Supplemental Information

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Arabidopsis Cryptochrome 2 Undergoes Blue Light-Dependent Interaction with the SPA1-COP1 Complex to Regulate Floral Initiation in Plants

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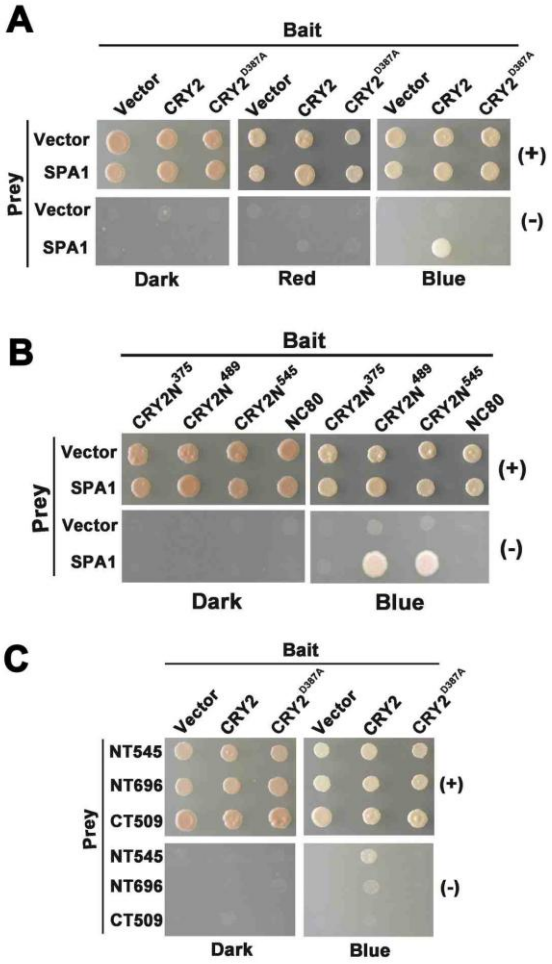

Figure S1 (related to Figure 1). Yeast two-hybrid assay showing the blue light-dependent CRY2-SPA1 interaction

(A) Auxotrophy assays showing the blue light-dependent CRY2-SPA1 interaction. The colonies formation of yeast cells expressing the indicated proteins were grown in the dark, red light (50 $\mu molem^{-2}s^{-1}$), or blue light (50 $\mu molem^{-2}s^{-1}$). (+): yeast cells grown on the medium containing no Trp and Leu. (-): yeast cells grown on the medium containing no Trp, Leu, and His.

(B-C) Auxotrophy assays showing interactions between SPA1 and different domains of CRY2 (A) or CRY2 with different domains of SPA1 (B). Nomenclature of the domains used are described in Fig. 2A. Yeast cells expressing indicated proteins were grown in dark or blue light ($50\mu molm^{-2s-1}$), on the medium described in A.

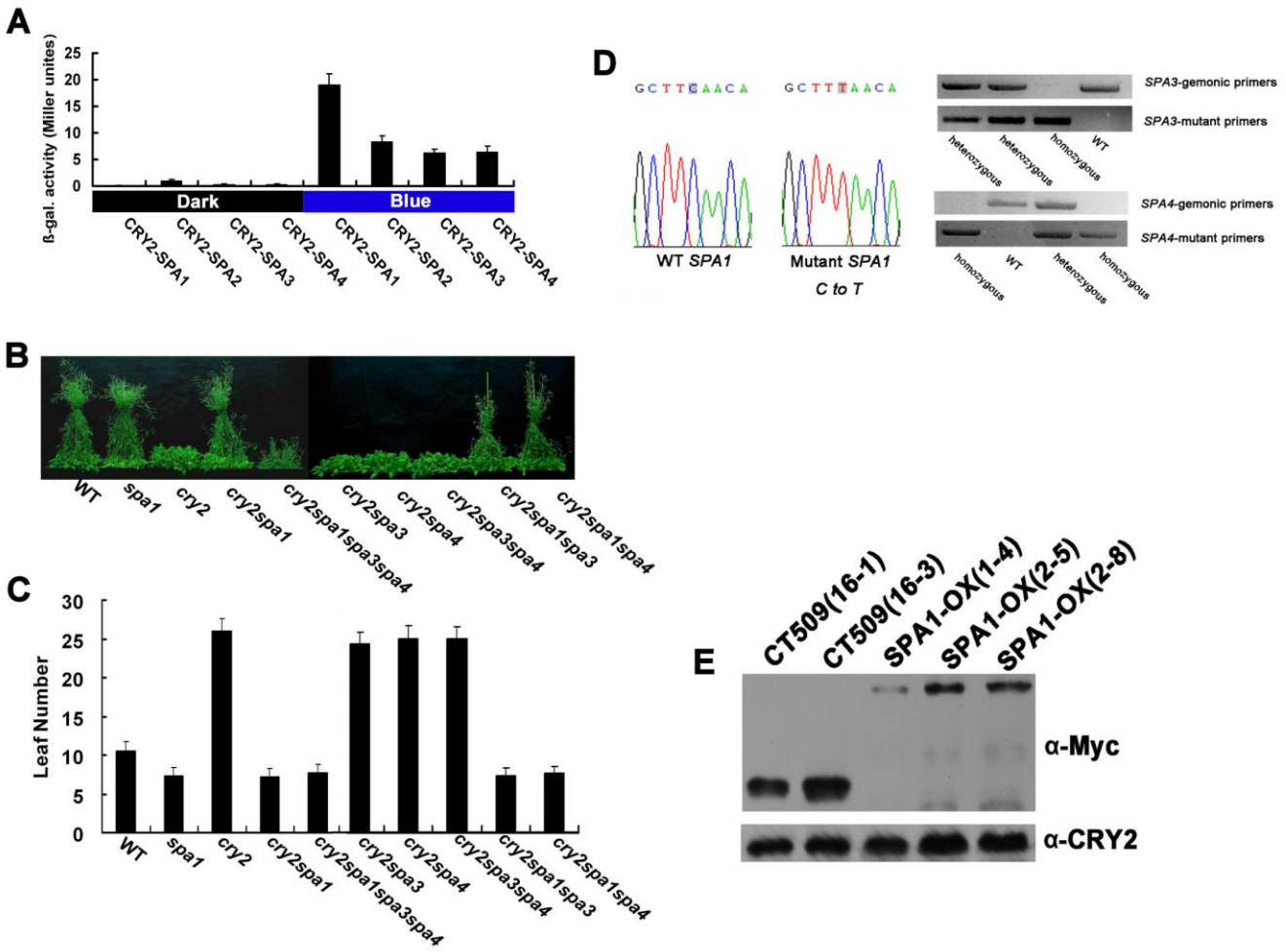

Figure S2 (related to Figure 2). A comparison of the function of the SPA quartet members in the regulation of flowering time

(A) β-galactosidase assay showing interaction of CRY2 with members of the SPA quartet family in the dark (Dark) or blue light (Blue).

(B-C) The *spa1*, but not other *spa* mutants, suppresses the late-flowering phenotype of the *cry2* mutant. The wild type (F1 of RLD x Col), *spa1*, *cry2 (*derived from *cry2* x *spa1), cry2spa1, spa3spa4, cry2spa3, cry2spa4, cry2spa3spa4, cry2spa1spa3,* and *cry2spa1spa4* mutant plants grown in long-day photoperiods (16hL/8hD) for 20 days (B) or until all plants flowered (C). The standard deviations (n>20) are shown. The results are from the same experiment shown in Fig. 3A-B; the data of WT, *spa1*, *cry2*, and *spa1cry2* are shown in Fig. 3A-B, but included here for comparison.

(D) The molecular confirmation of the genotypes used in B-C. The genomic DNAs isolated from wild-type and the indicated mutants plants were amplified by PCR using the indicated

primer pairs followed by DNA sequence analysis (*spa1*) or agarose gel electrophoresis (*spa3* and *4*).

(**E**) Immunoblot showing relative expression level of the CT509 and SPA1 protein in the transgenic lines used. Respective transgenic plants were grown in Long-day photoperiod, the protein extracts were fractionated, blotted, probed with Myc antibody (α-Myc), stripped, and re-probed with the anti-CRY2 antibody (α-CRY2).

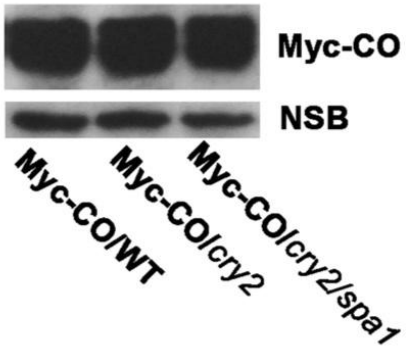

Figure S3 (related to Figure 3). Immunoblot showing the similar level of MycCO protein detected in MycCO/WT, MycCO/*cry2*, or MycCO/*cry2spa1* mutant plants in the light phase (short-day photoperiods, 8hL/16hD). Immunoblots were probed with Myc antibody (Myc-CO). A non-specific band was included as the loading control (NSB).

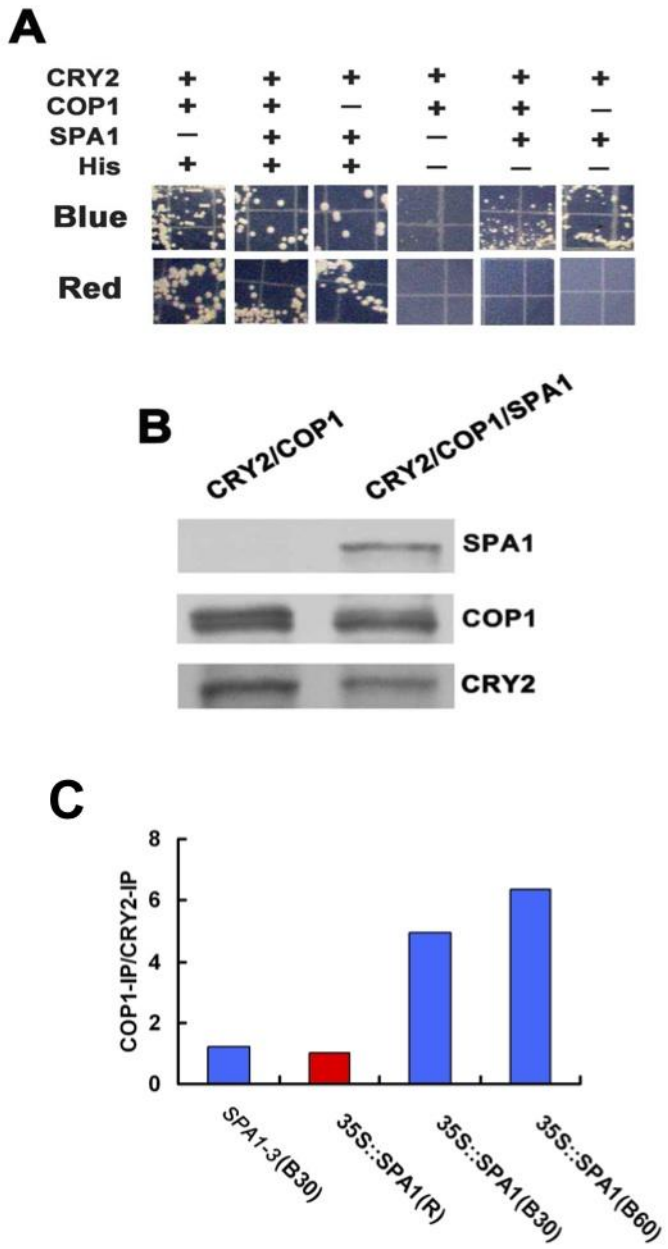

Figure S4 (related to Figure 4). Blue light and SPA1 stimulate the CRY2-COP1 interaction

(A) Auxotrophy assays showing a SPA1-dependent enhancement of the CRY2-COP1 interaction in response to blue light. The colony formation of yeast cells expressing indicated proteins were grown in the dark, red light (50 $\mu molem^{-2}s^{-1}$), or blue light (50 $\mu molem^{-2}s^{-1}$). (His +): yeast grown in the medium containing no Trp, Leu and Met. (His -): yeast grown on the medium containing no Trp, Leu, Met and His.

(B) Immunoblot showing the induction of expression of the bait-mate SPA1 protein in yeast cells cultured in the SD/-Trp/-Leu/-Met/+Asp medium (right), and lack of SPA1 expression in yeast cells grown in SD/-Trp/-Leu/-Asp/+Met medium (left).
(**C**) Quantification of the immunoblot blot shown in Fig. 4C. The relative CRY2-COP1 interaction in different samples was digitized, using Image J, and calculated by the formula [COP1-IP]/[CRY2-IP]. R: red light, B30: 30 min of blue light treatment, B60: 60 min of blue light treatment.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plant materials

spa1-3 (RLD accession), *cry2-1* mutant (Col accession), and transgenic plants expressing the *35S::MycSPA1* or *35S::MycCT509* transgene are reported previously [1-3]. The *cry2spa1* double mutant was prepared by a genetic cross of *cry2* and *spa1*, and its flowering time was compared with the "wild-type" control derived from a cross of the RLD and Col accession. The *spa3spa4, cry2spa3, cry2spa4, cry2spa3spa4, cry2spa1spa3,* and *cry2spa1spa4* mutant plants were prepared by a genetic cross of *cry2* and *spa134.* The genotype of *cry2spa1* was confirmed by DNA sequencing of genomic fragment of the *SPA1*, *SPA3 or SPA4* genes and by immunoblot for the absence of the CRY2 protein**.** Transgenic Arabidopsis expressing the *35S::MycCO* transgene were prepared by floral dipping method in different genetic backgrounds. The plasmid 35S::MycCO was prepared by cloning of the coding region of the CO cDNA into the pEGAD-Myc vector in the EcoRI and SalI sites downstream from the 35S promoter. The pEGAD-Myc vector was described previously [4].

Yeast two-hybrid assays

Yeast two-hybrid experiments use the Matchmaker system according to the manufacturer's instructions (Matchmaker user's manual, Clontech, California). The coding sequences of CRY2, CRY2^{D387A}, and different domains of CRY2 reported previously [5] were fused in-frame with the GAL4 DNA-binding domain (BD) of the bait vector pBridge (Clontech, Cat# 630404). The coding sequences of SPA1 and different domains of SPA1 were fused in-

frame with the GAL4 transcription-activation domain (AD) of the prey vector pGAD (Clontech, Cat# K1612-1)[3]. The bait plasmid and the prey plasmid were co-transformed into the yeast strain Y190.

The protein-protein interaction was analyzed using both the histidine auxotrophy assay and β-galactosidase assay. For the histidine auxotrophy assay, yeast colonies were patched in duplicate onto His- and His+ plates. One duplicate was grown under blue light (25 $\mu molem^{-2}s^{-1}$), at 28°C, for 2-3 days. The second duplicate was wrapped in aluminum foil to block the light, and grown in the same condition. The β-galactosidase analyses were performed according the manufacturer's recommendations (Clontech, yeast hand book, Protocol # PT3024-1, Version # PR742227), using chlorophenol red-β-D-galactopyranoside (CPRG) as the substrate. Light treatment and calculation of the β-galactosidase activity are as described [4].

Yeast three-hybrid assay

The prey vector PGADT7 (Clontech, Cat# K1612-1) expressing the prey-AD protein, and the bait vector pBridge (Clontech, Cat# 630404) expressing the bait-BD and the bait-mate proteins were used in the yeast three-hybrid assays. For the COP1-SPA1 interaction, CRY2 was used as the bait-mate. For the CRY2-COP1 interaction, SPA1 was used as the bait-mate. One pair of plasmids (expressing bait-mate) or the control pair of plasmids (expressing no bait-mate) was co-transformed to yeast strain MAV203 to analyze the effect of the bait-mate. Colonies were selected and cultured at 28^{O}C in the dark, shaking with 180rpm, until they reached OD_{600} of ~0.1 in a 100ml flask containing 40ml of the SD medium (–Leu/-Met/-Trp/+Asp). The yeast culture was then split and cultured at 160rpm/21^{O}C under different light conditions until OD_{600} of ~0.5~0.8. The relative bait-prey interaction was presented as arbitrarily unit (AU), which is calculated by the formula: AU=[miller units (light)]/[miller unit (dark)], with the AU of dark-treated samples set to 1. The standard deviations (n=3) are shown. Three repeats were performed for each experiment.

Immunoblot, Immunostaining, and Co-immunoprecipitation (co-IP)

Immunoblot and immunostaining are as described previously [4, 5]. Sub-cellular localizations of CRY2 and MycSPA1 were analyzed using immunostaining probed with the

CRY2 or Myc antibody. The immunoprecipitation experiments are as previously described with minor modifications [4, 5]. The anti-COP1 antibody was kindly provided by Dr. Xingwang Deng. The primary antibody (anti-CRY2) was coupled to protein A-conjugated Sepharose beads prior to the antibody-antigen interaction, which total incubation/washing time to about two hour. To prepare anti-CRY2-IgG-coupled protein-A Sepharose, 8 μl anti-CRY2 antiserum was incubated with 20 μl protein-A Sepharose beads in a 30 μl NEB buffer (20 mM HEPES, pH 7.5, 40mM KCl, 1mM EDTA), at 4^{O}C for 2hr, and used soon after. Tissues were grounded in liquid nitrogen, homogenized in NEB-T buffer (20 mM HEPES [pH 7.5], 40mM KCl, 1mM EDTA, 1% Triton X-100, 1mM PMSF) buffer, incubated at 4^{O}C for 15 min and centrifuged at 16,000g for 10 min. 1ml supernatant was mixed with 1x protease inhibitor cocktail (Roche), 58μl suspension of the anti-CRY2-IgG-coupled protein-A Sepharose beads, which was prepared prior in the same day, and incubated at 4^{O}C for 60 min. Spin at 1,500 rpm for 20 sec. Beads left in the tubes were washed three times with the wash buffer (20 mM HEPES, pH 7.5, 40mM KCl, 0.1% Triton X-100). The proteins were eluted from the Sepharose beads by mixing with ~1/4 volume of 4xSDS-PAGE sample buffer, boiling for 5min, and spinning at 12000rpm for 5min at room temperature. 10ul supernatant were fractioned by 10%SDS-PAGE, and the immunoblots carried out as previously described, using the antibodies indicated [4, 5].

mRNA expression Analyses

Total RNAs were isolated using the Illustra RNAspin Mini kit (GE healthcare). cDNA was synthesized from 1 μg total RNA using SuperScript first-strand cDNA synthesis system (Invitrogen). Brilliant SYBR Green qPCR Master Mix (Stratagene) or Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) was used for qPCR reaction, using the MX3000 System (Stratagene). The level of *ACTIN* mRNA expression (At3g18780) was used as the internal control. For qPCR analyses of *FT*, cDNA was denatured at 95^{O}C for 10 min, followed by a 60-cycle program (95^{o}C, 30 sec; 58^{o}C, 30 sec; and 72^{o}C, 30 sec per cycle). The qPCR results shown are representatives of the experiments repeated at least three times with similar results.

SUPPLEMENTAL REFERENCES

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