The Role of Cryptochrome 2 in Flowering in Arabidopsis[1]

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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* (L*er*), 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 L*er*. 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 monogenic 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), *GIGANTEA* (*GI*; Fowler et al., 1999), *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999), *FWA* (Soppe et al., 2000), *PHYA* (*PHYTOCHROME A*; Johnson et al., 1994), and *CRY2* (*CRYPTOCHROME 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.,

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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 = hy4* 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* (L*er*) 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*-L*er* 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 upregulate 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. *GA1*) 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 L*er*. 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 L*er* 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 L*er* 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 L*er* 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 L*er* 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 photoperiod 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.

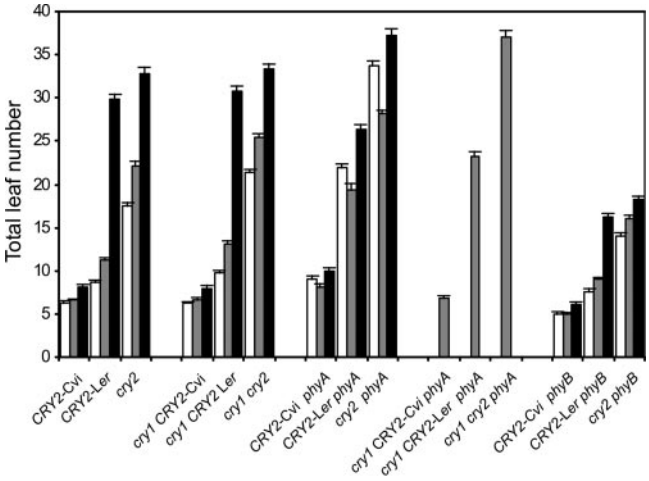

Figure 1. The effect of different day length treatments on the total leaf number (TLN) of single, double, and triple mutants of different photoreceptor genes. Plants were grown in three different photoperiod conditions: extended LD conditions (white bars), standard LD conditions (gray bars), and SD (black bars) climate chamber conditions. Mean and SE of 24 plants are shown.

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 L*er* 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 L*er* 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; Tóth 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 L*er* 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 L*er* and *CRY2*-Cvi was seen for *CRY2* in SD, where *CRY2* expression was 4-fold higher in L*er* than in *CRY2*-Cvi at the beginning of the light period. After lights were switched on, *CRY2* expression in L*er* 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*-L*er* 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-

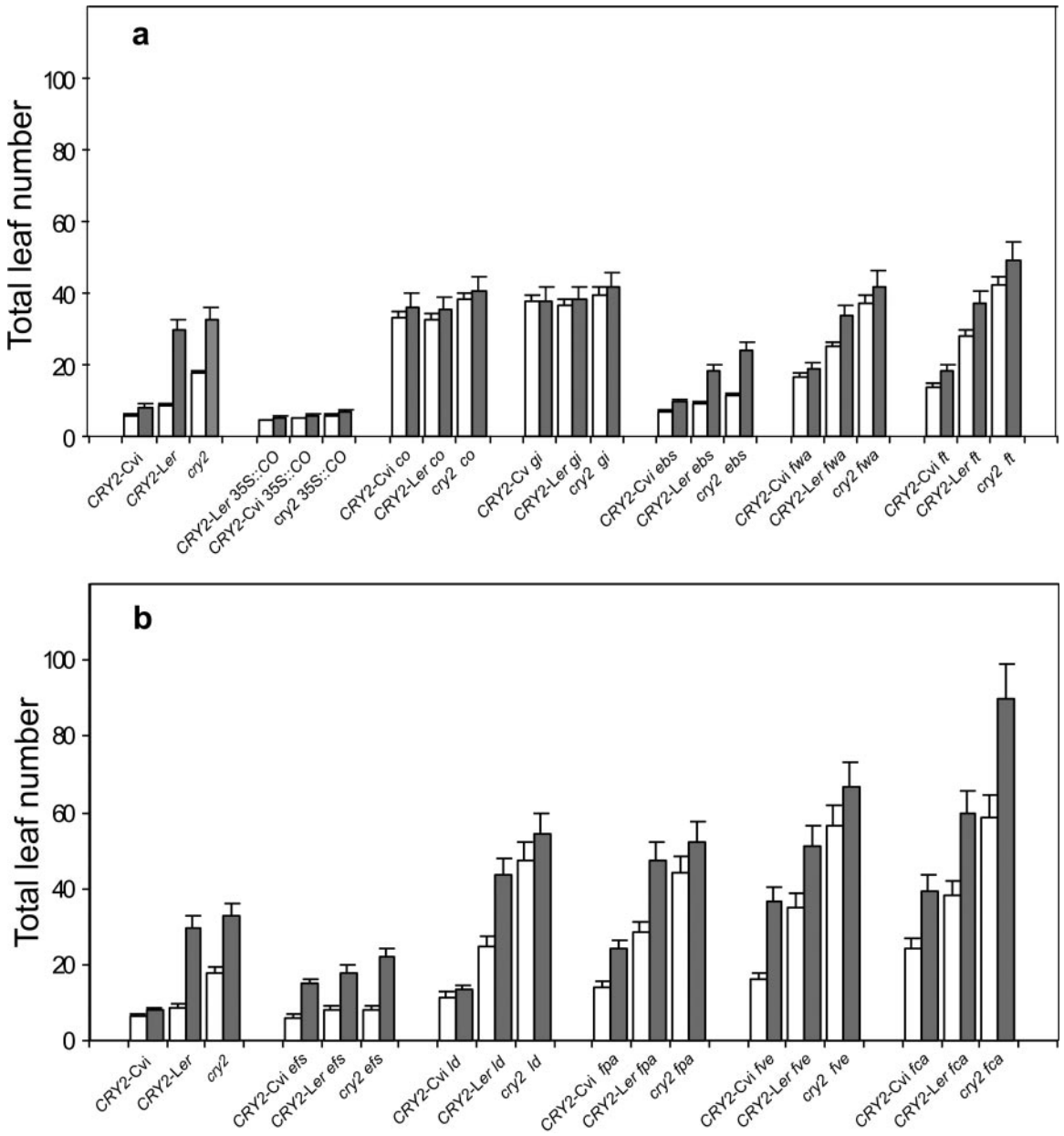

Figure 2. The effect of day length on the TLN of single and double mutants involving genes of the various flowering promotion pathways and *CRY2* alleles. Plants were grown in extended LD (white bars) and SD (gray bars) climate chamber conditions. a, Mutants of the photoperiod promotion pathway; b, mutants of the autonomous promotion pathway. Mean and SE of 24 plants per genotype are shown.

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

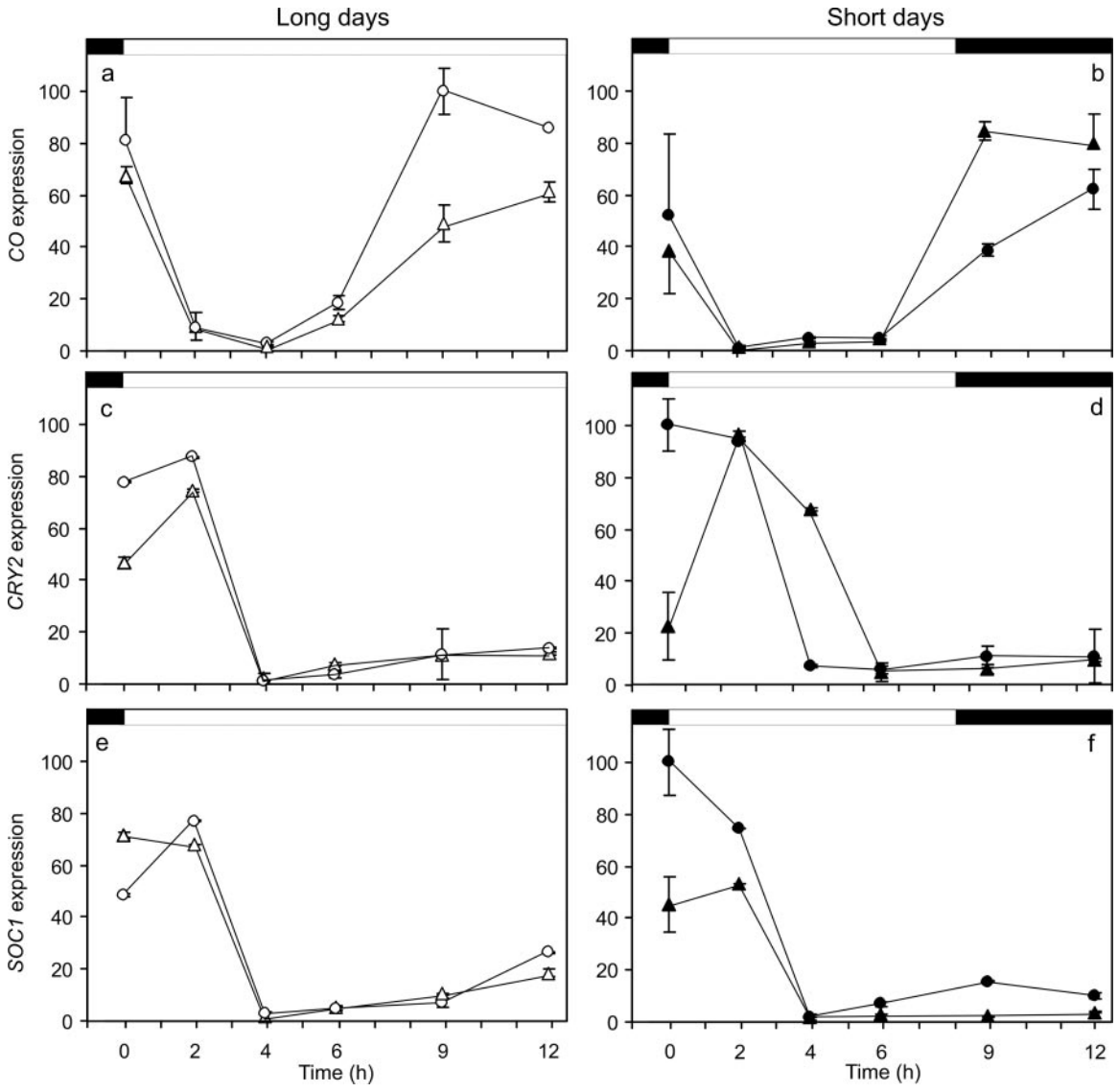

Figure 3. Diurnal mRNA expression of flowering time genes in 7-d-old seedlings of CRY2-L*er* (L*er* WT) and *CRY2*-Cvi. Plants were grown in extended LD (a, c, and d) or in SD (b, d, and f) conditions. a and b, *CO* mRNA; c and d, *CRY2* mRNA; e and f, *SOC1* mRNA. Samples were collected at the times indicated in the horizontal scale. mRNA amounts are estimated by quantitative reverse transcriptase (RT)-PCR and presented as the relative percentage from the highest level of each gene. Triangles, L*er*; circles, *CRY2*-Cvi.

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

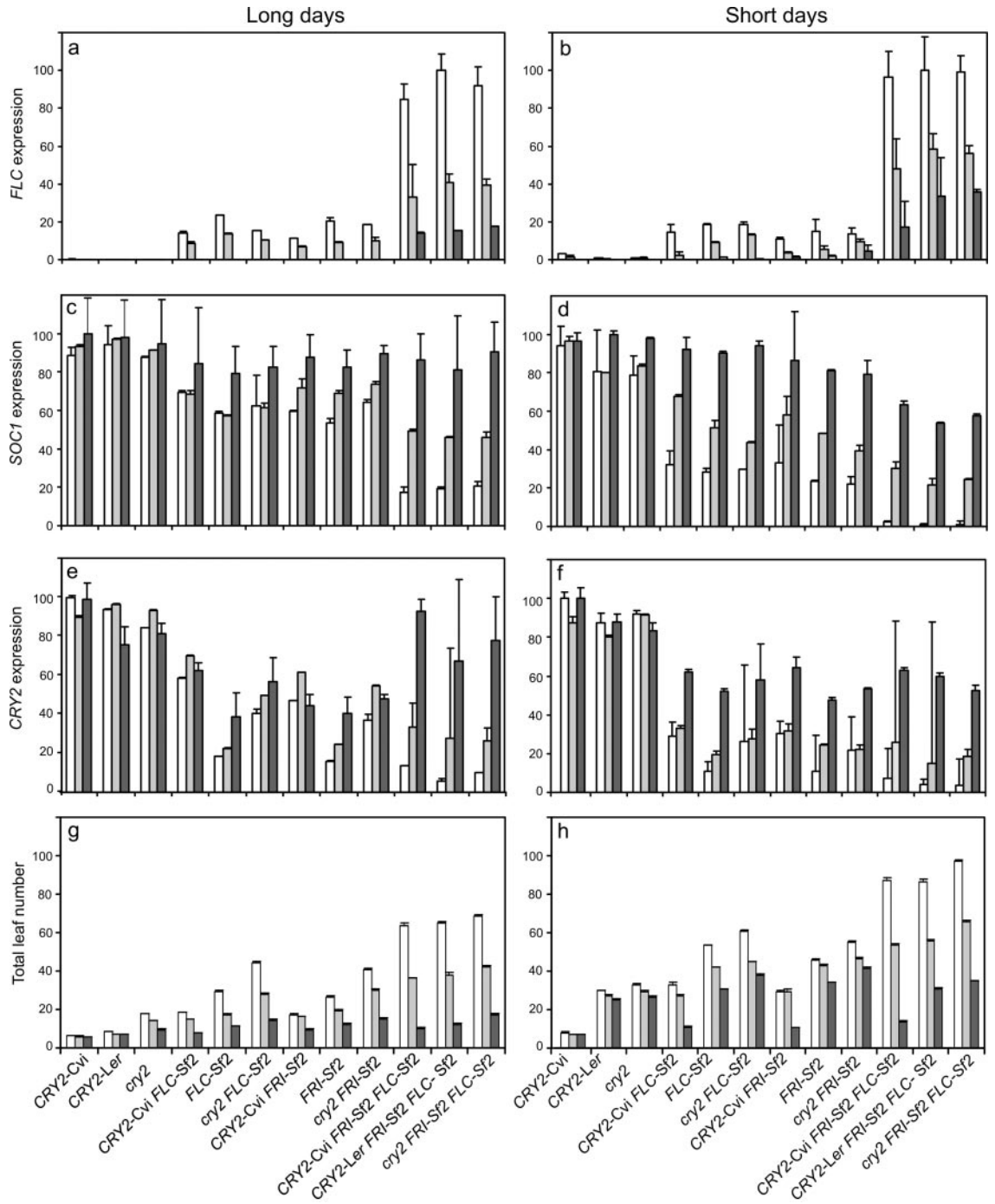

Figure 4. *FLC, SOC1*, and *CRY2* mRNA expression and the TLN in genotypes involving different alleles of the vernalization genes *FRI*, *FLC*, and *CRY2*. The symbols *FRI* and *FLC* refer to the active *FRI*-Sf-2 and *FLC*-Sf2 alleles that were used as described in experimental procedures. Plants were grown in extended LD (a, c, e, and g) and SD (b, d, f, and h) conditions. a and b, *FLC* mRNA expression; c and d, *SOC1* mRNA expression; e and f, *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.

downstream gene *SOC1* (Fig. 4, c and d) whose transcription has been shown to be negatively regulated by *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

that we had determined previously to correspond to the highest mRNA levels of these genes in L*er* and *CRY2*-Cvi (Fig. 3). As previously described (Sheldon et al., 1999; Michaels and Amasino, 2001), we found that lines carrying active *FLC-Sf2* or *FRI-Sf2* alleles show increased level of *FLC* transcript compared with L*er*, with this increase being 5 times higher when both *FRI*-Sf2 and *FLC*-Sf2 alleles were combined (Fig. 4, a and b). However, *FLC* RNA amount was strongly decreased by vernalization in a time-dependent manner. A 5-week treatment was more effective than 2 weeks.

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, L*er*, 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*-L*er* 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 negatively regulated by *FLC* in a manner dependent on the level of *FLC* expression. Minor effects of the *CRY2* genotype on *CRY2* expression were observed in an *FLC-Sf2* or an *FRI-Sf2* background, which must be explained by an additional regulatory mechanism involving cry2 self-regulation or downstream flowering time genes.

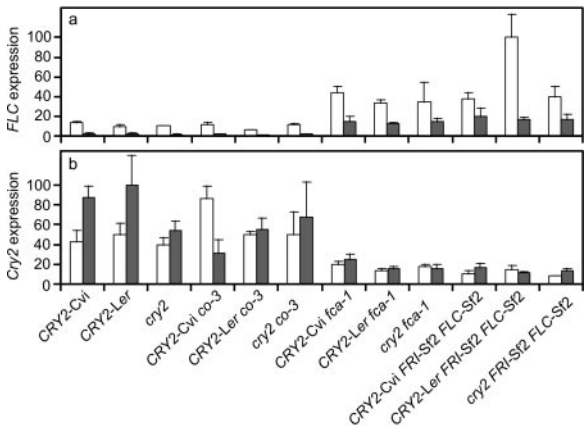

Figure 5. *FLC* and *CRY2* mRNA expression in some representative genotypes of each flowering promotion pathway. Plants were grown in extended LD (white bars) and SD (gray bars) conditions. mRNA amounts are estimated by quantitative RT-PCR from samples of 3-week-old seedlings collected 2 h after lights 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.

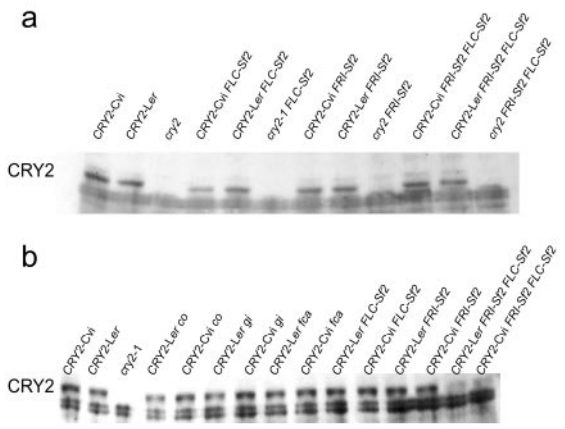

Figure 6. CRY2 protein expression in some representative genotypes of the autonomous and vernalization pathways. Total proteins of 3-week-old plants collected 2 h after lights were on by western blotting using the anti-CRY2 antibody. a, Plants grown in LD after a 2 weeks of vernalization treatment (mRNA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

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 L*er*, 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

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 L*er* 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*-L*er* 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*-L*er* allele, restores photoperiod sensitivity of *CRY2*-Cvi genotypes in an L*er* background. This is in agreement with the previous analysis of the L*er*/Cvi RILs in which lines carrying *CRY2*-Cvi, *FLF*-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.

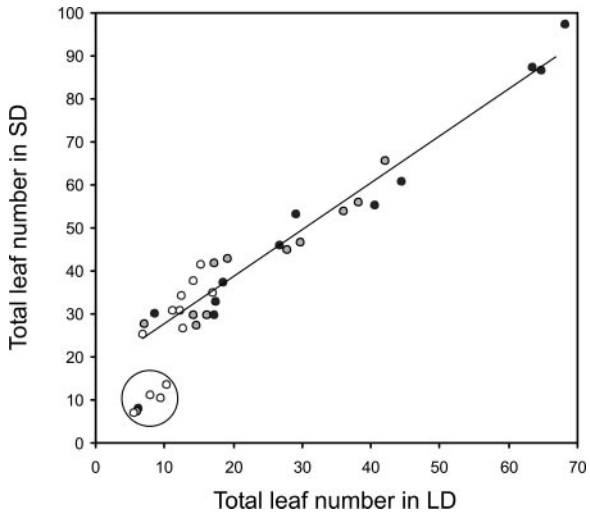

Figure 7. Correlation between the TLN in extended LD and SD conditions of the various genotypes differing for *FLC*, *FRI*, and *CRY2* alleles. Dark-gray circles, Non-vernalized plants; light-gray circles, 2-week vernalized plants; white circles, 5-week vernalized plants. The circle encloses the day length-insensitive genotypes, whereas the regression line is calculated for the other genotypes.

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, Suarez-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 (Suarez-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-

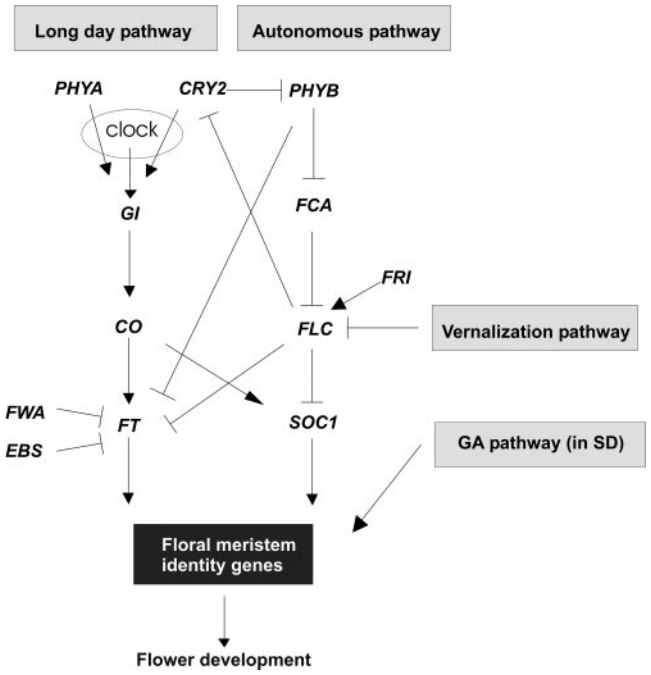

Figure 8. A model scheme of the interactions among the various components of the flowering promotion pathway. The flowering pathways are shown in boxes. →, Promotive effect; ⊥, Repressive effect.

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 *LFY* 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 L*er* genetic background, were used: *co-3*, *gi-3*, *ft-1*, *fwa-1*, *fca-1*, *fve-1*, *fpa-1* (Koornneef et al., 1991); *ld-1* mutation introgressed in L*er* (Koornneef et al., 1994); and the early flowering mutants 35S::*CO* (Simon et al., 1996), *efs* (Soppe et al., 1999), and *ebs* (Gomez-Mena et al., 2001). The photoreceptor null mutants *hy4-1 = cry1-1* (formerly called *hy4-2.23N*; Koornneef et al., 1980; Ahmad and Cashmore, 1993), *fha1-1 = cry2* (Koornneef et al., 1991; Guo et al., 1998), *phyA-201* (formerly *fre-1*; Nagatani et al., 1993), and *hy3-1 = 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 L*er* 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 *clavata* 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 Saint Feliu-2 introgressed in L*er* (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 F_2 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 *EDI* line. For the isolation of combinations of *CRY2* alleles with other photoreceptor mutants, the selection was performed by growing F_3 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 thecorresponding restriction enzyme, and the DNA fragments were separated in 2% (w/v) metaphore agarose gel (BMA, Rockland, ME). For the *CRY2*-Cvi allele marker, the primers used were 5′-CGGGGAAATAAGCGTCAGACACGT-3′ and 5′-CATTTCCATGGAAGGAGAAGAAACTTCC-3′, and the DNA was cleaved with the restriction endonuclease *Bfa*I. For the *fha1-1* marker, the primers used were 5′-GACAGTTTTATCCTGGAAGAGCTTCACCAT-3′ and 5′-GCTTTGCACAGAGATCCCACGTTCC-3′, and the DNA was digested with *Nco*I.

Growth Conditions and Light Treatments

Seeds were sown on filter paper (no. 595, Schleicher & Schull, Dassel, 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 in 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. En 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 Rneasy plant mini kit (Qiagen, Chatsworth, CA). Total RNA was resuspended in DnaseI buffer and treated with Rnase-free DnaseI (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 $dT_{12\text{-}18}$ 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 *CRY2* gene were: forward, 5′-TTGGCGGTTGATGCCAAT-3′; reverse, 5′-TCCAGCCCTAGTTCTTCAATCG-3′; and probe, 5′-CAAATCGCTTCAGCCGCTGCAGT-3′. Primer sequences used for the *CO* gene were: forward, 5′-AACGACATAGGTAGTGGAGAGAACAAC-3′; reverse, 5′-GCAGAATCTGCATGGCAATACA-3′; and probe, 5′-ACGACCCTGTGACACATGCCGGT-3′. Primer sequences used for the *SOC1* gene were: forward, 5′-AAATATGAAGCAGCAAACATGATGA-3′; reverse, 5′-TTTTCTCAAGCTGTTGCTCAATCT-3′; and probe, 5′-AAGCTTCTAAACGTAAACTCTTGGGAGAAGGCA-3′. Primer sequences used for the *FLC* gene were: forward, 5′-ACGCATCCGTCGCTCTTCT-3′; reverse, 5′-GCATGCTGTTTCCCATATCGA-3′; and probe, 5′-TCCGCCTCCGGCAAGCTCTACAG-3′.

The mRNA expression of each gene is given as the relative amount of PCR product. In samples collected at different times, the amount of RNA is presented relative to the highest value of the samples after normalization to PCR product of the constitutively expressed *ACTIN2* gene. In samples collected at a single time point (2 h after light turns on), the amount of a gene for each genotype is presented relative to the value of the highest genotype after normalization to the amount of PCR product of the *ACTIN2* gene. Primers for this internal control were: forward, 5′-GCTGAGAGATTCAGATGCCA-3′; reverse, 5′-GTGGATTCCAGCAGCTTCCAT-3′; and probe, 5′-AAGTCTTGTTCCAGCCCTCGTTTGTGC-3′.

Primers and probes labeled with 3′Tamra/5′6-FAM were manufactured by Isogen Bioscience (XXXX, 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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