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Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction

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SUMMARY

The *Arabidopsis* photoreceptors cry1, cry2 and phyB are known to play roles in the regulation of flowering time, for which the molecular mechanisms remain unclear. We have previously hypothesized that phyB mediates a red-light inhibition of floral initiation and cry2 mediates a blue-light inhibition of the phyB function. Studies of the *cry2/phyB* double mutant provide direct evidence in support of this hypothesis. The function of cryptochromes in floral induction was further investigated using the *cry2/cry1* double mutants. The *cry2/cry1* double mutants showed delayed flowering in monochromatic blue light, whereas neither monogenic *cry1* nor *cry2* mutant exhibited late flowering in blue light. This result suggests that, in addition to the phyB-dependent function, cry2 also acts redundantly with cry1 to promote floral initiation in a phyB-independent manner. To understand how photoreceptors regulate the transition from vegetative growth to reproductive development, we examined the effect of sequential illumination by blue light and red light on the flowering time of plants. We found that there was a light-quality-sensitive phase of plant development, during which the quality of light exerts a profound influence on flowering time. After this developmental stage, which is between approximately day-1 to day-7 post germination, plants are committed to floral initiation and the quality of light has little effect on the flowering time. Mutations in either the *PHYB* gene or both the *CRY1* and *CRY2* genes resulted in the loss of the light-quality-sensitive phase manifested during floral development. The commitment time of floral transition, defined by a plant's sensitivity to light quality, coincides with the commitment time of inflorescence development revealed previously by a plant's sensitivity to light quantity – the photoperiod. Therefore, the developmental mechanism resulting in the commitment to flowering appears to be the direct target of the antagonistic actions of the photoreceptors.

Key words: *Arabidopsis thaliana*, CRY, PHYB, Flowering, Photoreceptors, Photoperiodism

INTRODUCTION

Plant development is regulated by environmental conditions such as temperature, humidity, salinity, and light. The quality and quantity of light affect plant development mainly through two types of photoreceptors – the red/far red light receptors phytochromes and blue/UV-A light receptors cryptochromes (see reviews in Kendrick and Kronenberg, 1994). These photoreceptors regulate plant development throughout the life cycle of the plant from seedling growth to floral initiation. Phytochromes are proteins containing covalently bound linear tetrapyrrole chromophores which allow phytochrome molecules to reversibly interconvert between the red light-absorbing Pr forms and the far red light-absorbing Pfr forms (reviewed by Quail et al., 1995). Cryptochromes are flavin-type blue light receptors that share similarities in both protein structure and chromophore composition with the microbial DNA photolyases (reviewed by Sancar, 1994; Cashmore, 1997). Cryptochromes were first characterized in plants (Ahmad and Cashmore, 1993; Lin et al., 1998). Recently, this type of blue light receptor has also been found in Drosophila and mouse (Emery et al., 1998; Miyamoto and Sancar, 1998; Stanewsky et al., 1998; Thresher et al., 1998).

Arabidopsis has five different phytochromes – phyA, B, C, D, and E. The functions of phyA and phyB in plant photomorphogenesis have been studied extensively using *Arabidopsis* mutants impaired in each of the photoreceptor genes (reviewed by Furuya, 1993; Quail et al., 1995; Chory, 1997). Seedlings containing mutations in the *PHYA* or *PHYB* gene grew taller than the wild type under continuous far-red light or red light, respectively (Koornneef et al., 1980; Nagatani et al., 1991; Somers et al., 1991; Dehesh et al., 1993), demonstrating the function of phyA and phyB in the perception of the corresponding wavelength of light for the hypocotyl inhibition response. Recent studies indicated that phyC may be involved in the red-light-induced hypocotyl inhibition and leaf expansion (Halliday et al., 1997; Qin et al., 1997); phyD and phyE act in conjunction with phyB in the regulation of many shade avoidance responses (Halliday et al., 1994; Aukerman et al., 1997; Devlin et al., 1998). *Arabidopsis* also has multiple

blue light receptors including cryptochromes such as cry1 and cry2 (reviewed by Cashmore, 1997), and a protein kinase NPH1 (Huala et al., 1997; Christie et al., 1998). cry1 mediates blue-light induced inhibition of hypocotyl elongation (Koornneef et al., 1980; Ahmad and Cashmore, 1993), whereas cry2 regulates floral initiation in response to photoperiod (Koornneef et al., 1991; Guo et al., 1998). NPH1 is a flavoprotein possessing a light-regulated kinase activity required for the blue light-dependent phototropism (Liscum and Briggs, 1995). The functions of cry1 in regulating hypocotyl inhibition and anthocyanin biosynthesis have been studied extensively using mutant plants (*hy4*) impaired in the *CRY1* gene (Koornneef et al., 1980; Liscum and Hangarter, 1991; Ahmad and Cashmore, 1993; Jackson and Jenkins, 1995; Lin et al., 1995a,b, 1996a; Bruggemann et al., 1996; Ahmad and Cashmore, 1997). The function of cry2, which shares about 50% amino-acid sequence identity with cry1 (Hoffman et al., 1996; Lin et al., 1996b, 1998), has been studied using the *Arabidopsis cry2* mutant isolated recently. The *cry2* mutant showed a defect in hypocotyl inhibition under low intensities of blue light (Lin et al., 1998), which indicates that although cry1 is the major blue light receptor mediating blue light inhibition of hypocotyl elongation, cry2 is also involved in this response.

Floral initiation represents another developmental process regulated by light. The flowering time of many plants is determined by the daily duration of light and/or darkness – the photoperiod. Plants in which flowering requires or is accelerated by short-day (SD) or long-day (LD) photoperiods are referred to as short-day plants or long-day plants, respectively. Plants that flower at about the same time regardless of LD or SD conditions are known as day-neutral plants. The molecular mechanism of plant photoperiodism is not clear. Plant physiology studies in the last half century have indicated that phytochromes play major roles as the photoreceptors regulating flowering-time in plants (reviewed by Thomas and Vince-Prue, 1997). This notion is supported by the more recent genetic studies, which indicate that both phyA and phyB are involved in the photoperiodic sensing and flowering time determination. For example, it is known that phyA promotes flowering. A phyA-deficient mutant (*fun1*) of garden pea (*Pisum sativum*, a LD plant) flowered later than the wild type in LD but not in SD, resulting in reduced sensitivity of the mutant plants to photoperiod (Weller et al., 1997a,b). phyA also plays a role in the flowering-time regulation in the facultative long-day plant *Arabidopsis*. *Arabidopsis* transgenic plants overexpressing *PHYA* showed photoperiod-insensitive early flowering under day-extension conditions (Bagnall et al., 1995), while the *phyA* mutant flowered significantly later than the wild type in response to daylength extensions (Johnson et al., 1994). phyB has been found to inhibit floral initiation, and the function of phyB in the photoperiodic regulation of flowering has been illustrated by genetic studies in different plant species. A phyB-deficient mutant (*lv*) of pea flowered earlier than the wild type in SD but not in LD (Weller and Reid, 1993, 1997b). More interestingly, the *phyB* mutant (ma_3^R) of the SD plant sorghum (*Sorghum bicolor*) flowers earlier than the wild type, but the early-flowering phenotype of the sorghum *phyB* mutant plants is more pronounced in LD than in SD photoperiods (Childs et al., 1997). Therefore, *phyB* mutations result in accelerated floral initiation and decreased responsiveness to photoperiod in both LD and SD plants. *Arabidopsis phyB* mutants flowered earlier than the wild type (Goto et al., 1991; Reed et al., 1993; Bagnall et al., 1995). The early flowering of the *phyB* mutant is more pronounced in SD than in LD, although the role of phyB in the photoperiodic flowering of *Arabidopsis* has not been explicitly defined (Goto et al., 1991; Bagnall et al., 1995; Koornneef et al., 1995). Since both *phyB/phyD* and *phyB/phyE* double mutants flowered earlier than the *phyB* monogenic mutant (Aukerman et al., 1997; Devlin et al., 1998), phyB, phyD, and phyE, which form a subgroup of the *Arabidopsis* phytochrome family (Goosey et al., 1997), may have a redundant function in the regulation of photoperiodic flowering in *Arabidopsis*.

Arabidopsis cry1 and cry2 are both known to positively regulate floral initiation. In addition to its major function in hypocotyl inhibition and anthocyanin biosynthesis, *Arabidopsis* cry1 has been shown to play a role in the regulation of flowering time based on the observation that some *hy4* alleles cause late flowering in SD (Bagnall et al., 1996; Mozley and Thomas, 1998). However, the function of cry1 in the photoperiodic response remains unclear. cry2, on the other hand, is apparently the primary blue light receptor regulating flowering time of *Arabidopsis* in response to photoperiod (Koornneef et al., 1991; Guo et al., 1998). The *cry2* mutant, which flowers significantly later than the wild-type in LD but not in SD, was found to be allelic to a previously isolated photoperiod-insensitive flowering-time mutant *fha* (Koornneef et al., 1991; Guo et al., 1998). It has also been demonstrated that cry2 is a positive regulator of the flowering-time gene, *CO*, for which the expression is regulated by photoperiod (Putterill et al., 1995; Guo et al., 1998). The late-flowering of *cry2* mutants under continuous white light can be phenocopied by continuous red-plus-blue light, but not by continuous red light or blue light alone (Guo et al., 1998). Together with the discovery that the early-flowering phenotype of the *phyB* mutant is dependent on red light, it was proposed that phyB mediates red light inhibition of flowering whereas cry2 mediates blue light inhibition of the phyB function (Guo et al., 1998).

To further investigate the seemingly complex actions of different photoreceptors in the course of the developmental transition of plants from vegetative to reproductive growth, we prepared *cry2/phyB* and *cry2/cry1* double mutants and analyzed the floral initiation of various mutants impaired in photoreceptors under different light conditions.

MATERIALS AND METHODS

Plant Materials

The *Arabidopsis thaliana* photoreceptor mutants used for this report are in the Columbia ecotype background, except *hy3* and *fha* which are in the L*er* (Landsberg *erecta*) ecotype background. *cry2-1, cry1-301, cry1-304*, and *hy4-B104* were isolated from fast-neutron mutagenized populations (Bruggemann et al., 1996; Guo et al., 1998). *cry1-301* and *cry1-304* are newly isolated alleles of the *hy4* mutant (Fig. 1). *hy4-101, hy4-102, hy4-103*, and *hy4-105* were isolated from an EMS-mutagenized Columbia ecotype background (Liscum and Hangarter, 1991; Bagnall et al., 1996). *phyB-9* is a null *phyB* mutant allele (M. Neff and J. Chory, personal communications). For the other flowering time mutants used, *elf3* (Zagotta et al., 1996), *gi-1* and *gi-2* are in the Columbia background, the rest are all in the L*er* background (ABRC, Ohio State University).

Seeds were sown on soil, cold treated for 4 days in the dark, and exposed to white light for 4 hours to enhance germination. Following white light treatment, plants were moved to the respective conditions employed for each experiment in temperature-controlled growth chambers or dark rooms and grown at approximately 22-25°C. Light for experiments involving continuous blue illumination was provided by Bili Blue fluorescent bulbs (F48T12/B-450/HO; Interlectric Corp., Warren, PA) filtered through a blue plexiglass filter (2424 Blue; Polycast Technology Corp., Stamford, CT). Continuous red light was provided by red fluorescent bulbs (F48T12/R-660/HO; Interlectric) filtered through a red plexiglass filter (2423 Red; Polycast). The wavelengths of the emission peak for the blue light and red light are 436 nm and 658 nm, respectively, with a half bandwidth of less than 25 nm for both the blue light and red light (Interlectric Corp., Warren, PA). Red-plus-blue light studies were conducted using alternating red and blue fluorescent bulbs and the light was transmitted through a deflecting neutral density filter. Trays of plants (22 in. × 11 in.) were rotated 90 degrees each day to further ensure even exposure of light. Cool white fluorescent lights (F48T12/CW/HO; General Electric, Cleveland, OH) were used as the white light sources in all photoperiodic studies. Fluence rates were measured using a Li250 quantum photometer (Li-Cor, Inc., Lincoln, NE).

Photoperiod studies were conducted in either a Conviron E7/2 (CMP4030) growth chamber (Controlled Environments Limited, Winnipeg, Canada), or in a temperature-controlled dark room in which the lights are controlled by a programmable timer. Plants grown in LD [6 hours of darkness and 18 hours of white light (75-100 μmole/second·m^2)] or SD [15 hours dark/9 hours light (150-200 μmole/second·m^2)] received a similar amount of total irradiance per 24 hours. Hypocotyl lengths were measured manually to a 0.5 mm increment. The flowering times were measured as both the "days to flower" and the "leaf number". The "days to flower" were the days between the date plants were placed under light to the date the first flower bud appeared. The "leaf number" was scored as the number of rosette leaves at the day the first flower bud appeared.

Genetic analysis

cry2/phyB double mutants were constructed using the null alleles of *cry2* (*cry2-1*) and *phyB* (*phyB-9*). F_2 plants derived from a cross between *cry2-1* and *phyB-9* were grown under white light and leaf tissues from individual plants were assayed for CRY2 protein by immunoblot as described by Lin et al. (1998). F_3 seedlings from the F_2 plants that lacked CRY2 were screened for those that grew uniformly taller than the wild type under red light. *cry2phyB* double mutants were confirmed by analysis of F_3 and F_4 progenies using immunoblot and hypocotyl assays. In addition, the *cry2phyB* double mutants were also verified by the extreme elongated hypocotyl phenotype as compared to either *cry2-1* or *phyB-9* parents, when grown under red-plus-blue light.

cry2/cry1 double mutants were constructed using *cry2-1* (Guo et al., 1998) with different cry1 mutant alleles including *hy4-B104* (Bruggemann et al., 1996) and *cry1-301*. We also prepared and analyzed the double mutants *cry2-1*/*cry1-304* and *hy4-2.23N/fha-1* which both showed similar phenotypes as the other allele combinations (not shown). F_2 seedlings from the crosses between *cry2* and *cry1* were grown under blue light and extremely tall seedlings were selected for transplantation to soil. *cry2/cry1* double mutants were identified by immunoblots probed with both anti-CRY1 and anti-CRY2 antibodies, respectively, and the genotypes were confirmed in F_3 and F_4 by immunoblot and hypocotyl analysis.

RESULTS

cry2 suppresses the phyB inhibition of floral induction

We prepared a double mutant defective in both the *CRY2* and *PHYB* genes. The *cry2/phyB* double mutant expressed no CRY2 protein (Fig. 1A). As expected, young seedlings of the double mutant showed a long hypocotyl phenotype similar to the *cry2* mutant under blue light and to the *phyB* mutant under red light (Fig. 2). Namely, the double mutant seedlings grew taller than the wild type under either blue light (Fig. 2 Blue) or red light (Fig. 2 Red). Under red-plus-blue light, *cry2/phyB* double mutant seedlings (approximately 9 mm) grew significantly taller than either the *cry2* (approximately 3 mm) or *phyB* (approximately 5 mm) monogenic mutant parents (Fig. 2 Red+Blue), indicating an additive or synergic effect of the two photoreceptors in the hypocotyl inhibition response. It is not known whether the two photoreceptors are associated with the same or different signaling mechanisms in mediating hypocotyl inhibition. Nevertheless, this new phenotype of the double mutant provided a convenient means of identifying it.

The *cry2/phyB* double mutant grown in LD flowered significantly earlier than the *cry2* monogenic mutant (Figs 3, 4A,B). The *cry2/phyB* double mutant grown in SD flowered at about the same time as the *phyB* monogenic mutant, and both flowered significantly earlier than the wild type (Figs 3, 4A,B). To further investigate the functional interaction of cry2 and phyB, we examined the flowering time of different mutant lines in response to red light or blue light. As reported previously, wild-type *Arabidopsis* plants grown under continuous blue light flowered significantly earlier than plants grown under a similar fluence rate of red light (Brown and Klein, 1971; Eskins, 1992; Guo et al., 1998) (Fig. 4C,D). *cry2* mutant plants flowered late in white light or red-plus-blue light, but showed normal flowering in monochromatic blue light or red light; and the *phyB* mutant flowered early in continuous red light (Guo et al., 1998) (Fig. 4C,D). These findings were interpreted collectively as *phyB* mediates a red-light inhibition of floral

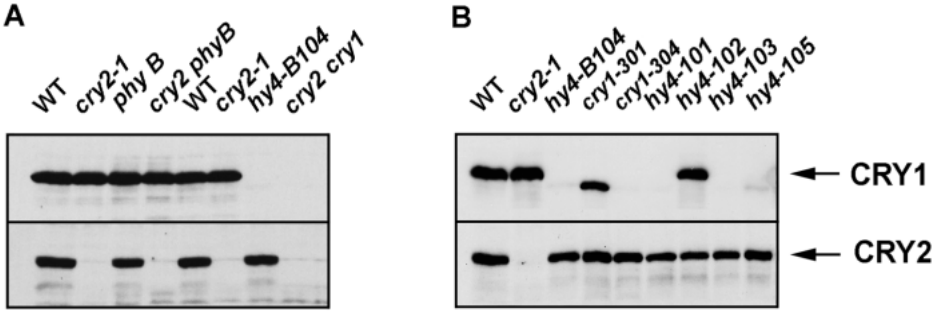

Fig. 1. Molecular characterization of *Arabidopsis* photoreceptor mutations. (A) Samples from cry2, *phyB*, *cry2phyB* mutant, *cry1*, and *cry2cry1* (*cry2-1/hy4-B104*) mutant and Columbia wild-type (WT) plants were prepared and analyzed using immunoblot as described by Lin et al. (1998). (B) Samples from Columbia wild type (WT), *cry2* and *cry1* (*hy4*) mutant plants (*hy4-B104, cry1-301, cry1-304, hy4-101, hy4-102, hy4-103, hy4-105*) were prepared and analyzed using immunoblot as in A. Blots were probed with anti-CRY2 antibody (lower), and were then stripped and re-probed with the anti-CRY1 antibody (upper).

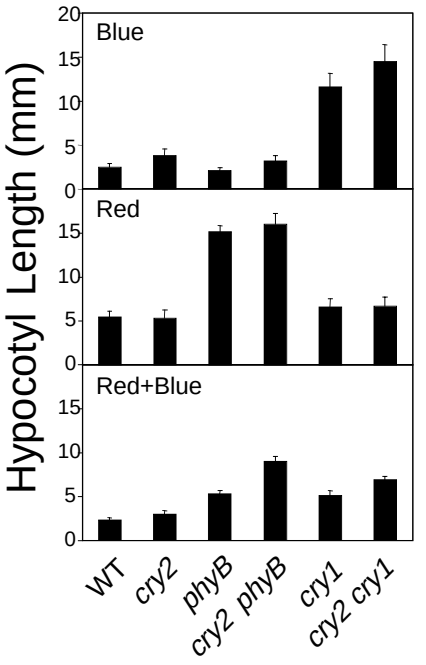

Fig. 2. Hypocotyl inhibition response of *cry2phyB* and *cry1cry2* double mutants. Columbia wild type (WT), *cry2*, *cry1*, *cry2cry1*, *phyB*, and *cry2phyB* mutant seedlings were germinated as described in Methods and grown under continuous blue light (Blue; approx. 60 μmole/second/m^2), continuous red light (Red; approx. 50 μmole/second/m^2), or red-plus-blue light (Red + Blue; approx. 55 μmole/second/m^2 with a fluence ratio of red light to blue light of approximately 2.5:1.0) for 4 days. Hypocotyl lengths of 20-30 seedlings of each sample were measured. The means and standard errors of 4, 5 and 9 independent experiments are shown for "Red", "Blue" and "R+B" panels, respectively.

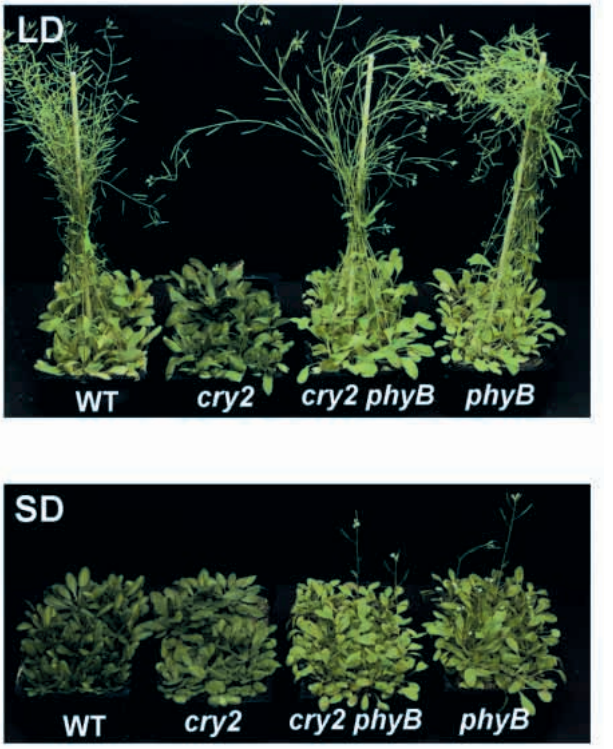

Fig. 3. 38-day-old plants of the wild-type Columbia (WT), *cry2*, *cry2phyB*, and *phyB* mutants grown under long day (LD) and short day (SD) conditions.

initiation, whereas cry2 mediates a blue light inhibition of phyB function (Guo et al., 1998), which is depicted in a model shown in Fig. 5. According to our model, it is expected that mutations in the *CRY2* gene should not affect the early-flowering phenotype of the *phyB* mutant in red light, because the phyB-mediated red light inhibition of floral initiation is dependent on neither cry2 nor blue light (Fig. 5). On the other hand, according to this model, mutations in the *PHYB* gene could suppress the late-flowering phenotype of the *cry2* mutant in red-plus-blue light if this particular aspect of cry2 function is dependent on not only blue light but also red light and phyB (Guo et al., 1998). The results shown in Fig. 4C,D are consistent with this prediction. Under red light, *cry2/phyB* mutant plants flowered at about the same time as *phyB* mutant plants (Fig. 4C,D, Red). Clearly, the *cry2* mutation did not suppress the early-flowering of *phyB* under red light. Under red-plus-blue light, *cry2/phyB* double mutant plants also flowered at about the same time as the *phyB* mutant but significantly earlier than the *cry2* mutant parent (Fig. 4C,D, Red+blue). Thus, the mutation in the *PHYB* gene suppressed the late-flowering phenotype of the *cry2* mutant, or the mutation of *CRY2* in the *phyB* background no longer caused delayed flowering in red-plus-

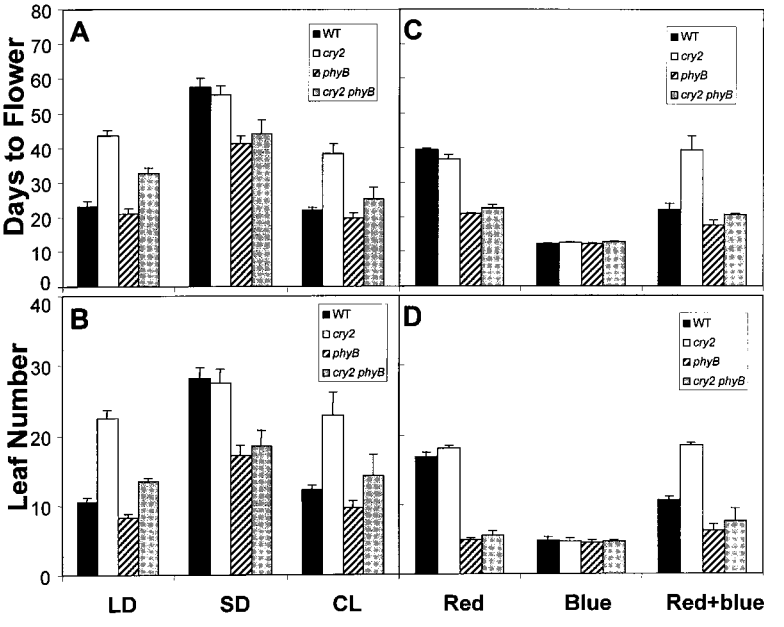

Fig. 4. Effects of different wavelengths of light and photoperiods on the flowering time of *Arabidopsis cry2*, *phyB*, and *cry2phyB* mutants. The "days to flower" (A,C) and "leaf number" (B,D) were measured as described in the Materials and Methods. Individual samples containing between 19 and 58 plants. Means of 5 (A,B) or 4 (C,D) independent experiments and the corresponding standard errors are shown.

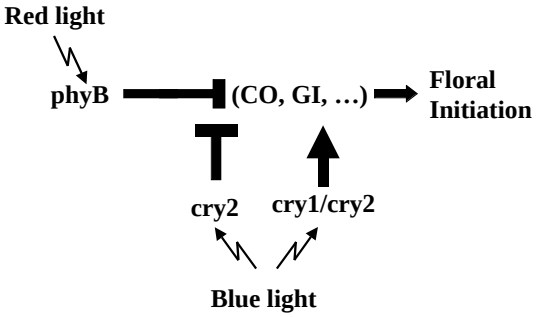

Fig. 5. A model for the regulation of floral initiation by *Arabidopsis* photoreceptors. Possible functional interactions between photoreceptors that regulate floral initiation in *Arabidopsis* are depicted. The arrows represent stimulatory effect and the lines terminated with a bar denote inhibitory effect.

blue light. We conclude that cry2 mediates a blue light-dependent inhibition of the phyB suppression of floral initiation. No alteration in flowering time was found for either *cry2*, *phyB*, or *cry2/phyB* double mutant plants under blue light (Fig. 4C,D, Blue), which is also as predicted by our model (Fig. 5).

A redundant function of cry1 and cry2 in promoting floral initiation

Arabidopsis mutants impaired in the *CRY1* gene are defective in the hypocotyl inhibition response under a wide range of blue light intensities (Koornneef et al., 1980; Ahmad and Cashmore, 1993), whereas the impaired hypocotyl inhibition of the *cry2* mutant is restricted largely to low intensities of blue light (Lin et al., 1998). Although cry2 is a primary blue-light receptor regulating floral induction in response to light, it has been suggested that cry1 may also be involved in the regulation of floral induction by blue light (Bagnall et al., 1996; Zagotta et al., 1996). To investigate the relationship of cry1 and cry2 in plant development, we constructed and analyzed several *cry2/cry1* double mutants. Results of the *cry2/cry1* double mutant constructed using the *hy4-B104* (Bruggemann et al., 1996) and *cry2-1* (Guo et al., 1998) null alleles, which accumulated neither CRY1 nor CRY2 protein (Fig. 1A), are shown in Figs 2 and 6. The *cry2/cry1* double mutant showed normal hypocotyl inhibition in red light (Fig. 2 Red), but grew slightly (approximately 20%) taller than the *cry1* mutant under blue light (Fig. 2, Blue) and red-plus-blue light (Fig. 2, Red+blue). These results indicate that although cry2 only plays a minor role in the blue light inhibition of hypocotyl elongation, its action does contribute to the overall blue light sensitivity of young seedlings. It is interesting that a different *Arabidopsis cry2/cry1* double mutant was reported to have been isolated from an EMS-mutagenized *cry1* mutant line by screening for second-site mutations that caused the loss of the phototropic response (Ahmad et al., 1998), but none of our *cry2/cry1* double mutant lines showed an easily discernible defect in the phototropic response (unpublished observation).

The flowering time of the *cry2/cry1* double mutant was very similar to that of the *cry2* monogenic mutant in both LD and SD photoperiods (Fig. 6A,B), suggesting no apparent interaction of cry1 and cry2 in photoperiodic flowering. We further analyzed the flowering response of the mutant plants to different wavelengths of light. Interestingly, although the *cry1* and *cry2* monogenic mutants flowered at about the same time as the wild type in both red light and blue light (Fig. 6C), the *cry2/cry1* double mutant plants flowered significantly later than the wild type or the *cry1* and *cry2* monogenic mutant parents under blue light (Fig. 6C, Blue). The late-flowering phenotype of *cry2/cry1* double mutations under blue light was further confirmed by the analysis of the flowering time of three additional *cry2/cry1* double mutants (not shown) and six additional *cry1* (*hy4*) alleles (Fig. 7A) (T. C. M. and C. Lin, unpublished data). Most of these *cry1* (*hy4*) mutations accumulated no CRY1 protein, except *cry1-301* and *hy4-102*, which synthesized a truncated CRY1 or apparently normal-sized CRY1, respectively (Fig. 1B). The *cry2/cry1* double mutant consistently showed an approximately 70% delay in flowering time under different intensities of blue light. As shown in Fig. 7A, under continuous blue light, the wild-type Columbia, *cry2* mutant, and all plants with the 7 *hy4* (*cry1*) alleles flowered in approximately 13 days. In contrast, the *cry2/cry1* double mutants (*cry2hy4-B104* and *cry2cry1-301*) did not flower until approximately 24 days. This observation indicates that cry2 possesses two blue-light-dependent functions in the regulation of flowering time, of which one is dependent on red light and phyB, whereas the other is independent of red light and phyB (Fig. 5). The red-light-dependent function of cry2 is to suppress phyB action, as

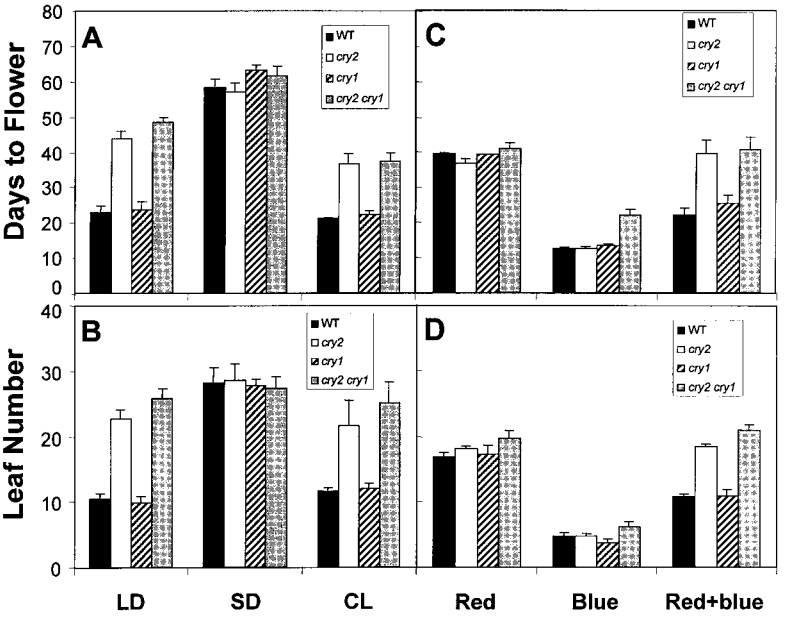

Fig. 6. Effects of different wavelengths of light and photoperiods on the flowering time of *Arabidopsis cry2*, *cry1*, and *cry2/cry1* mutants. The flowering times (A,C) and leaf number (B,D) for Columbia wild type (WT), *cry2*, *cry1*, *cry2/cry1* mutant plants were assayed as described in Fig. 4. Growth conditions were identical to those described for Fig. 4. The means of 4 independent experiments with individual samples containing between 11 and 39 plants (A,B) or between 11 and 63 plants (C,D), and the standard errors are shown.

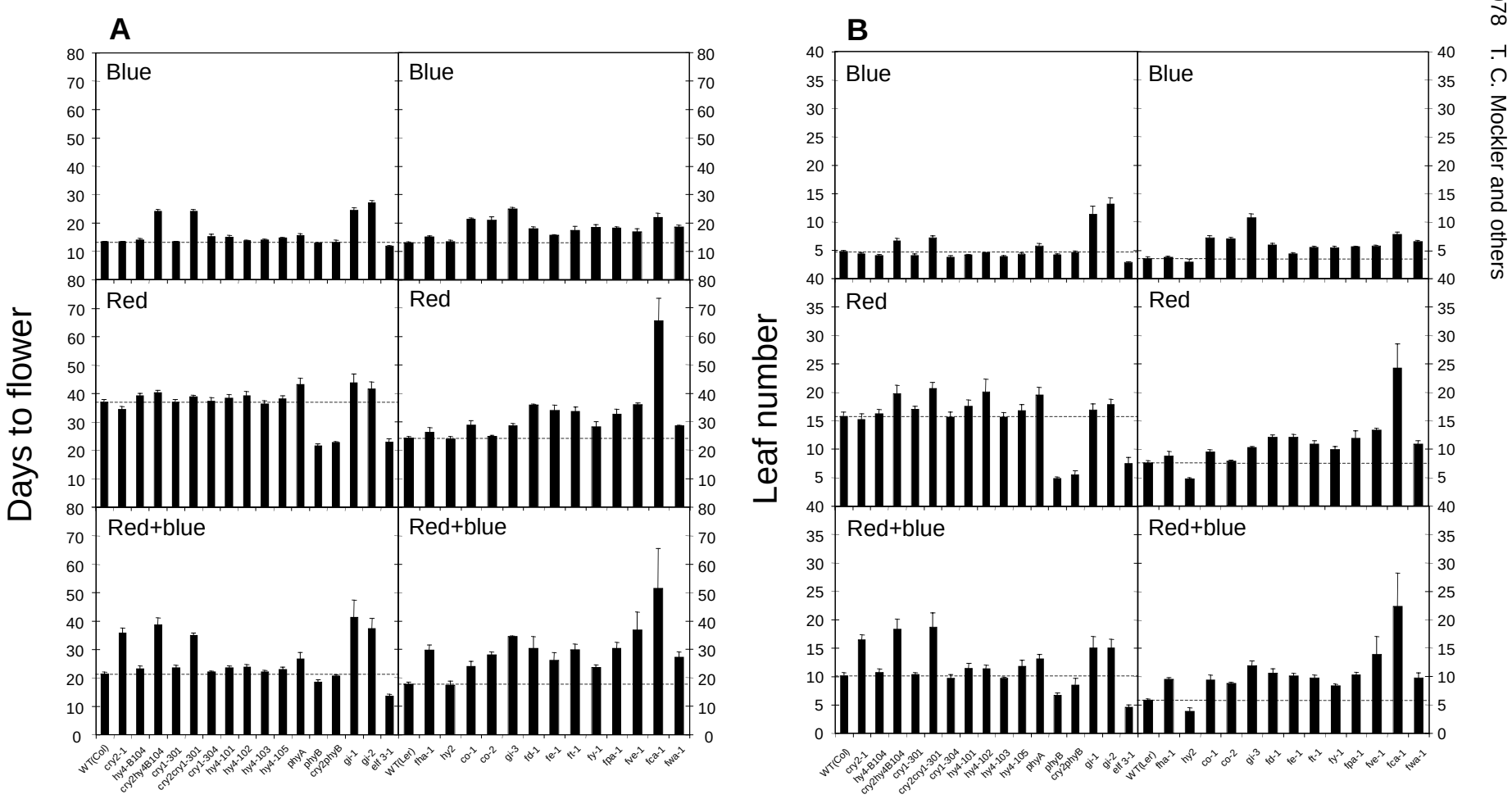

Fig. 7. The effects of different wavelengths of light on the flowering time of wild type and various *Arabidopsis* mutants. Days to flower (A) and number of rosette leaves (B) were measured as described in Materials and Methods. The dotted line in each panel corresponds to the mean of the wild type. Blue: plants were grown under continuous blue light (65-85 μmole/second·m^2). Means of between 3 to 21 independent experiments, depending on the genotype, and the standard errors are shown. Individual samples contained between 8 and 52 plants, depending on the genotype. The minimum number of total plants assayed for a single line was 43, and the maximum was 389. Red: plants were grown under continuous red light (65-85 μmole/second·m^2). Means of between 3 to 15 independent experiments and the standard errors are shown. Individual samples contained between 8 and 63 plants, and the minimum number of total plants assayed for a single line was 45, and the maximum was 371. Red+Blue: plants were grown under continuous red light (55-75 μmole/second·m^2 with a fluence ratio of red light to blue light of approximately 2:3). Means of between 3 and 17 independent experiments and the standard errors are shown. Individual samples contained between 10 and 57 plants. The minimum number of total plants assayed for a single line was 47, and the maximum was 364.

described in the previous section, and it is readily revealed in the *cry2* monogenic mutant grown under white light or blue-plus-red light (Fig. 4C,D). The red-light-independent function of cry2 is to mediate a direct blue-light promotion of floral initiation, but this function of cry2 is carried out redundantly by cry1 (Fig. 5) such that delayed flowering under blue light was only observed in the *cry2/cry1* double mutant plants (Fig. 6C,D). Despite the different modes of actions, both red-light-dependent and red-light-independent functions of cry2 result in the same biological consequence – the blue-light-dependent promotion of floral initiation. It should be pointed out that the *cry2/cry1* double mutant grown in continuous red light developed a slightly larger number of rosette leaves (approx. 20-25%) than the wild type (Fig. 7B), although the double mutant flowered at about the same time as the wild type (Fig. 7A). The cause of this deviation from the generally observed correlation between flowering time and leaf number (Koornneef et al., 1991) remains to be examined.

To investigate the genetic mechanisms of blue-light-dependent promotion of floral initiation, we examined the flowering response of various *Arabidopsis* mutants under different wavelengths of light (Fig. 7). Most of the non-photoreceptor flowering time mutants analyzed (*fd, fe, fy, fpa, fve, fca, fwa, elf3, co, gi*) showed altered flowering-time under all the light conditions tested (Fig. 7A,B). This is consistent with the suggestion that the corresponding genes may function in either the signaling of both phytochromes and cryptochromes (Zagotta et al., 1996; Koornneef et al., 1998b) or pathways not directly associated with day-length perception (Koornneef et al., 1998b). However, some of these mutations (*fd, fe, fpa, fve, fca*) appeared to have a more severe flowering-time defect in red light than in blue light, whereas others (*co, gi*) exhibited more pronounced late-flowering in blue light than in red light (Fig. 7A,B). For example, in red light, the flowering time of *co* and *gi* was only slightly delayed, except for one of the *co* alleles that showed no alteration (Fig. 7, Red). This could be due to the flowering-promotion activities (or the expressions) of *CO* and *GI* being suppressed by red light, possibly mediated by phyB (Fig. 5), so that mutations in either gene may result in little alteration of flowering time under red light. This interpretation is consistent with the result of a previous study of the *co/phyB* double mutant, which demonstrated that CO was required for the phyB function and that phyB might suppress the CO activity (Putterill et al., 1995). On the other hand, the fact that *co* and *gi* mutants still showed some degree of late flowering in red light suggests that the function of CO and GI may also be controlled by phytochromes other than phyB (Halliday et al., 1994; Koornneef et al., 1998a). Under continuous blue light, all three *gi* alleles (*gi-1* and *gi-2* in Columbia background, *gi-3* in L*er* background) and two *co* alleles (*co-1* and *co-2* in L*er* background) flowered significantly (P<0.01) later than the corresponding wild-type plants (Fig. 7). The flowering times of these *gi* and *co* alleles were similar to that of the *cry2/cry1* double mutant lines under blue light (Fig. 7). One interpretation for these observations is that the function (or expression) of CO and GI may be associated with not only the phyB-dependent flowering-time regulation (Putterill et al., 1995; Simon et al., 1996; Guo et al., 1998), but also the phyB-independent regulation of flowering time mediated redundantly by cry1 and cry2 (Fig. 5). This hypothesis, however, remains to be tested directly.

Only the phyB-dependent function of cry2 requires the simultaneous presence of blue light and red light

According to the model depicted in Fig. 5, it is conceivable that the so-called phyB-dependent function of cry2 could be detected only when plants are illuminated simultaneously with red light and blue light, provided a relatively short half-life of the signal generated by the action of either photoreceptor. To test this speculation, we examined the effect of sequential illumination with blue light and red light on the flowering time of different genotypes. In this experiment, plants were first grown under continuous blue light. Samples of various genotypes were then transferred to continuous red light at different times, and allowed to grow in continuous red light until flowering was completed. As demonstrated in Fig. 8, *cry2* mutant plants flowered at about the same time as the wild type regardless of when the plants were transferred from blue light to red light. Thus, the function of cry2 appears to require a

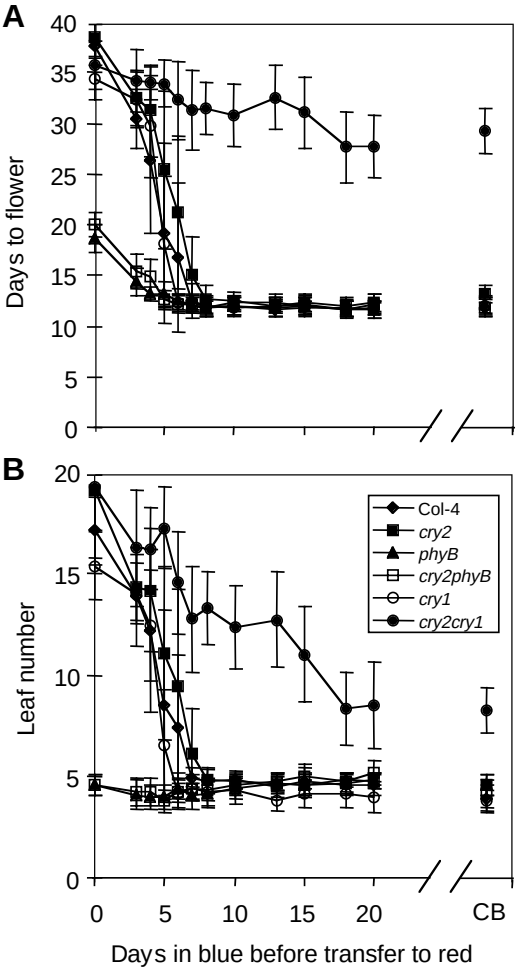

Fig. 8. The flowering time of *Arabidopsis* photoreceptor mutants in response to sequential exposure to blue and red light at different developmental stages. Plants were grown under continuous blue light (~105 μmole/second·m^2) and then transferred to continuous red light (~105 μmole/second·m^2) at the time shown on the abscissa, and allowed to flower. The flowering times (A) and leaf numbers (B) shown are the means of at least 13 plants per sample with standard deviations.

simultaneous presence of red light and blue light. In a separate experiment, we also grew plants first in red light, then transferred them to blue light at different times to grow until flowering (Fig. 9). In this experiment, the *cry2* mutant also showed a profile of flowering time similar to that of the wild type (Fig. 9), indicating again that the phyB-dependent function of cry2 requires the simultaneous presence of red light and blue light.

In contrast to *cry2*, the *cry2/cry1* double mutant showed a very different profile of flowering time (Fig. 8). The *cry2/cry1* double mutant flowered constitutively late, irrespective of when the change of light quality occurred (Figs 8, 9). The constitutive late flowering of the *cry2/cry1* double mutant under conditions of sequential illumination with red light and blue light demonstrated that the double mutant lost its blue light sensitivity with respect to floral induction. This observation is consistent with our model for photoreceptor interactions (Fig. 5). According to the model, both cryptochromes act downstream of the phyB signaling pathway to antagonize the phyB suppression of floral initiation (Fig. 5). It is expected that removal of both antagonistic factors to the phyB function should result in the constitutive late-flowering regardless of when light quality is changed.

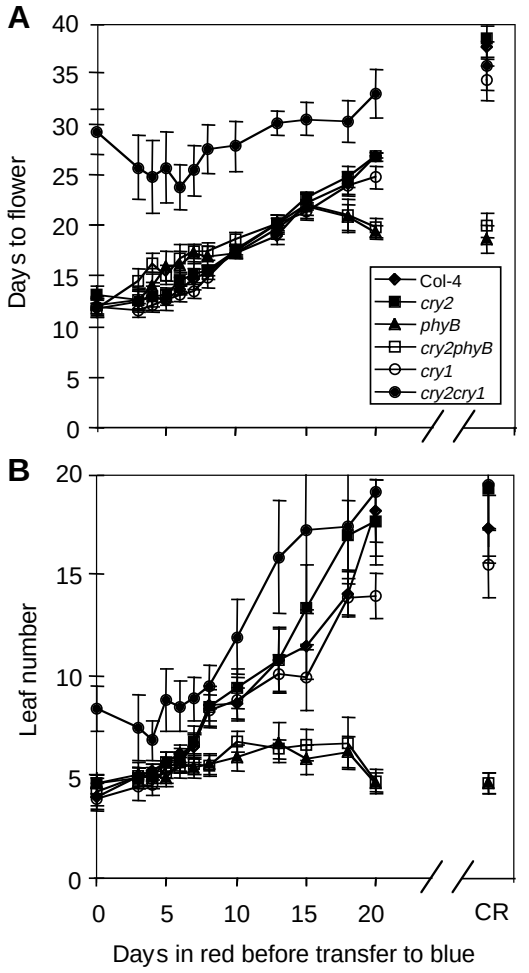

Fig. 9. The flowering time of *Arabidopsis* photoreceptor mutants in response to sequential exposure to red and blue light at different developmental stages. Plants were grown under continuous red light (~105 μmole/second·m^2) and then transferred to continuous blue light (~105 μmole/second·m^2) at the time shown on the abscissa. The flowering times (A) and leaf numbers (B) shown are the means of at least 13 plants per sample with standard deviations.

Developmental mechanisms associated with commitment to floral transition may be direct targets of photoreceptor actions

By treating plants with different wavelengths of light at different developmental stages, and comparing the flowering time of different photoreceptor mutations, we were also able to assess how the actions of photoreceptors affect the developmental transition. An interesting discovery made by this experiment is that there is a developmental stage, referred to as light-quality-sensitive phase, during which the light quality had the most dramatic influence on the flowering time of plants (Fig. 8). Only during the light-quality-sensitive phase, which was between 1-7 days after germination (Fig. 8), did the change of light quality from the flowering-promotive blue light to the flowering-inhibitory red light result in a dramatic delay in the flowering time for wild-type plants. As shown in Fig. 8A, for 7-day-old or younger plants, changing from blue light to red light resulted in a steady gradient of accelerated flowering: the longer the seedlings were kept in blue light, the earlier they flowered. For plants that were older than about 7 days, changing the light source from blue to red had little effect on the flowering time (Fig. 8).

The light-quality-sensitive phase is clearly dependent on the function of both phyB and cry2/cry1. Mutations in either the *PHYB* gene alone or both the *CRY1* and *CRY2* genes appeared to all but eradicate the existence of the light-quality-sensitive phase in floral development (Fig. 8). The *phyB* mutant and *phyB/cry2* double mutant flowered constitutively early regardless of when the plants were transferred from blue light to red light (Fig. 8). Conversely, the *cry2/cry1* double mutant flowered constitutively late; irrespective of the time when the wavelength of light was changed (Fig. 8). In contrast, the flowering onset of the *cry1* and *cry2* monogenic mutants showed a light-quality-sensitive phase almost identical to that of the wild type (Fig. 8), suggesting a redundancy of the two cryptochromes in conditioning the light quality sensitivity of the floral transition process.

Interestingly, the light-quality-sensitive phase is only obvious when plants were grown first in the flowering-stimulatory blue light. A very different profile of the flowering responses was observed if plants were treated first with the flowering-inhibitory red light and then transferred to blue light, although the correlation between flowering time and leaf number for some of the mutant lines was somewhat obscured again in this case (Fig. 9). There was no obvious light-quality-sensitive phase discernable in the wild-type *Arabidopsis* (and mutant lines) under this condition (Fig. 9). The order in which the plants were treated with alternate red light and blue light had such dramatically different effects on the pattern of the flowering time suggested again the different functions of phytochromes and cryptochromes in *Arabidopsis* floral development.

The results shown in Fig. 8 suggest that by about 7 days after germination, plants grown under blue light have been fully committed to floral transition, and the inhibitory environmental factors such as red light could not affect floral initiation any

more. Intriguingly, the commitment time to floral initiation defined by the light-quality-sensitive phase described here appears to coincide with the commitment time estimated by the sequential treatment of plant with LD and SD photoperiods (Bradley et al., 1997). It was found that *Arabidopsis* was most sensitive to the changing of photoperiods, with respect to flowering time, at about days 5-8 after germination in LD photoperiods (Bradley et al., 1997). Day 7 post germination was referred to as the commitment time, in which the apical meristem of *Arabidopsis* is committed to floral development (Bradley et al., 1997). The maximum sensitivities to the quality and quantity of light are both manifested prior to or during the commitment time of *Arabidopsis* plants when the first pair of true leaf is expanded (T. C. M. and C. Lin, unpublished data). Therefore, mechanisms associated with the developmental commitment to floral transition may be direct targets of the actions of photoreceptors.

DISCUSSION

We have investigated the complex relationships of different photoreceptors in the regulation of floral induction in response to the changing light environment. Our results indicate that the blue/UV-A light receptors cry1 and cry2 and the red/far red light receptor phyB function antagonistically in the regulation of flowering time. Specifically, phyB mediates the red light inhibition of floral initiation, whereas cry2 mediates a blue light inhibition of the phyB action. In addition, cry1 and cry2 also mediate a direct blue-light promotion of flowering in a redundant manner that is independent of the phyB function. Consistent with a previously proposed genetic model (reviewed by Koornneef et al., 1998b), the physiological analysis presented here indicates that signal transductions of different photoreceptors may converge to the same downstream factors such as CO, GI or other factors. A test of this hypothesis will require the direct analysis of the expression or activity of the *CO*, *GI*, or other flowering time genes in different genotypes under different light conditions.

The mechanisms underlying photoreceptor-regulated floral induction are almost certain to be more complicated than we have demonstrated. For example, the recent studies of phyD and phyE indicated that these two photoreceptors may participate in the regulation of flowering time in a way similar to that of phyB (Aukerman et al., 1997; Devlin et al., 1998). It remains to be examined whether phyB, phyD and phyE have the same mode of action with respect to flowering-time regulation, and whether the same downstream factor, i.e. CO, is involved in the signaling of multiple phytochromes in different photoperiods. Furthermore, the exact function of cry1 in the regulation of flowering time is not very clear. It has been reported that *hy4* alleles impaired in the *CRY1* gene flowered late in SD photoperiods and that this phenotype of *hy4* mutants could be phenocopied in the wild type by night breaks with blue light (Bagnall et al., 1996; Mozley and Thomas, 1998). We call this function of cry1 a cry2-independent function to distinguish it from the one revealed here by the delayed flowering of the *cry2/cry1* double mutant under continuous blue light. The function of cry1 in flower development is further complicated by the inconsistency of this cry2-independent late flowering phenotype of *cry1* (*hy4*) mutants in SD photoperiods. For example, the *hy4* allele in the L*er* ecotype background was found to flower normally or later in SD in different reports (Goto et al., 1991; Bagnall et al., 1996; Mozley and Thomas, 1998). The *hy4* alleles in other ecotype backgrounds such as Columbia or Wassilewskija (Ws) also displayed similar inconsistency in flowering time; they flowered earlier, later, or at the same time as the corresponding wild type (Bagnall et al., 1996; Zagotta et al., 1996) (T. C. M. and C. Lin, unpublished results). These discrepancies may be the consequences of different experimental conditions, although it is more likely to indicate that the mechanisms underlying the action of cryptochromes in floral induction are of additional complexity.

Individual photoreceptors function in response to only a specific portion of the visible spectrum, whereas the spectrum of light changes little throughout the year. It is unclear how, in nature, the actions of different photoreceptors affect changes of flowering time in response to different photoperiods. To start addressing this question, we analyzed how light quality affects the induction of floral transition. Our results suggest that the actions of photoreceptors may target the commitment mechanism. Although the molecular mechanism determining the commitment time is not clear, it may be related to the time when genes essential for floral transition start to accumulate. For example, the promoter activity of a floral meristem identity gene *LFY* could be detected in 4-day-old seedlings (Blazquez et al., 1997), and the expression of *LFY* is dependent on the function of flowering time gene *CO* (Simon et al., 1996). It remains to be tested directly whether the expression of flowering-time genes and/or floral meristem identity genes could be inhibited by the action of phyB and stimulated by the action of cry1 and cry2. However, it has been suggested that photoreceptors may influence photoperiodic flowering by the regulation of circadian rhythm (reviewed in Thomas and Vince-Prue, 1997). This may be the case for phyA, phyB and cry1, because all three photoreceptors have been demonstrated to mediate light-regulated rhythmic responses in *Arabidopsis* (Somers et al., 1998). Recently, cryptochromes have also been isolated from animals including *Drosophila*, mouse, and human; and this type of photoreceptor has been shown to regulate the entrainment of the circadian clock in animals (Hsu et al., 1996; Emery et al., 1998; Stanewsky et al., 1998; Thresher et al., 1998). Surprisingly, the *Arabidopsis cry2* mutant had no significant defect in circadian period length control, which suggested that the function of cry2 may be more likely involved in the gating mechanism of the clock than in the entrainment of the clock (Somers et al., 1998). Apparently, exactly how cry2 regulates photoperiodic flowering remains to be elucidated.

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