The Role of Cryptochrome 2 in Flowering in Arabidopsis

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We have investigated the genetic interactions between cry2 and the various flowering pathways in relation to the regulation of flowering by photoperiod and vernalization. For this, we combined three alleles of CRY2, the wild-type CRY2-Landsberg erecta (Ler), a cry2 loss-of-function null allele, and the gain-of-function CRY2-Cape Verde Islands (Cvi), with mutants representing the various photoreceptors and flowering pathways. The analysis of CRY2 alleles combined with photoreceptor mutants showed that CRY2-Cvi could compensate the loss of phyA and cry1, also indicating that cry2 does not require functional phyA or cry1. The analysis of mutants of the photoperiod pathway showed epistasis of co and gi to the CRY2 alleles, indicating that cry2 needs the product of CO and GI genes to promote flowering. All double mutants of this pathway showed a photoperiod response very much reduced compared with Ler. In contrast, mutations in the autonomous pathway genes were additive to the CRY2 alleles, partially overcoming the effects of CRY2-Cvi and restoring day length responsiveness. The three CRY2 alleles were day length sensitive when combined with FRI-Sf2 and/or FLC-Sf2 genes, which could be reverted when the delay of flowering caused by FRI-Sf2 and FLC-Sf2 alleles was removed by vernalization. In addition, we looked at the expression of FLC and CRY2 genes and showed that CRY2 is negatively regulated by FLC. These results indicate an interaction between the photoperiod and the FLC-dependent pathways upstream to the common downstream targets of both pathways, SOC1 and FT.

The mechanisms that control the timing of floral initiation have been studied extensively in Arabidopsis by the isolation and characterization of mono- genic mutants and by the analysis of “natural variants” that flower earlier or later than the wild type (WT; for review, see Martinez-Zapater et al., 1994; Koornneef et al., 1998b; Simpson et al., 1999; Mouradov et al., 2002). The genetic control of the transition to flowering in Arabidopsis has been shown to be complex, as indicated by the large number of genes known to affect this process. These allelic variants have been classified physiologically on the basis of their responsiveness to environmental factors such as day length, light quality, and vernalization. Based on this phenotypic analyses and the genetic epistasis among these mutations, flowering time genes have been grouped into several signal transduction pathways. These transmit either the developmental or environmental signals that regulate the expression of the floral meristem identity genes controlling the formation of the floral meristems (Simpson et al., 1999; Mouradov et al., 2002). The models for initiation of flowering that have been established include a photoperiod promotion pathway that promotes flowering under long-day (LD) conditions, an autonomous promotion pathway that promotes flowering independently of the effect of photoperiod, and a vernalization promotion pathway that promotes flowering at low temperatures.

The photoperiod promotion pathway (Simpson et al., 1999; Mouradov et al., 2002), also called the LD promotion pathway (Koornneef et al., 1998b), relates photoperiodic timing signals to the floral initiation process. Mutations in genes in this pathway reduce the responsiveness to photoperiod and delay the flowering of Arabidopsis plants grown in LD but do not substantially alter the flowering time of plants grown in short days (SDs; Koornneef et al., 1991). Mutations in genes such as CONSTANS (CO; Putterill et al., 1995), GIANTANIA (GI; Fowler et al., 1999), FT (Kardailsky et al., 1999; Kobayashi et al., 1999), PHMA (PHOTOCROME A; Johnson et al., 1994), and CRY2 (CRYPTOCROME 2; Guo et al., 1998) belong to this class. Cryptochromes (cry1 and cry2) are blue light (BL) photoreceptors, and it has been suggested that cry2 is the predominant photoreceptor in perception of the LD photoperiod signal in the control of flowering (Guo et al.,...
1998). This is based on the observation that cry2 mutants (and the allelic photoperiod-insensitive flowering time mutants fha1) flower significantly later than WT in LD but not in SD (Koornneef et al., 1991; Guo et al., 1998). A lesser role for cry1 has been proposed on evidence that some cry1 = hyd4 mutants are only slightly late flowering in SD and extended LD (Mozley and Thomas, 1995; Bagnall et al., 1996). In addition to induced mutants, a naturally occurring allele of CRY2 also has been identified in an accession from Cape Verde Islands (Cvi). Plants carrying this variant (originally designated EDI for Early Day length Insensitive) in a Landsberg erecta (Ler) genetic background flower early in both LD and SD and become day length insensitive (Alonso-Blanco et al., 1998). Molecular analyses of the CRY2-Cvi allele have shown that it is a gain-of-function allele, which in SD conditions maintains a high level of CRY2 protein for a longer time after the onset of the light period than the CRY2-Ler allele (El-Assal et al., 2001; Mockler et al., 2003). This protein stability in SD correlates with early flowering in SD and, therefore, day length insensitivity. In addition to the crys, other photoreceptors also play a role in the control of flowering. Mutants deficient in the far-red light sensor phytochrome A (phyA) are late flowering under certain LD conditions and, thus, resemble mutants of the photoperiod promotion pathway (Johnson et al., 1994). In contrast, a deficiency in the major red light sensor phytochrome B (phyB) leads to early flowering, indicating that phyB inhibits flowering (Goto et al., 1991). However, because phyB mutants are still delayed in flowering by SD (Koornneef et al., 1995), phyB does not appear to act specifically in day length perception.

Mutations affecting autonomous pathway genes such as FCA (Macknight et al., 1997), FVE (Koornneef et al., 1991), FPA (Schomburg et al., 2001), and LD (Lee et al., 1994a) delay flowering irrespective of photoperiod (Koornneef et al., 1991, 1998b). It is thought that the autonomous pathway promotes flowering by reducing the expression of the FLC gene, which encodes a repressor of flowering (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999).

Vernalization, appears to act together with the autonomous pathway to repress FLC expression (Michaels and Amasino, 1999; Sheldon et al., 1999). The FRI gene confers a vernalization requirement in many naturally occurring late-flowering accessions by increasing the level of FLC expression (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). Ultimately, the photoperiod promotion pathway and the FLC-mediated pathways seem to converge to control the expression of a limited number of target genes, which include FT (Kardailsky et al., 1999) and SOC1 (Hepworth et al., 2002; Samach et al., 2000). Ultimately, these genes converge to up-regulate the floral meristem identity genes such as LEAFY and APETALA1 (Mouradov et al., 2002).

In addition to the genes described above, several other less well-characterized loci are associated with flowering, including EFS (EARLY FLOWERING IN SHORT DAYS; Soppe et al., 1999), EBS (EARLY BOLTING IN SHORT DAYS; Gomez-Mena et al., 2001), and VIP4 (VERNALIZATION INDEPENDENCE 4; Zhang and van Nocker, 2002). Mutations in these genes accelerate flowering, mainly under SD photoperiods, but they also participate in other processes. EFS is involved in the autonomous promotion pathway (Soppe et al., 1999), EBS appears to regulate FT expression (Gomez-Mena et al., 2001), and VIP4 may be involved in the vernalization pathway (Zhang and van Nocker, 2002). Because mutants in GA biosynthesis (e.g. GAI) and response (e.g. GAI) genes are also required for flowering under SD (Wilson et al., 1992), the existence of an additional GA pathway also has been proposed.

The current models of flowering induction constitute an appropriate framework for the analysis of flowering at the level of mRNA and protein of the genes involved. However, the models are still far from complete and many questions remain, including the interaction of pathways at levels upstream of the common target genes such as SOC1 and FT. An indication that the photoperiod pathway may depend on the FLC-mediated pathways comes from the observation that in plants of the Cvi accession, despite that they carry the CRY2-Cvi allele, day length sensitivity is present, and this is only reduced compared with Ler. It was shown that this is genetically due to the presence of Cvi alleles at two other loci identified on chromosome 5, called FLF and FLG, of which FLF most likely is FLC (Alonso-Blanco et al., 1998). These results suggest that the presence of an active FLC-mediated pathway partly restores the photoperiod response in lines with the CRY2-Cvi allele. It has been shown that CRY2 affects the expression of FT but not of CO (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). In addition, it has been proposed that day length regulation of flowering is at least partly determined by the external coincidence of light, perceived by the two photoreceptors cry2 and phyA, and the day length circadian expression of CO. This coincidence will only be achieved in LD but not in SD photoperiods and would lead, by an unknown mechanism, to the activation of CO function and the induction of FT expression (Yanovsky and Kay, 2002, 2003). Furthermore, the lability of the phyA and cry2 proteins in SD (El-Assal et al., 2001; Mockler et al., 2003) and the activation of cry2 by phosphorylation (Shalitin et al., 2002) add other levels of complexity to the regulation of flowering by photoperiod.

Thus far, no interaction between the photoperiod and the FLC-mediated pathways has been proposed except at the level of their downstream targets FT
and SOC1 (Samach et al., 2000). In this work, we aim to further investigate the role of cry2 in flowering by analyzing the genetic interactions between CRY2 alleles and genes involved in the various flowering pathways. For that, the three different CRY2 alleles currently available were used; the WT Ler allele, the fha1-1 mutant, which is an artificially induced null mutant of the CRY2 gene (Koornneef et al., 1991; Guo et al., 1998) and is called cry2 hereafter, and the EDI = CRY2-Cvi allele, which is the naturally occurring variant of CRY2 present in the accession Cvi (Alonso-Blanco et al., 1998; El-Assal et al., 2001). A set of double and triple mutants involving these CRY2 alleles and mutations in 15 other loci were obtained and analyzed in an Ler genetic background. We have studied the effect of the various mutations representing the different flowering pathways in the three CRY2 genetic backgrounds and in different photoperiod conditions. In addition, the response to a vernalization treatment was tested in a limited set of these genotypes. Furthermore, we analyzed the transcriptional expression of CO and FLC genes, known to be controlled by environmental factors, and of SOC1, whose expression is regulated by CO and FLC. These analyses provide new insights on the function of CRY2 in the regulation of flowering induction and especially in the control of flowering by photoperiod.

RESULTS

To understand the role of cry2 in flowering and day length perception, we investigated the way in which the effect of allelic differences for CRY2 depended on other flowering genes and vice versa. To do this, a number of different triple and double “mutants” were constructed, and their flowering phenotypes were analyzed under different photoperiod and light quality and vernalization conditions.

Interactions between CRY2 and Other Photoreceptor Genes

Interaction between CRY1 and CRY2 Photoreceptor Genes

As shown in Figure 1, the cry2 null mutant flowers later than Ler WT plants under extended LD, standard LD (greenhouse), and SD conditions, whereas plants carrying the gain-of-function allele CRY2-Cvi flower much earlier than Ler under all three photoperiodic conditions and are essentially day length insensitive. In contrast, loss of the cry1 photoreceptor in the cry1 null mutant had little effect under any of the photoperiodic conditions, in agreement with previous observations (Bagnall et al., 1996). However, the cry1 cry2 double mutants flowered slightly later than cry2 in LD conditions, indicating some functional redundancy of cry1 and cry2 in promoting flowering time. However, the monogenic CRY2-Cvi line and the cry1 CRY2-Cvi line flowered with about the same number of leaves and are very early both in SD and LD (Fig. 1), indicating that in this gain-of-function cry2 background, cry1 has no detectable effect.

Interaction between the CRY2 and PHYA Genes

As shown in Figure 1, flowering of the Arabidopsis phyA mutant occurs at almost the same time as WT in SD but is delayed under LD conditions. Extended LD conditions delay flowering of phyA mutants more than the standard LD provided by white light in the greenhouse as shown before by Johnson et al. (1994) and Mockler et al. (2003).

In a cry2 mutant background, the phyA mutation showed an additive effect in all three photoperiodic conditions (Fig. 1). However, the CRY2-Cvi phyA line flowered almost at the same time as the monogenic CRY2-Cvi line, suggesting that although phyA promotes flowering in LD, this photoreceptor is not essential for cry2 function.

We also analyzed triple mutants for cry1, cry2, and phyA. In standard LD conditions, the triple mutant cry1 cry2 phyA flowered later than the monogenic parent lines and also later than the cry2 phyA and cry1 phyA double mutants. In contrast, the CRY2-Cvi phyA cry1 line flowered much earlier than the phyA cry1 double mutant and similarly to the CRY2-Cvi phyA and CRY2-Cvi lines. These results show that the more active CRY2-Cvi allele can fully compensate for the loss of phyA and cry1 in LD conditions and further support the conclusion that the CRY2 gene does not require functional phyA for its effect on flowering.
Interaction between the **CRY2** and **PHYB** Genes

As shown in Figure 1, the **PHYB** gene inhibits floral initiation because phyB-deficient mutants flower earlier than the WT, an effect that is especially significant in SD conditions (Goto et al., 1991; Bagnall et al., 1996; Weller et al., 1997). Compared with the phyB and cry2 single mutants, the cry2 phyB double mutant showed an intermediate flowering time in LD. However, in SD conditions, the cry2 phyB double mutants flowered at about the same time as the phyB single mutant and significantly earlier than WT. The CRY2-Cvi phyB line was significantly earlier than the phyB mutant and even earlier than the CRY2-Cvi line in all three photoperiodic conditions. These results confirm that the day length response is not controlled exclusively through phyB because the phyB-deficient mutant still shows a photoperiod response. The early flowering phenotype of phyB mutant is essentially epistatic to cry2 in SD, whereas in LD, the cry2 mutation has a clear effect in a phyB background, suggesting that the flowering promotive effect of cry2 does not depend solely on phyB.

Interactions between CRY2 and Genes of the Photoperiod Promotion Pathway

To study the genetic interactions between CRY2 and genes of the so-called photoperiod promotion pathway to which CRY2 is assigned, a number of genotype combinations between the two CRY2 alleles with opposing effects (CRY2-Cvi and cry2) and mutants of the CO, GI, FWA, FT, and EBS genes were made and analyzed (Fig. 2). Interestingly, when the CRY2-Cvi allele was combined with the co and gi mutants, these genotypes flowered late and practically with the same number of leaves as the monogenic co and gi mutants in both LD and SD conditions. In addition, the cry2 gi and cry2 co double mutants were only slightly later than the monogenic gi and co mutants (Fig. 2). These results showed that the co and gi mutants are essentially fully epistatic to CRY2. This conclusion is consistent with the observation that the double mutants CRY2-Cvi 35S::CO and cry2 35S::CO flowered as early as the monogenic 35S::CO line. This genetic interaction implies that cry2 requires the product of CO and GI genes to promote flowering and, therefore, acts upstream of CO and GI in the photoperiod promotion pathway.

The double mutants CRY2-Cvi fwa and CRY2-Cvi ft flowered with a TLN intermediate between CRY2-Cvi and the two monogenic fwa and ft mutants. In addition, the double mutants cry2 fwa and cry2 ft flowered somewhat later than the monogenic parents, showing that FWA and FT are not direct regulatory targets for cry2 or at least not the only target. In addition, the additive effects of CRY2-Cvi and ebs and the intermediate phenotype of the cry2 ebs double mutant suggests an independent action of CRY2 and EBS (Fig. 2A). All double mutants showed a photoperiod response very much reduced compared with Ler and similar to the reduced response of the parental monogenic mutants (Fig. 2A).

Because the promoting effect of cry2 on flowering depends on CO, it was important to analyze if this interaction occurs through transcriptional activation of CO by cry2, as suggested by Guo et al. (1998). Taking into account the circadian rhythm reported for this gene (Harmer et al., 2000; Suarez-Lopez et al., 2001), 1-week-old Ler and CRY2-Cvi plants were grown under extended LD and SD conditions and sampled at 2-h intervals for the analysis of CO and CRY2 transcript abundance by quantitative real-time PCR (see “Materials and Methods”). Expression of SOC1 was also examined because this gene is known to be regulated by CO. As shown in Figure 3, all three genes showed diurnal variation in expression, similar to previous descriptions (Harmer et al., 2000; Suarez-Lopez et al., 2001; Töth et al., 2001). However, in both LD and SD, the transcript levels of all three genes showed a peak at the beginning of the light period and a subsequent rapid decline, reaching a minimum 6 to 8 h after the onset of light. After this point, the expression of CO increased rapidly in both LD and SD conditions. In contrast, CRY2 and SOC1 expression increased only slightly in LD and did not change in SD. No major differences in the transcript level of CO, CRY2, and SOC1 were observed between Ler and CRY2-Cvi at 2 h after lights on. However, quantitative differences were found at several other time points. In most cases, plants carrying CRY2-Cvi alleles appeared showing higher levels of the gene expressions (Fig. 3). The largest difference between Ler and CRY2-Cvi was seen for CRY2 in SD, where CRY2 expression was 4-fold higher in Ler than in CRY2-Cvi at the beginning of the light period. After lights were switched on, CRY2 expression in Ler decreased slowly over the first 6 h, whereas in CRY2-Cvi, it rose to a sharp peak at 2 h before dropping rapidly to a low level by 6 h (Fig. 3). Previous analyses of the same samples for CRY2 protein abundance showed a difference between CRY2-Ler and CRY2-Cvi only in SD (El-Assal et al., 2001), which, thus, might be partly determined by CRY2 transcriptional regulation.

Interactions between CRY2 and the Autonomous Promotion Pathway Genes

The late-flowering genes LD, FPA, FVE, and FCA are presumed to promote flowering under both LD and SD and, therefore, have been placed in the so-called constitutive or autonomous promotion pathway. To study the interactions between CRY2 and genes in the autonomous pathway, we constructed and analyzed combinations of CRY2 alleles with the ld, fpa, fve, and fca mutants (Fig. 2B). All combinations with the CRY2-Cvi allele showed flowering times intermediate between those of the corresponding sin-
gle mutants both in extended LD and in SD, indicating an additive effect of this CRY2 allele with the autonomous pathway genes. A similar behavior was seen in lines combining CRY2-Cvi and the early flowering efs mutant. These results support the previous conclusions about the relationship between cry2 and the autonomous promotion pathway (Koornneef et al., 1998a). The results also show that in contrast to mutations in the photoperiod pathway, mutations in the autonomous pathway genes can partially overcome the effects of CRY2-Cvi and restore day length responsiveness. This is especially the case for fca, which is the latest mutant of this group (Fig. 2B). The only exception is the ld mutant, which in combination with the CRY2-Cvi allele flowers very early in SD.

Interaction between CRY2 and the Vernalization Promotion Pathway Genes

To study the genetic interactions between CRY2 and the vernalization pathway, we analyzed the effects of different CRY2 alleles in genetic backgrounds.
with high FLC expression due to the presence of active FLC-Sf2 and/or FRI-Sf2 alleles. The various genotypes were grown in extended LD and SD and without or with a vernalization treatment of 2 or 5 weeks.

As shown in Figure 4, double mutants involving the CRY2 alleles (CRY2-Cvi or cry2) and FLC-Sf2 or FRI-Sf2 all showed an intermediate leaf number between the monogenic parental lines in LD and SD (Fig. 4, g and h), indicating that CRY2-Cvi and cry2 are additive with FRI-Sf2 and FLC-Sf2. However, as previously described by Lee et al. (1994a), the genotype FRI-Sf2 FLC-Sf2 flowers much later than the parental monogenic genotypes, indicating a synergistic interaction between FRI-Sf2 and FLC-Sf2. Interestingly, the lines CRY2-Cvi FLC-Sf2 FRI-Sf2 and cry2 FLC-Sf2 FRI-Sf2 flowered at about the same time as the FLC-Sf2 FRI-Sf2 in both extended LD and SD conditions, indicating epistasis of active FLC and FRI genes to CRY2 alleles. Furthermore, all these genotypes were day length sensitive, except the CRY2-Cvi-containing lines carrying FLC-Ler and FRI-Ler alleles, unless the delay of flowering caused by active FRI-Sf2 and FLC-Sf2 alleles was reduced by a 5-week vernalization treatment (Fig. 4, g and h). These results suggest that the effect of cry2 is not expressed when both FLC-Sf2 and FRI-Sf2 are present, and this prompted us to look at the expression of FLC and CRY2 genes in this series of genotypes (Fig. 4, a, b, e, and f). We also examined the expression levels of the
downstream gene \textit{SOC1} (Fig. 4, c and d) whose transcription has been shown to be negatively regulated by \textit{FLC} (Samach et al., 2000; Michaels and Amasino, 2001). The mRNA abundance of these genes was determined by quantitative RT-PCR (see “Materials and Methods”) at a single time point 2 h after dawn.

**Figure 4.** \textit{FLC}, \textit{SOC1}, and \textit{CRY2} mRNA expression and the TLN in genotypes involving different alleles of the vernalization genes \textit{FRI}, \textit{FLC}, and \textit{CRY2}. The symbols \textit{FRI} and \textit{FLC} refer to the active \textit{FRI-Sf-2} and \textit{FLC-Sf2} alleles that were used as described in experimental procedures. Plants were grown in extended LD (a, c, e, and g) and SD (b, d, f, and h) conditions. a and b, \textit{FLC} mRNA expression; c and d, \textit{SOC1} mRNA expression; e and f, \textit{CRY2} mRNA expression; g and h, TLN. White bars, Plants without vernalization; gray bars, plants with 2 weeks of vernalization; black bars, plants with 5 weeks of vernalization treatment. mRNA amounts are estimated by quantitative RT-PCR from samples of 3-week-old seedlings collected 2 h after lights switch on. Expressions are presented as the relative percentage from the highest level of each gene in each photoperiod condition. Bars = mean of two RT-PCR sample replicates and SE. TLNs are provided as the mean and SE of 18 plants.
These results indicate that increased strongly when vernalization was applied. FRI-Sf2 when both LCRY2 produced, especially in genotypes containing WT expression in LD than SD conditions. However, the genicant effect of day length, with higher level of expression (Fig. 4, c and d) and in agreement with previous mach et al., 2000; Michaels and Amasino, 2001) but controlled not only by the FLC-mediated pathway (Samach et al., 2000; Michaels and Amasino, 2001) but also by photoperiod independently of cry2. The level of CRY2 mRNA (Fig. 5) was similar and substantially not affected by day length in genotypes where FLC mRNA was practically absent (i.e. in CRY2-Cvi, Ler, and cry2) in agreement with our previous observations (El-Assal et al., 2001). However, in the presence of either FLC-Sf2 or FRI-Sf2, CRY2 expression is reduced, especially in genotypes containing WT CRY2-Ler alleles. CRY2 mRNA levels were even lower when both FRI-Sf2 and FLC-Sf2 were present but increased strongly when vernalization was applied. These results indicate that CRY2 expression is neg-

The expression of SOC1 was reduced by the presence of FLC-Sf2 or FRI-Sf2 and increased by vernalization in a manner opposite to that of FLC expression (Fig. 4, c and d) and in agreement with previous reports (Samach et al., 2000; Michaels and Amasino, 2001). In addition, SOC1 expression showed a significant effect of day length, with higher level of expression in LD than SD conditions. However, the genotype at CRY2 had no effect on SOC1 transcript levels. These results suggest that SOC1 expression is controlled not only by the FLC-mediated pathway (Samach et al., 2000; Michaels and Amasino, 2001) but also by photoperiod independently of cry2. The level of CRY2 mRNA (Fig. 5) was similar and substantially not affected by day length in genotypes where FLC mRNA was practically absent (i.e. in CRY2-Cvi, Ler, and cry2) in agreement with our previous observations (El-Assal et al., 2001). However, in the presence of either FLC-Sf2 or FRI-Sf2, CRY2 expression is reduced, especially in genotypes containing WT CRY2-Ler alleles. CRY2 mRNA levels were even lower when both FRI-Sf2 and FLC-Sf2 were present but increased strongly when vernalization was applied. These results indicate that CRY2 expression is neg-

Figure 5. FLC and CRY2 mRNA expression in some representative genotypes of each flowering promotion pathway. Plants were grown in extended LD (white bars) and SD (gray bars) conditions. mRNA amounts are estimated by quantitative RT-PCR from samples of 3-week-old seedlings collected 2 h after lights switch on. Expressions are presented as the relative percentage from the highest amount of each gene for each gene; therefore, only samples for the same gene are directly comparable.

Figure 6. CRY2 protein expression in some representative genotypes of the autonomous and vernalization pathways. Total proteins of 3-week-old plants collected 2 h after lights were on by western blotting using the anti-CRY2 antibody. a, Plants grown in LD after a 2 weeks of vernalization treatment (mRNA expression data in Fig. 4); b, plants grown in SD photoperiod without vernalization (mRNA expression data in Fig. 5).

Figure 6. CRY2 protein expression in some representative genotypes of the autonomous and vernalization pathways. Total proteins of 3-week-old plants collected 2 h after lights were on by western blotting using the anti-CRY2 antibody. a, Plants grown in LD after a 2 weeks of vernalization treatment (mRNA expression data in Fig. 4); b, plants grown in SD photoperiod without vernalization (mRNA expression data in Fig. 5).

To further confirm this negative regulation of CRY2 gene expression by FLC, we also looked by quantitative RT-PCR at the expression level of both genes in genetic backgrounds carrying the mutant alleles with the strongest effects representing the autonomous and the photoperiod flowering promotion pathways, i.e. co and fca (Fig. 5). We looked at these expressions in LD and SD photoperiods at 2 h after dawn. Genotypes carrying the fca mutation showed high expression of FLC, whereas genotypes in a co mutant background did not differ from Ler, confirming previous observations that mutations in the autonomous pathway genes increase FLC transcript level (Sheldon et al., 1999; Michaels and Amasino, 2001). In addition, the level of CRY2 expression was also strongly reduced in the fca mutant background but not in the co mutant background (Fig. 5).

To examine whether these differences in CRY2 mRNA were reflected in CRY2 protein abundance, 3-week old plants of selected genotypes with high FLC and low CRY2 mRNA were grown under SD conditions, and the amount of CRY2 protein was analyzed by western blotting. As shown in Figure 6, a significantly lower amount of CRY2 was only detected in genotypes FRI-Sf2 FLC-Sf2, which have very high levels of FLC mRNA and very low levels of CRY2 mRNA. The amount of CRY2 protein in genotypes with intermediate FLC expression (such as those carrying fca or either FRI-Sf2 or FLC-Sf2) did not differ significantly from the amount in genotypes

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with low FLC expression, indicating that differences in mRNA levels might not be fully reflected in protein abundance. However, it is possible that differences in CRY2 protein level might be more pronounced at earlier stages of development as previously shown for Ler and CRY2-Cvi genotypes, which strongly differ in the amount of CRY2 only in 1-week-old seedlings but not at later times (El-Assal et al., 2001).

Taken together, these analyses indicate a negative relationship between the levels of FLC expression and the levels of SOC1 and CRY2 that also correlates with the photoperiod response. The negative regulation of CRY2 and SOC1 by FLC might occur directly or indirectly via other genes regulated by FLC. We have analyzed the expression of CO in the same plant materials to see if CO expression is controlled by FLC or CRY2. Interestingly, we found no correlation between the levels of CO and FLC expression (data not shown). CO expression at this sample time depended on the photoperiod but showed no correlation with flowering time or vernalization response, as previously reported by Suarez-Lopez et al. (2001).

**DISCUSSION**

In this work, we have investigated the genetic interactions between the BL photoreceptor cry2 and genes in the various floral induction pathways in relation to the regulation of flowering by photoperiod and vernalization. For this, we combined the three available alleles of CRY2, the WT CRY2-Ler allele, the loss-of-function null allele cry2, and the gain-of-function CRY2-Cvi, with different mutants representing the various photoreceptors and flowering pathways.

The flowering analysis of various CRY2 and CRY1 genotypes in LD and SD conditions has shown that cry1 can play a minor role in promoting flowering in the absence of cry2 (Bagnall et al., 1996; Mockler et al., 1999), but this is not seen when WT cry2 is present. In the presence of the gain-of-function CRY2-Cvi allele, no effect of the absence of cry1 is observed.

As reported by Johnson et al. (1994) and Mockler et al. (2003), our analysis also showed that the Arabidopsis phyA mutant flowers almost at the same time as WT in SD, but LD conditions failed in promoting its flowering. The CRY2-Cvi allele was able to fully compensate for the loss of phyA and cry1 in LD conditions. Furthermore, the additive effects of the mutations in the various double and triple mutants of CRY2 and PHYA in LD conditions suggested that although phyA is important for the promotion of flowering in LD conditions, the effect of cry2 does not depend on phyA.

The earliness of the phyB-deficient mutants described before by Goto et al. (1991) implies that the phyB inhibits flowering but does not control the photoperiod response exclusively because SD still delays flowering in the phyB mutant. However, it is likely that phyD and phyE are responsible for this residual effect because plants impaired in both PHYB and the PHYD genes flowered earlier than the phyB monogenic mutation in both LD and SD conditions (Aukerman et al., 1997), and the phyB phyE double mutants flowered earlier than the phyB mutant in SD conditions (Devlin et al., 1998).

The present work shows that the cry2 phyB double mutants were almost insensitive to photoperiod. The delay of the phyB mutant in the cry2 background and its earlier flowering in CRY2-Cvi background in LD indicates that the phyB mutation is not fully epistatic to CRY2. Therefore, the flowering promotion of cry2 does not depend only on its inhibitory effect on the phyB-dependent inhibition as was also proposed by Mockler et al. (2003). However, it cannot be excluded that phyD and phyE acting in a partially redundant manner with phyB might be responsible for a residual effect of cry2. Furthermore, an effect of phyB via the autonomous pathway is suggested by the epistasis of fca to phyB (Koornneef et al., 1995). Because this epistasis of fca is much stronger in SD, it appears that the flowering inhibitory effect of phyB via the autonomous pathway is much more important in SD, probably because no suppression of the phyB inhibition by cry2, which is rapidly degraded in SD (El-Assal et al., 2001), can take place.

The flowering time analysis of combinations of CRY2 alleles with the photoperiod promotion pathway mutants co and gi showed that in LD and SD conditions, the co and gi mutants are completely epistatic to all CRY2 variants. Moreover, the double mutants CRY2-Cvi 35S::CO and cry2 35S::CO flowered as early as the monogenic line 35S::CO, in agreement with the epistatic relationship described above.

The additive phenotype of the double mutants between CRY2 alleles and the photoperiod promotion pathway-related genes (FT and FWA) indicates that FT or FWA are not controlled exclusively by cry2 and also that cry2 function does not depend only on these genes. That FT is not an exclusive target of CO was also suggested by Samach et al. (2000). In addition, the independent effect of CRY2 and the EBS gene supported the independence of the CRY2 and FT genes because Gomez-Mena et al. (2001) have shown that the EBS gene mediates the repression of flowering through FT, probably independently from the CO gene. Recently, it has been shown that in phyB mutants, the FT gene is up-regulated (Cerdan and Chory, 2003; Halliday et al., 2003). Although the effect on FT might be via CO, Cerdan and Chory (2003) provided evidence for a “light quality” pathway independent of CO via the PFT1 gene. However, it is unknown whether this FT activation might be related to CRY2 activity.

The molecular and genetic analysis of the FLC gene, which is a common target of the autonomous...
and the vernalization pathways, provide important results concerning the control of the photoperiod response and its relationship with the FLC-mediated pathways. We observed that an active FLC allele such as FLC-Sf2, which is characterized by higher expression than the FLC-ler allele, restores photoperiod sensitivity of CRY2-Cvi genotypes in an Ler background. This is in agreement with the previous analysis of the Ler/Cvi RILs in which lines carrying CRY2-Cvi, FLC-Cvi (which most likely is an active allele of FLC), and FLG-Cvi respond to photoperiod (Alonso-Blanco et al., 1998). Therefore, plants with increased levels of FLC respond more to photoperiod, i.e. FLC increases photoperiod sensitivity, which can be reverted by FLC down-regulation through vernalization (Fig. 7). Several arguments support this observation, such as the fact that CRY2-Cvi combined with mutations in the autonomous pathway, which are known to increase the FLC mRNA levels (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999), recover photoperiod sensitivity (present work) and the fact that mutants deficient in the photoperiod response pathway like co and gi respond considerably to photoperiod when combined with mutations in the autonomous pathway, especially with fca (Koornneef et al., 1998a). This effect of FLC on photoperiod response might be through its effects on downstream integrator genes such as FT and SOC1 or by interacting with a central regulator of the photoperiod pathway such as CO. Interactions between pathways may occur by the regulation of gene transcription of one pathway by the other pathway (by changing either its amount or its cyclic pattern) or by posttranscriptional regulation affecting, for instance, protein stability. Our results indicate that FLC affects the transcriptional regulation of CRY2 by either reducing its transcription or changing the time of its highest expression during the day. In genetic backgrounds with high FLC expression (such as FLC-Sf2, FRI-Sf2, or a mutation in the autonomous pathway), there are reduced levels of CRY2 mRNA. Therefore, the specific features of the CRY2-Cvi allele that causes reduction of photoperiod sensitivity by increasing its effect in SD because of its higher protein stability becomes less relevant. In addition, we do not know if FLC also might affect photoperiod response through the regulation of other genes.

The observation that at low levels of cry2 (in FLC-expressing lines and in the cry2 mutant background) a photoperiod effect is observed suggests that LDs are not exclusively perceived by cry2. Interestingly, Suárez-Lopez et al. (2001) proposed that CO mediates between the circadian oscillator and the activation of the flowering time gene FT, supporting the hypothesis that CO mediates the day length perception from different sources parallel to cry2. Also, it has been shown previously by Samach et al. (2000) that CO and FLC control the transcription of SOC1 and FT, FLC being able to bind directly to the SOC1 promoter (Hepworth et al., 2002). Thus, it has been suggested that the expression level of SOC1 and FT might be determined by a balance of CO and FLC activity. This is in agreement with our observation that SOC1 transcription is altered by photoperiod independently of the CRY2 genotype, indicating that another sensor affects also SOC1 transcription and, as suggested above, might be through CO. A candidate for this is phyA, which we found to act independently of cry2. It has been shown that the expression of both cry2 and phyA changes in response to photoperiod (El-Assal et al., 2001; Mockler et al., 2003) and that both photoreceptors are necessary for the CO-mediated induction of FT expression that occurs under LD conditions (Yanovsky and Kay, 2002).

In Figure 8A, schematic representation of the different effects of the various flowering pathways, deduced from previous observations and from the genetic interactions between CRY2 alleles and the flowering promotion pathways described in this work, is shown. This scheme indicates that cry2 is affecting flowering through its effect on CO (which acts downstream of GI) probably by affecting its activity (Suárez-Lopez et al., 2001; Yanovsky and Kay, 2003) and not by promoting CO transcript expression. On the other hand, cry2 is also postulated to participate in removing a phyB-induced flowering inhibitor (Mockler et al., 1999). In view of this model, the autonomous and the photoperiod pathways are suggested to regulate photoperiod sensitivity and response. The photoperiod pathway is speculated to promote flowering mainly in LDs with the participation of CRY2, PHYA, GI, and CO genes, whereas the autonomous pathway is speculated to affect photo-
Figure 8. A model scheme of the interactions among the various components of the flowering promotion pathway. The flowering pathways are shown in boxes. →, Promotive effect; ↓, Repressive effect.

period response by its partial repression, mainly in SDs controlled by phyB and involving FCA and FLC genes. Cross regulation between both pathways is becoming clear as illustrated by the repression of the phyB flowering inhibition by cry2 and, reciprocally, with the repression of CRY2 transcription by FLC. Furthermore, integrators of both mechanisms must involve the circadian clock, which might regulate the differential expression of both pathways in different photoperiods. A detailed analysis of the expression of FLC and target genes such as SOC1, FT, and LEY and several other genes involved in photoperiod sensitivity such as LHY, CCA, and CO in genotypes specifically constructed for this purpose might shed more light on these complex interactions of flowering pathways.

MATERIALS AND METHODS

Construction of Genotypes

The following mutant alleles, all in the Lr genetic background, were used: co-3, gr-3, ft-1, fua-1, fca-1, fve-1, fja-1 (Koornneef et al., 1991); ld-1 mutation introgressed in Lr (Koornneef et al., 1994); and the early flowering mutants 35S::CO (Simon et al., 1996), dfb (Soppe et al., 1999), and cbs (Gomez-Mena et al., 2001). The photoreceptor null mutants hyd-1 = cry2-1 (formerly called hyd-2.23N; Koornneef et al., 1980; Ahmad and Cashmore, 1993), fha1-1 = cry2 (Koornneef et al., 1991; Guo et al., 1998), phyA-201 (formerly fve-1; Nagatani et al., 1993), and hyg3-1 = phyB-1 (former isolation number Bo64; Koornneef et al., 1980; Reed et al., 1993). The line with the CRY2-Cvi allele used carries about 7 cM of the top of chromosome 1 from Cvi introgressed into an Lr genetic background (El-Assal et al., 2001). This line is characterized by the first siliques often showing three ovaries, which resembles a weak phenotype of clevata mutants, and is probably due to a Cvi allele at a closely linked locus to CRY2 because transgenic plants containing the CRY2-Cvi alleles do not show this phenotype (El-Assal et al., 2001). Two introgression lines containing the FRI-Sf2 or FLC-Sf2 alleles from the accession L. sativum Feliu-2 introgressed in Lr (Lee et al., 1994b) were used as active FRI and FLC alleles.

Double mutants between CRY2-Cvi or cry2 (fha1-1) and the above-listed alleles were preliminarily selected from the F2 progenies derived from crosses between the single mutants. The selection of plants carrying the CRY2-Cvi alleles was assisted by the described fruit phenotype of the edl line. For the isolation of combinations of CRI2 alleles with other photoreceptor mutants, the selection was performed by growing F3 lines in various broad wavelength color cabinets (Weller et al., 2000) and selecting the lines that were tall in BL for cry1, in red light for phyB, or in far-red light for phyA mutants, and, thus, homozygous lines for the corresponding photoreceptor mutant alleles were obtained. The allele at the CRY2 locus of all the genotypes used was finally identified by PCR using two derived Cleaved Amplified Polymorphic Sequence markers specifically designed for the CRY2-Cvi and fha1-1 alleles (Neff et al., 1998). Basically, DNA was isolated from a few leaves of the candidate lines following the protocol of Bernatzky and Tanksley (1986), and the CRY2 gene was amplified by PCR using either the fha1-1 or the CRY2-Cvi primers depending on the genotypes. The amplified DNA was then cleaved with the corresponding restriction enzyme, and the DNA fragments were separated in 2% (w/v) metaphore agarose gel (BMA, Germany) soaked with water in plastic petri dishes, and stored in a climate room for 3 d for germination (25°C, 16 h of light for extended LD experiments; 25°C, 8 h of light for SD experiments). Thereafter, seedlings were planted in soil.

Three kinds of photoperiodic light conditions were used: SD, extended LD (both performed in growth chambers), and standard LD (performed in greenhouses). SD and extended LD experiments were carried out in similar growth chambers, whereby the light treatment provided by fluorescent tubes was the same for the first 8 h in both treatments. However, LD was extended for 8 h with four incandescent lamps alone as a source of low-fluence rate light at the end of the main photoperiod (Koornneef et al., 1995). Ten plants per two 10- × 10-cm pots were used for each genotype/treatment combination. Individual pots were randomized and grown in extended LD and SD cabinets.

For standard LD conditions plants were grown in an air-conditioned greenhouse supplemented with additional light from the middle of September until the beginning of April, providing a day length of at least 14 h. Day temperature was 22°C to 25°C and night temperature 16°C to 19°C. Two groups of 12 plants were grown in single pots per genotype in a row. Each experiment, plants were grown in two blocks, the genotypes being randomized within the blocks.

For vernalization treatments, seeds were surface sterilized with 20% (v/v) bleach (4% [w/v] hypochloride) in 96% (v/v) ethanol and rinsed twice with ethanol. After drying, they were sown on Murashige and Skoog agar medium containing 2% (w/v) Suc and stored in darkness at 4°C for 2 or 5 weeks before planting in the climate chambers described above.

Measurement of Flowering

Flowering initiation was measured as TLN because this trait and the time (number of days) from sowing until flowering (flowering time) are tightly correlated traits (Koornneef et al., 1991). The final number of rosette and cauline leaves in the main inflorescence (not including leaves on axillary inflorescences) was counted on the day that the first petals became visible.
Analysis of Gene Expression by Real-Time Quantitative RT-PCR (QPCR)

RNA for quantitative RT-PCR analysis was isolated from 1- or 3-week-old plants with the Rnasea plant mini kit (Qiagen, Chatsworth, CA). Total RNA was resuspended in Dnase buffer and treated with Rnase-free Dnase (Life Technologies/Gibco-BRL, Cleveland). For first strand cDNA synthesis, 5 μg of total RNA was used, and cDNA synthesis was primed by using the standard d12, α adapter primer (Life Technologies/Gibco-BRL) and reverse transcribed with Moloney murine leukemia virus (Life Technologies/Gibco-BRL). Thereafter, the cDNAs were diluted to 200 μL with water, and a 5-μl aliquot was quantitatively analyzed for the expression of each gene by the fluorogenic 5‘-nuclease PCR assay (Livak et al., 1995). Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) during 40 cycles. Specific primers and probes for different flowering time genes were designed by Primer Express software (Perkin-Elmer Applied Biosystems), to avoid detecting homologous genes. Primer sequences used for the CR2 gene were: forward, 5‘-TGCGCGGTGTAGTGC-CAAT-3‘; reverse, 5‘-TACCGGTCAGTGTTCTCAATCT-3‘; and probe, 5‘-CAATGCTGCCACCTGGACCCATTATAT-3‘. Primer sequences used for the CO gene were: forward, 5‘-AACGGCAATAGTGAGGGAGAAGGCA-3‘; reverse, 5‘-GCCAAGCTGTCAATGCAATAA-3‘; and probe, 5‘-AGGACCTCTGTACACATTGCGGT-3‘. Primer sequences used for the SOC1 gene were: forward, 5‘-AAATATGAGGAGCAAAAATGATGA-3‘; reverse, 5‘-TTTCTTCAGCGTTCGTCACATCT-3‘; and probe, 5‘-AAGGTTCTATAACGTTAACCTTGGAGAGGAGAACA-3‘. Primers and probes labeled with 6-FAM were manufactured by Isogen Bioscience (XXX, XX). Each PCR analysis was performed twice on one sample of cDNA, and the mean and se of both measurements were estimated.

Cry2 Protein Analysis

Cry2 protein was analyzed by western blot using an anti-CRY2 antibody as described by El-Assal et al. (2001). Total proteins were extracted from duplicated samples collected at the same time as the samples for RNA analyses described above.

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LITERATURE CITED


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CORRECTION


Plant Physiology regrets that due to an editorial error, the last name of the first author was incorrectly indicated in the running head. The correct name is El-Assal.