting chromosomal enhancers. The vector was transformed into the phyB mutant of Arabidopsis by the Agrobacterium tumefaciens-mediated method, and 336...
independent PBT lines were established. We first examined fluorescence of phyB-GFP in light-grown seedlings under a conventional fluorescence microscope. Fluorescence was observed in 74 of the 336 PBT lines. Characteristic phyB-GFP speckles (Kircher et al., 1999; Yamaguchi et al., 1999; Gil et al., 2000) were observed in the nuclei of these lines. The spatial patterns of phyB-GFP expression are summarized in Table 1.

Expression of phyB-GFP under the Control of the Authentic Promoter

As controls, lines that expressed phyB-GFP under the control of the authentic PHYB promoter, referred to as Bpro lines, were established. The phyB mutant was transformed with a vector carrying the authentic PHYB promoter and the PHYB-GFP gene (Figure 1A). We obtained more than 30 Bpro lines that exhibited normal flowering under white light. Observations of the light-grown Bpro seedlings under a conventional fluorescence microscope revealed phyB-GFP expression in all organs and tissues, including vascular bundles, stomata, and trichomes. The overall patterns were consistent with those reported for the PHYB promoter-GUS fusion gene expression (Somers and Quail, 1995; Goosey et al., 1997). On the basis of the immunoblotting analysis, we chose Bpro7 as a representative of Bpro lines (data not shown).

PhyB-GFP in Cotyledons Complements phyB-Deficient Early Flowering Phenotype

We examined the flowering phenotype of the 74 GFP-positive PBT lines under continuous white light. Under these conditions, the phyB mutant, which was used as the host plant for the transformation, clearly exhibited the early flowering phenotype (Reed et al., 1993). Among these PBT lines, 56 lines exhibited normal flowering time (Table 1). These included the lines expressing phyB-GFP in almost all organs (20 lines), in cotyledons and other organs (23 lines), and in cotyledons (13 lines). The patterns in the other 18 lines that flowered as early as the phyB mutant were as follows: five root-specific lines, two vascular bundle-specific lines, two leaf primordia-specific lines, six trichome-specific lines, and three stomata-specific lines (Table 1). To confirm that the expression of phyB-GFP was properly monitored, 50 PBT lines that were negative for the GFP fluorescence were chosen at random and subjected to the flowering time analysis. As expected, all the 50 PBT lines flowered early. Thus, evidence suggests that phyB-GFP in cotyledons is crucial in the regulation of flowering.

Interestingly, all of the lines that exhibited normal flowering time appeared normal with respect to seedling and rosette leaf morphology. By contrast, all of the noncomplemented lines were indistinguishable from the phyB mutant (Table 1). Hence, phyB in cotyledons appears to be important, not only for flowering time regulation, but also for seedling and rosette leaf morphogenesis.

PhyB-GFP in Mesophyll Cells Delays Flowering

Initial analysis of PBT lines suggests that cotyledons are the photoreceptive sites for the regulation of flowering by phyB (Table 1). To further confirm this and to examine the signal transduction processes within cotyledons, we chose representative lines and analyzed them in more detail. These lines were as follows: PBT48 and PBT56 as cotyledon-specific lines, PBT6 and PBT239 as vascular bundle lines, and PBT133 and PBT390 as root-specific lines. In agreement with previous reports (Goto et al., 1991; Reed et al., 1993), flowering was accelerated in phyB mutants under continuous (Figure 1B), long-day (Figure 1C), and short-day light treatments (Figure 1D). As expected, lines expressing phyB-GFP in cotyledons (Bpro7, PBT48, and PBT56) flowered as late as the wild type, whereas the others (PBT6, PBT239, PBT133, and PBT390) flowered early. In parallel, complementation of long hypocotyl and small cotyledon phenotypes was observed in the normal flowering lines (Figures 1E and 1F).

We observed the expression pattern of phyB-GFP in more detail under a laser scanning confocal microscope. In the mesophyll cells of cotyledons, the phyB-GFP speckles were detected in Bpro7, PBT48, and PBT56 but not in other lines (Figures 2A to 2F and 2H). By contrast, phyB-GFP in the vascular bundles of the cotyledon was observed only in Bpro7, PBT6, and PBT239 (Figures 2I to 2N and 2P). We then examined these lines to determine whether phyB-GFP was expressed in other parts of the seedlings. In the shoot apex, only Bpro7 expressed a significant level of phyB-GFP (Figures 3A to 3F and 3H). In the hypocotyl, the expression was observed in Bpro7, PBT6, and PBT239 (Figures 3I to 3N and 3P). It should be noted that the expression of PhyB-GFP was restricted to the vascular bundles in PBT6 and PBT239 (Figures 3I to 3N and 3P).

### Table 1. PhyB-GFP Expression Patterns in PBT Lines

<table>
<thead>
<tr>
<th>GFP</th>
<th>Flowering Time</th>
<th>Expression Pattern</th>
<th>Number of Lines</th>
<th>Short Hypocotyl Lines</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
<td>Normal (74 lines)</td>
<td>Whole seedling</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Early (18 lines)</td>
<td>Multiple organs, including cotyledons</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Only cotyledons</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Negative</td>
<td>Early (262 lines)</td>
<td>Roots</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vascular bundles</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf primordia</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trichomes</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stomata</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Not examined</td>
<td></td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>212</td>
<td>Not examined</td>
</tr>
</tbody>
</table>
PBT239. PhyB-GFP was observed in roots in Bpro7, PBT6, PBT239, PBT133, and PBT390 (Figures 3Q to 3V and 3X). Again, the expression was restricted to the vascular bundles in PBT6 and PBT239. These observations indicate that flowering is delayed, in the presence of phyB-GFP in mesophyll cells, to a greater extent than it is when phyB-GFP is present in vascular bundles.

In addition to mesophyll expression, a low but detectable level of phyB-GFP was observed in the epidermis in PBT48 and PBT56 (Figures 2Q to 2V and 2X). Thus, it is possible that phyB-GFP in the epidermis regulates flowering. To examine this possibility, an additional line, PBG10 (Figures 2G, 2O, 2W, 3G, 3O, and 3W), in which phyB-GFP was expressed under the control of the CaMV 35S promoter, was employed. PhyB-GFP was expressed in specific parts of the seedlings in the PBG10, which is probably a result of the positional effect (Lippman et al., 2004). Confocal microscopic observations revealed that phyB-GFP epidermal expression was significantly higher in PBG10 than in PBT56 (Figure 4), although a higher level of expression was

Figure 2. Confocal Microscopic Detection of phyB-GFP Fluorescence in the Cotyledon.

PhyB-GFP was detected in nuclei (Yamaguchi et al., 1999). Seedlings were grown for 5 d under continuous white light (50 μmol m⁻² s⁻¹). Green fluorescence from GFP and red fluorescence from chlorophyll were overlaid electronically. A differential interference contrast image was overlaid as well for epidermis. Arrows and arrowheads indicate phyB-GFP fluorescence in mesophyll and vascular bundle cells, respectively. (A) to (H), (I) to (P), and (Q) to (X) show phyB-GFP fluorescence in mesophyll, vascular bundle, and epidermis, respectively. PhyB-GFP fluorescence in PBT48 (A), (I), and (Q), PBT56 (B), (J), and (R), PBT6 (C, K, and S), PBT239 (D, L, and T), PBT133 (E, M, and U), PBT390 (F, N, and V), PBG10 (G, O, and W), and Bpro7 (H, P, and X). Bars = 50 μm.
observed for PBT48 (data not shown). Nevertheless, flowering time was normal in PBT56, yet early in PBG10 (Figures 1B to 1D). Hence, we concluded that the effect of phyB on flowering is much more significant when it is expressed in the mesophyll than it is when present in the epidermis.

To confirm the observed expression patterns and to estimate the expression levels of phyB-GFP in PBT lines, seedlings were separated into four parts, cotyledons, shoot apex, hypocotyls, and roots, and subjected to immunoblotting analysis. A band of predicted size (150 kD) was detected with anti-phyB antibody in all lines (Figure 5). As expected, the levels of phyB-GFP accumulated in the Bpro7 seedlings were comparable to those in the wild-type seedlings, demonstrating that Bpro7 seedlings accumulated phyB-GFP at normal levels. Consistent with our microscopic observations, phyB-GFP protein was detected in cotyledons but not in other plant parts in the PBT48 and PBT56 lines. By contrast, phyB-GFP was detected only in the roots in PBT133 and PBT390. In the vascular bundle lines PBT6 and PBT239, low levels of phyB-GFP were detected in cotyledons, hypocotyls, and roots.

Figure 3. Confocal Microscopic Detection of phyB-GFP Fluorescence in Shoot Apex, Hypocotyls, and Roots.

Seedlings were grown for 5 d under continuous white light (50 μmol m⁻² s⁻¹). Green fluorescence from GFP, red fluorescence from chlorophyll, and a differential interference contrast image were overlaid electronically. (A) to (H), (I) to (P), and (Q) to (X) show phyB-GFP fluorescence in shoot apex, hypocotyls, and roots, respectively. PhyB-GFP fluorescence in PBT48 ([A], [I], and [Q]), PBT56 ([B], [J], and [R]), PBT133 ([C], [K], and [S]), PBT6 ([D], [L], and [T]), PBT239 ([E], [M], and [U]), PBT390 ([F], [N], and [V]), PBG10 ([G], [O], and [W]), and Bpro7 ([H], [P], and [X]). Bars = 50 μm.
PhyB-GFP in Mesophyll Cells Suppresses FT Expression

It has been proposed that phyB has an effect on flowering by regulating gene expression of a key regulator (FT) in response to shade (Mockler et al., 1999; Simpson and Dean, 2002). Accordingly, FT expression is increased in the phyB mutant (Cerdan and Chory, 2003; Halliday et al., 2003). To further verify that FT is involved in the regulation of flowering by phyB, we established a phyB ft double mutant and examined its flowering time under continuous white light. In agreement with the above research findings, the early flowering phenotype of the phyB mutant was greatly suppressed by the ft mutation (Figure 6).

In continuous white light, the APETALA1 gene, a marker for floral determination, is expressed in shoot apical meristems by day 12 (Liljegren et al., 1999). Hence, we examined FT expression by RT-PCR during the first week of seedling development (i.e., day 2 through 7) in the wild type, the phyB mutant, PBT48, PBT56, and PBT6 under continuous light (Figure 7A). FT expression was increased continuously until day 7. Throughout the experimental period, FT expression was lower in PBT48, PBT56, and the wild type than it was in PBT6 and the phyB mutant. Hence, phyB-GFP effectively delayed FT expression when it was expressed in the mesophyll (PBT48 and PBT56) but not when it was expressed in vascular bundles (PBT6).

The FT gene is expressed mainly in leaves but not in the shoot apex or the stem (Takada and Goto, 2003). To examine organ-specific regulation of FT expression, Arabidopsis seedlings were grown for 5 d under continuous white light and then separated into cotyledons, shoot apex, and the remainder of the plant (i.e., hypocotyls and roots) and analyzed by RT-PCR. As expected, FT expression was much higher in cotyledons than in other plant parts (Figure 7B). Furthermore, FT expression was suppressed in cotyledons by phyB (cf. the wild type versus the phyB mutant). A similar pattern of suppression was observed in PBT48 and PBT56 but not in PBT6. Hence, we concluded that phyB-GFP in mesophyll cells regulated FT expression in cotyledons in a manner similar to that of endogenous phyB.

**Isolation of Mesophyll Cells and Vascular Bundles**

To examine PHYB-GFP and FT expression at the tissue level, we isolated mesophyll protoplasts and vascular bundles from cotyledons (Figures 8A and 8B). Mesophyll protoplasts were prepared according to Jin et al. (2001). The purity of mesophyll protoplasts was estimated to be >90% by microscopic observation. To isolate vascular bundles, cotyledons were dipped into cell wall digesting enzyme solution and sonicated gently several times.

**Figure 4.** Quantitative Analysis of the phyB-GFP Fluorescence in Epidermal Cells.

PhyB-GFP fluorescence in epidermis was observed by confocal microscopy with different photomultiplier settings. The gain was set to 650 (top), 620 (middle), or 590 (bottom). Numbers ± SE below indicate relative intensity of the phyB-GFP fluorescence in epidermal cells. The gain was set to 650, and the fluorescence intensity within the nuclear region was integrated for each nucleus. n = 10 to 14. Bar = 20 μm.

**Figure 5.** Immunoblot Detection of phyB-GFP and Endogenous phyB in Cotyledons, the Shoot Apex, the Hypocotyls, and the Roots.

Proteins were extracted from 5-d-old seedlings grown under continuous white light (50 μmol m⁻² s⁻¹) and subjected to immunoblotting analysis with anti-Arabidopsis phyB antibody, mBA2. Closed and open arrowheads indicate positions of phyB-GFP and endogenous phyB, respectively. Asterisks indicate bands that are presumed to be a degradation product of phyB-GFP. MW, molecular weight (k). Each lane contained 20 μg of total proteins.
times. Most of the mesophyll and epidermal cells were broken, whereas vascular bundles remained intact for the large part because of their hardness. Little mesophyll cell contamination was observed in the preparations.

The expression of marker genes, RbcS for mesophyll cells and Sultr for vascular bundles (Yoshimoto et al., 2003), was examined to further confirm that the separation of the tissues was efficient (Figure 8C). The expression of RbcS in mesophyll protoplasts was approximately four times higher than that in the vascular bundle samples. Conversely, the Sultr expression in the vascular bundle sample was much higher than in the mesophyll protoplasts. We examined the PHYB-GFP mRNA in the same samples to confirm the tissue-specific expression of phyB-GFP. Consistent with the confocal microscopic observations, mesophyllic expression in PBT48 and PBT56 and vascular bundle expression in PBT6 were comparable to those in Bpro7, whereas vascular bundle expression in PBT48 and PBT56 and mesophyll expression in PBT6 were low (Figure 8D).

PhyB-GFP in Mesophyll Cells Suppresses FT Expression in Vascular Bundles of Cotyledons

The FT gene is expressed mainly in vascular bundles but much less in the mesophyll in wild-type seedlings (Takada and Goto, 2003). Nevertheless, phyB-GFP expressed in mesophyll was much more effective in suppressing FT expression in cotyledons than that in vascular bundles (Figure 7B). Hence, we examined whether the mesophyllic phyB-GFP indeed suppressed FT expression in vascular bundles. Mesophyll protoplasts and vascular bundles were prepared from the cotyledons of the seedlings grown under continuous white light for 5 d followed by RT-PCR analyses. Consistent with a previous promoter analysis (Takada and Goto, 2003), FT expression was higher in vascular bundles than it was in mesophyll cells in wild-type seedlings (Figure 8E). The expression was higher compared with the wild type in vascular bundles, but not in the mesophyll in the phyB mutant, indicating that the endogenous phyB mainly suppresses FT expression in vascular bundles.

Expression of FT in the vascular bundles was suppressed in PBT48 and PBT56, in which phyB-GFP was expressed in the mesophyll but not in the vascular bundles, compared with the phyB mutant (Figure 8E). By contrast, FT expression in vascular bundles was as high in the parental phyB mutant as it was in the vascular bundles of PBT6. Hence, phyB-GFP in mesophyll cells suppressed FT expression in vascular bundles, whereas phyB-GFP in the vascular bundles did not.

DISCUSSION

The phyB-GFP Expression in PBT Lines

In this study, we expressed a phyB-GFP fusion protein using a CaMV 35S minimal promoter-based enhancer-trap system (Figure 1A). Consequently, we established 336 PBT lines, 74 lines of which exhibited phyB-GFP expression under a conventional fluorescence microscope (Table 1). The ratio of the fluorescent lines to the total lines was consistent with results reported for the enhancer-trap lines in which the GUS gene was used as a reporter (He et al., 2001). As we expected, the expression of phyB-GFP was restricted to particular parts of the seedlings in many of these lines (Table 1). However, not all the organs/tissues were
covered by this set of PBT lines. For example, we could not find lines that specifically expressed phyB-GFP in the epidermis, shoot apex, and hypocotyl.

In this study, expression patterns of phyB-GFP were determined by fluorescence microscopy. Although the sensitivity of fluorescence microscopy was relatively high, background fluorescence interfered with the observations in some cases. However, in the case of phyB-GFP, background interference did not hamper our observations because phyB-GFP forms characteristic nuclear speckles in the light (Kircher et al., 1999; Yamaguchi et al., 1999; Gil et al., 2000). We could easily observe phyB-GFP in the Bpro7 lines (Figures 2 and 3), in which the level of phyB-GFP was comparable to that of the authentic phyB in the wild type (Figures 2, 3, and 5). Furthermore, phyB-GFP was observed even in heterozygous progeny of Bpro7, PBT56, and PBT6 (data not shown), which indicates that relatively low levels of expression were observable.

All of the GFP-negative lines examined for the flowering phenotype flowered as early as the phyB mutant. In addition, the lines that expressed phyB-GFP only in vascular bundles, roots, leaf primordia, trichomes, or stomata flowered earlier as well. By contrast, all of the lines that expressed phyB-GFP in the cotyledon, except the vascular bundle lines, exhibited a normal flowering phenotype (see below). Hence, a good correlation was observed between the expression patterns and the complementation of the phyB mutant phenotype. This indicates that the phyB-GFP expression was properly monitored.

PhyB in Cotyledons Is Sufficient to Complement Flowering and Seedling Phenotype of the phyB Mutant

The results of our analyses of PBT lines suggest that the cotyledons are the major sites of phyB action in the regulation of flowering time as well as seedling morphogenesis. We examined flowering phenotype in all the PBT lines in which phyB-GFP was detected. Of these lines, 56 PBT lines exhibited complementation of the early flowering phenotype. All these lines expressed phyB-GFP in cotyledons, except the vascular bundle lines, which exhibited a normal flowering phenotype (see below). Hence, a good correlation was observed between the expression patterns and the complementation of the phyB mutant phenotype. This indicates that the phyB-GFP expression was properly monitored.

**Figure 8. FT and PHYB-GFP Expression in Mesophyll and Vascular Bundles.**

Mesophyll protoplasts and vascular bundles were isolated from cotyledons. Seedlings were grown for 5 d under continuous white light (50 \( \mu \)mol m\(^{-2} \) s\(^{-1} \)). TUB2/TUB3 was used as a control. a.u., arbitrary unit.

(A) Mesophyll protoplasts isolated from the cotyledons. Bar = 100 \( \mu \)m.

(B) Vascular bundles isolated from the cotyledons. Bar = 1 mm.

(C) Expression of RbcS (a mesophyll maker) and Sultr (a vascular bundle marker) in the mesophyll and vascular bundle samples. Total RNA was extracted from \( \sim 10^4 \) protoplasts or from vascular bundles prepared from 20 cotyledons and subjected to the analysis. The samples were analyzed by relative quantification using real-time PCR. RNA extraction was performed three times independently. Mean \( \pm \) SE (\( n = 3 \)).

(D) Expression of PHYB-GFP in the mesophyll protoplasts and vascular bundles. The samples were analyzed by relative quantification using real-time PCR. RNA extraction was performed three times independently. Mean \( \pm \) SE (\( n = 3 \)).

(E) Expression of FT in the mesophyll protoplasts and vascular bundles. The samples were analyzed by relative quantification using real-time PCR. RNA extraction was performed three times independently. Mean \( \pm \) SE (\( n = 3 \)).
Flowering Regulation by Mesophyll phyB

(Figure 1D), in which true leaves grow larger before the floral determination takes place. In such plants, phyB-GFP was detected both in mesophyll and vascular bundles in PBT48 and PBT56 (data not shown). Hence, it is possible that expression in true leaves is important under short-day conditions. Nevertheless, the vascular bundle expression alone was not sufficient to delay the flowering. PBT6 and PBT239 flowered earlier than the wild type under the short-day conditions (Figure 1D) regardless of the fact that phyB-GFP was expressed in vascular bundles of both cotyledons and true leaves in these lines (data not shown).

Isolation of Mesophyll Cells and Vascular Bundles

For isolation of mesophyll cells, we prepared protoplasts from cotyledons of the seedlings (Figures 8A and 8B). Because mesophyll cells are easily detached from leaves by the enzyme digestion compared with other tissues, cells collected at an early phase of the digestion are enriched in mesophyll cells. Indeed, contamination of other types of cells has been shown to be small in such samples (Sheen, 2001). We modified a method to prepare bundle sheath cells in C. sativus plants (Edwards et al., 1970; Kanai and Edwards, 1973a, 1973b) for the isolation of vascular bundles from Arabidopsis cotyledons. We found that sonication of Arabidopsis cotyledons in the cell wall digesting enzyme solution yielded vascular bundle specimens of good quality (Figures 8A and 8B), which is further confirmed by the gene expression analysis (Figure 8C). As demonstrated by this work, it is very important to locate the sites of signal transduction events at organ/tissue levels. This type of technique should be very useful to address such problems.

Interorgan and Intertissue Signals for the Regulation of Flowering

The importance of cotyledon as a photoperiod perceptive site for the regulation of flowering has previously been established by physiological experiments. For example, inhibition of flowering under long-day conditions was substantially reduced when the cotyledons are covered with plastic envelopes (Black and Shuttleworth, 1974). Evidence from studies of far-red light spot-irradiation treatments on different parts of cucumber or mustard (Sinapis alba) plants suggests that the leaves adjacent to the internodes are the photoreceptive sites for the regulation of hypocotyl elongation (Lechery, 1979; Casal and Smith, 1988a, 1988b). This work, together with preceding physiological analyses, highlights the importance of leaves for the regulation of hypocotyl elongation.

The above notion leads to the view that long-distance signal is transmitted from leaves to the hypocotyl. Involvement of phytohormones is suspected in this process. Several hormones affect the hypocotyl elongation (Vandenbussche et al., 2005). Especially interesting is auxin because it is transported from the shoot apex and cotyledons to the hypocotyl (Estelle, 1998). Evidence suggests that auxin is involved in the regulation of hypocotyl elongation by phytochrome (Morelli and Ruberti, 2002; Tanaka et al., 2002). However, it is equally possible that an as yet unknown substance mediates this long-distance signal. Further study is needed to elucidate how the phytochrome in the mesophyll can affect the elongation growth in the hypocotyl.

Potentialities of This Type of Approach

These findings reveal a new layer of complexity in the regulatory mechanism of flowering by light. This aspect of light responses in plants has probably been overlooked because of a lack of proper
techniques. Although plant photoreceptors are expressed in various organs/tissues (Toth et al., 2001), many of the responses may not be cell-autonomous. We anticipate that various yet unknown intracellular/organ signaling mechanisms play critical roles in various light responses. This type of analysis should greatly help us to examine the complexities of light signal transduction in plants.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis thaliana mutants used were phyB-1, phyB-5 (Reed et al., 1993), and ft-2 (Kardailsky et al., 1999; Kobayashi et al., 1999) in the Landsberg erecta (Ler) background. phyB-5 was used as the host for transformation. PBT6 was derived from the transformation of the phyB-1 mutant, which was outcrossed two to three times with the wild type (ecotype Columbia). The original PBT6 was additionally outcrossed three times with the phyB-5 mutant in the Ler background. We confirmed that the phyB mutation in the resultant PBT6 was phyB-5 using derived cleaved amplified polymorphic sequences. Preparation of PBG10 is described elsewhere (Yamaguchi et al., 1999).

Seeds were surface-sterilized and sown on 0.6% agar plates containing MS medium without sucrose. The plates were kept in the dark at 4°C for 24 h and then placed under white light conditions, as specified in the figure legends. For hypocotyl length measurements, the seedlings were grown on MS agar plates for 5 d at 22°C and then pressed gently onto the surface of agar medium before photographs were taken. Hypocotyl lengths and cotyledon area were determined by NIH image software (Bethesda, MD). Sterilized seeds, sown on soil, were used for the measurement of flowering times. Flowering times were measured by counting rosette leaf numbers (Koomneef et al., 1991).

Plasmid Construction and Plant Transformation

The binary vectors pPZP211/PBT and pPZP211/Bpro used for the expression of phyB-GFP were derived from the binary plant transformation vector pPZP211 (Hajdukiewicz et al., 1994). A 1.4-kb fragment containing the CaMV 35S promoter and neomycin phosphotransferase II (nptII) gene was amplified from pPZP211 by PCR and cloned into pGEM7zf (Promega, Madison, WI) (pGEM7zf/35S-nptII). A 0.2-kb fragment flanking the right border (RB) was amplified from pPZP211 and cloned into the vector pGEM3zf (Promega) (pGEM3zf/RB). A 70-bp fragment containing CaMV 35S minimal promoter was amplified from the vector pPZP211 and cloned into pGEM3zf/RB (pGEM3zf/RB-m35S). The fragment containing the RB and CaMV 35S minimal promoter was digested from pGEM3zf/RB-m35S and subcloned into the multiple cloning site of pGEM7zf/35S-nptII (pGEM7zf/RB-m35S-35S-nptII). A 0.4-kb fragment containing the nopaline synthase terminator (nosT) was released from pBI-Hyg/CaMV 35S-nosT (Yamaguchi et al., 1999) and inserted between the CaMV 35S minimal promoter and CaMV 35S of the vector pGEM7zf/RB-m35S-35S-nptII (pGEM7zf/RB-m35S-nosT-35S-nptII). The sequence containing the RB and the minimal promoter was designed: TUB2 gene was used as an internal control. The following primers were designed: TUB2 primer that spanned an exon–exon junction.

Immunochemical and Microscopic Detection of phyB-GFP

phyB and phyB-GFP proteins were detected by immunoblotting the protein extracts from seedlings. Protein extraction, SDS-PAGE, protein blotting, and immunodetection were performed as described by Yamaguchi et al. (1999). Proteins were extracted from 5-d-old seedlings grown under continuous white light and subjected to immunoblotting analysis with anti-Arabidopsis phyB antibody.

Five-day-old seedlings grown under continuous white light were examined with a confocal laser scanning microscope (Zeiss LSM510; Jena, Germany). Green fluorescence from GFP (observation, 500 to 530 nm; excitation, 488 nm) and red fluorescence from chlorophyll (observation, >560 nm; excitation, 543 nm) were overlaid electronically.

Isolation of Mesophyll Cells and Vascular Bundles

Mesophyll protoplasts were isolated as described by Jin et al. (2001). Protoplasts were prepared using 50 cotyledons from seedlings grown for 5 d under continuous white light. We developed a novel technique to isolate vascular bundles by modifying the bundle sheath isolation method of Edwards et al. (1970) and Kanai and Edwards (1973a, 1973b). Cotyledons were processed with a sonicator (Heat Systems Ultrasonics, Farmingdale, NY) in a 1.5-mL plastic tube with 1 mL of cell wall digesting enzyme solution (Jin et al., 2001). The vascular bundles that were detached from the other tissues were collected using a needle and tweezers under a binocular. The purity of mesophyll protoplasts was estimated to be >90% by microscopic observation, and little mesophyll cell contamination was observed in the vascular bundle preparations.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Arabidopsis seedlings were grown under continuous white light. Total RNA was extracted from the whole seedlings or separated organs using Sepazol super (Nacalai Tesque, Kyoto, Japan) following the manufacturer’s instructions. For tissue specificity analysis, total RNA was extracted from −10° mesophyll protoplasts or the vascular bundles prepared from 20 cotyledons using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) following the manufacturer’s instructions. Total RNA was digested with RNase-free DNasel (Qiagen, Tokyo, Japan) following the manufacturer’s instructions.

Reverse transcription was performed with oligo(dT) primer using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The TUB2/ TUB3 gene was used as an internal control. The following primers were designed: TUB2 primer that spanned an exon–exon junction. Exception GFP primers, all the primer sets included at least one primer that spanned an exon–exon junction.

PCR was performed in 200-μL tubes with a Rotor-Gene RG-3000A (Corbett Research, Sydney, Australia) using SYBR Green to monitor double-stranded DNA synthesis. Reaction mixtures contained 7.5 μL of SYBR Premix Ex Taq (TaKaRa, Otsu, Japan), 1 μL of cDNA, and 200 nM each gene-specific primer in a final volume of 15 μL. The following
standard thermal profile was used for all PCRs: 95°C for 10 s, 55 cycles of 95°C for 5 s, and 60°C for 20 s. Data were analyzed using Rotor-Gene 6.0.16 software (Corbett Research). We examined negative template controls in these experiments, and no signal was observed (data not shown).

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