國立臺灣大學生命科學院植物科學研究所

碩士論文

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Master Thesis

阿拉伯芥中 FIN219 與 Cryptochrome 2 在藍光及茉莉酸訊息傳遞途徑中之功能性探討

Functional Studies of FIN219 and Cryptochrome 2 in the Integration of Blue Light and Jasmonate Signaling in *Arabidopsis*

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中文摘要

光和植物荷爾蒙在植物的生長發育調控過程中皆扮演重要角色，植物整合這兩方面的訊息傳遞路徑之機制仍有待探究。阿拉伯芥中的隱花色素 2（Cryptochrome 2（CRY2））為其藍光受體之一，參與藍光下光形態發生和開花時間的調控，藍光活化的 CRY2 將受到 CONSTITUTIVE PHOTOMORPHOGENIC1（COP1）的泛素化標記和分解。Far-red insensitive 219 (FIN219)是參與茉莉酸訊息傳遞中的酵素，能將茉莉酸（Jasmonic Acid（JA））合成為具有生物活性的 Jasmonoyl- isoleucine (JA-Ile)。它亦在光敏素 A 介導的遠紅光訊息傳遞路徑中作為一個正調控因子。前人研究顯示 FIN219 同時參與在 CRY2 相關的藍光訊息傳遞路徑中。大量表現 FIN219 蛋白質的阿拉伯芥幼苗在藍光下呈現較短的下胚軸，而在 CRY2 缺失突變株的背景中大量表現 FIN219 蛋白質時，植株幼苗並不會表現對藍光敏感，且 FIN219 蛋白質的大量表達亦會促進阿拉伯芥提早開花。為了進一步了解 FIN219 與 CRY2 的交互作用對茉莉酸及藍光訊息傳遞的影響，本研究重新確認了 FIN219 與 CRY2 在藍光下存在直接的交互作用，且發現了 FIN219 蛋白質的大量表現可以增加 CRY2 蛋白質在藍光下的穩定性，而外加甲基茉莉酸（MeJA）則會使 CRY2 蛋白質在藍光下更不穩定。另外，發現了 FIN219 與 CRY2 共同調控藍光下阿拉伯芥下胚軸的光形態形成，在有 FIN219 蛋白質存在時，CRY2 蛋白質的存在能抑制外加甲基茉莉酸對下胚軸伸長的抑制。在對於開花時間的調控方面，短日照條件下，大量表現 *FIN219* 基因的阿拉伯芥植株中可以偵測到更多的 *Flowering Locus T* (*FT*)基因。然而，我們仍需要更多的證據來證實 FIN219 在其中所扮演的角色。綜合上述，FIN219 參與在 CRY2 所調控的藍光訊息傳遞路徑中，並促進阿拉伯芥之光形態發生。

關鍵字：FIN219、隱花色素 2、茉莉酸、藍光

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ABSTRACT

Light and plant hormones play important roles in the regulation of plant growth and development. Cryptochrome 2 (CRY2) is one of the blue light (BL) receptors that mediate de-etiolation primarily under weak blue light and photoperiodic control of flowering in *Arabidopsis*. Under BL stimulus, CRY2 is quickly ubiquitinated and degraded by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1). FAR-RED INSENSITIVE219 (FIN219) catalyzes the biosynthesis of Jasmonoyl-isoleucine (JA-Ile), a bio-active form of Jasmonic Acid (JA), and is a positive regulator of phytochrome A (phyA) mediated far-red light signaling. Previous studies showed that FIN219 was also involved in CRY2-mediated blue light signaling. The overexpression of FIN219 would resulted in a hypersensitive phenotype in response to blue light, ectopic expression of *FIN219* in *cry2* mutant did not exhibit obvious phenotype, and overexpression of FIN219 would lead to early flowering phenotype. This study verified the physical interaction between CRY2 and FIN219 protein under blue light, and observed that the CRY2 protein stability decreased with MeJA treatment but increased with the overexpression of FIN219 under blue light. CRY2 and FIN219 functioned together in BL-mediated inhibition of hypocotyl elongation, and CRY2 alleviated the inhibition of hypocotyl elongation caused by MeJA depending on FIN219. In short day (SD) condition, more *Flowering Locus T* (FT) gene was detected in *FIN219* overexpression line. However, more evidence was needed to elucidate the role of FIN219 in CRY2-mediate regulation of flowering. Taken together, FIN219 is involved in CRY2-mediated blue light signaling pathway, and promotes photomorphogenesis in *Arabidopsis.*

Key words: FIN219, Cryptochrome 2, Jasmonic Acid, Blue Light

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ABBREVIATION

aa	Amino Acid
AP2	APETALA2
AVG	Average
BiFC	Bimolecular Fluorescence Complementation
BL	Blue Light
CBP	Chitin binding protein
CCT2	Cryptochrome2 C-terminal
CIB	CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX
CRY	Cryptochrome
CO	CONSTANS
COI1	Coronatine-Insensitive1
Col	Columbia
COP1	CONSTITUTIVELY PHOTOMORPHOGENIC1
Dex	Dexamethasone
E. coli	*Escherichia coli*
FIN219	FAR-RED INSENSITIVE219
FLC	FLOWERING LOCUS C
FT	FLOWERING LOCUS T
FR	Far-Red light
GUS	β-glucuronidase
GST	Glutathione-S-Transferase
HFR1	LONG HYPOCOTYL IN FAR-RED1
HY5	Long Hypocotyl 5
JA	Jasmonic Acid
JA-Ile	(+)-7-iso-jasmonoyl-L-isoleucine
JAR1	JASMONATE RESISTANT1
JAZ	JASMONATE ZIM-domain
LB	Luria-Bertani broth
LD	Long Day
MeJA	Methyl-Jasmonate

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pGR219	pGR::FIN219
PHOT	Phototropin
phyA	Phytochrome A
phyB	Phytochrome B
PIF	PHYTOCHROME INTERACTING FACTOR
qPCR	Quantitative real-time Polymerase chain reaction
RL	Red Light
RT	Room Temperature
SD	Short Day
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
S.E.M	Standard Error of Mean
SPA	SUPPRESSOR OF PHYA-105
TOE	TARGET OF EARLY ACTIVATION TAGGED
UV-A	Ultraviolet-A
UV-B	Ultraviolet-B
UVR8	UV RESISTANCE LOCUS 8
WL	White Light
YC	C-terminal region of YFP
YN	N-terminal region of YFP
ZTL	ZEITLUPE

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FIGURE INDEX

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INTRODUCTION

1. Light and Plants

Plants are sessile living organism and have to regulate their growth and development in response to surrounding environment. Light is one of the most important signals for plants to perceive the ambient environment, in addition to being the major energy source for photosynthesis.

Plants have evolved several classes of photoreceptors to sense light signals. The phytochromes (PHYs) perceive red light (RL) and far-red light (FR), cryptochromes (CRYs), phototropins (PHOTs) and the ZEITLUPE family photoreceptors (ZTL) perceive blue light (BL) and ultraviolet-A (UV-A), and UV RESISTANCE LOCUS 8 (UVR8) is the ultraviolet-B (UV-B) receptor (Kronenberg and Kendrick, 1994; Deng and Quail, 1999; Briggs and Christie, 2002; Rizzini *et al*., 2011). Responding to different intensity, color, direction and periodicity of lights, these photoreceptors conduct various regulation of plant growth and development, including germination, de-etiolation, shade avoidance, phototropism, stomatal opening, and floral initiation, etc（Sullivan and Deng, 2003).

2. Photomorphogenesis

Plant seedlings undergo photomorphogenesis under light, which forms a short hypocotyl, open cotyledons and mature green chloroplast phenotype. Besides, dark-grown etiolated seedlings with the rapid elongated hypocotyl, apical hook, and enclosed

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pale yellow cotyledons are named skotomorphogenesis. The process of photomorphogenesis, especially the extent of hypocotyl elongation, has been widely used for studying light sensing and signaling regulation (Arsovski *et al*., 2012).

In *Arabidopsis*, the photoreceptors phytochromes and cryptochromes act to inhibit the key repressor, CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), which forms a complex with SUPPRESSOR OF PHYA-105 (SPA) and functions as a CULLIN4 (CUL4)-based E3 ubiquitin ligase, conducting the ubiquitination and degradation of long hypocotyl 5 (HY5), a basic leucine zipper (bZIP) transcription factor that regulates transcriptional changes for photomorphogenesis in light. In the light, the interaction between COP1 and HY5 is disrupted, leading to accumulation of HY5 and inhibition of hypocotyl elongation (Oyama *et al*., 1997, Osterlund *et al*., 2000).

In addition to HY5, a number of basic helix-loop-helix (bHLH) transcription factors also regulate hypocotyl growth. The PHYTOCHROME INTERACTING FACTORs (PIFs) could promote hypocotyl growth directly, and stimulates COP1-catalyzed ubiquitination and degradation of phyB, leading to light-hypersensitivity in *pifq* mutants. (Shin *et al*., 2009; Leivar and Quail, 2011; Jang *et al*., 2010). The LONG HYPOCOTYL IN FAR-RED1 (HFR1), similarly degraded in the dark by COP1-catalyzed ubiquitination light and stabilized in light, promotes photomorphogenesis (Fairchild *et al*., 2000; Duek *et al*., 2004). HFR1 also interacts with PIF4 and PIF5 directly to inhibit their activation

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of target genes (Hornitschek *et al*., 2009).

In addition to light, the plant hormones, including auxin, brassinosteroids, gibberellins, ethylene, and cytokinin, also play a role in the regulation of hypocotyl elongation, primarily through the interaction between their responsive genes and central photomorphogenic transcription factors such as HY5, PIFs or DELLAs (Arsovski *et al*., 2012).

3. Flowering

In *Arabidopsis*, there are at least five distinct pathways controlling flowering, including the photoperiod pathway, the vernalization pathway, the autonomous floral initiation, the gibberellic acid pathway, and the age pathway. It is generally believed that endogenous signals regulate flowering time through the autonomous and gibberellic acid pathways, and environmental signals regulate flowering time through the photoperiod and vernalization pathways (Fornara *et al*., 2010; Andrés and Coupland, 2012). Furthermore, other factors such as cold, drought, salinity and pathogen infection, also affect the timing of flowering (Bolouri Moghaddam and Van den Ende, 2013; Riboni *et al*., 2014). Finally, signaling information from all these pathways is integrated into flowering genetic networks through a small group of floral pathway integrators, such as FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), and LEAFY, which accumulate in the shoot apical meristem (SAM), promotes the SAM switching

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from vegetative to reproductive development (Srikanth and Schmid, 2011; Song *et al*., 2013).

Among these floral integrators, the best characterized one is FT that encodes an evolutionarily conserved small globular protein translocated from the leaves to the SAM to initiate floral transition (Kardailsky *et al*., 1999). Under favorable conditions, light signaling and the circadian clock coordinately increase the activity of CONSTANS (CO), which directly regulates the transcriptional expression of FT to promote flowering (Putterill *et al*., 1995; Fornara *et al*., 2009). On the other hand, the expression of FT is negatively regulated by several transcriptional repressors, such as FLOWERING LOCUS C (FLC), SHORT VEGETATIVE PHASE, TEMPRANILLO1, and SCHLAFMZE (SMZ). Under unfavorable conditions for flowering, or during the juvenile developmental phases, these repressors bind to specific cis-elements in the FT locus and repress its expression to prevent precocious flowering (Helliwell *et al*., 2006; Mathieu *et al*., 2009; Song *et al*., 2013).

4. Cryptochromes and BL signaling

Cryptochromes (CRYs) are photoreceptors that present in multiple species. They are flavoproteins that share sequence similarity to DNA photolyases, which repair UV-induced DNA damage by removing pyrimidine dimers from DNA under blue light. Plant and animal CRYs have lost DNA-repair activity and evolved other biochemical functions.

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Plants CRYs could regulate photomorphogenic growth and development, and both plants and animals' CRYs could regulate circadian clock (Sancar 2003). CRYs have also been reported to serve as magnetoreceptors in animals (Ritz *et al*. 2010).

Plant CRYs regulate a variety of blue-light-induced responses, including de-etiolation, photoperiodic flowering, guard cell development, stomata opening, leaf senescence, pathogenic responses, etc. Three CRYs, CRY1, CRY2 and CRY3, are encoded in the *Arabidopsis* genome. CRY1 and CRY2 are nuclear proteins that have functions as major blue light receptors, regulating blue light induced de-etiolation and photoperiodic flowering, which is the main concern of this work. CRY3 is detected in mitochondria or chloroplasts and has functions in UV-induced damage repair on single-stranded DNA (Pokorny *et al*. 2008).

Both CRY1 and CRY2 consist of two major domains: the N-terminal Photolyase Homologous Region (PHR) domain and the cryptochrome C-terminal Extension (CCE) domain. The PHR domain binds non-covalently with the CRY chromophore flavin adenine dinucleotide (FAD), which allows CRYs to absorb blue light (Malhotra *et al*. 1995). The PHR domain might possess a second chromophore, the 5,10-methenyltetra-hydrofalate, which could not be detected after purification (Hoang *et al*. 2008). CRYs function as dimers in plant cells. In addition to blue light perceiving, the PHR domain is responsible for CRY dimerization and protein interaction (Song *et al*. 2005; Rosenfeldt *et*

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al. 2008), and the PHR domain of CRY2 has been reported as the binding interface between CRY2/SPA1 and CRY2/CIBs (Zuo *et al*. 2011; Liu *et al*. 2008). The PHR domain of CRY1 also has been reported to mediate hypocotyl inhibition and flowering phenotype in spite of the direct interaction with COP1, suggesting that its interaction with the protein remains unknown presently (He *et al*. 2015). The CCE domains vary considerably in their length and sequence among plant CRYs. For *Arabidopsis* CRY1, the CCE domain is 180 amino-acid (aa) in length and for CRY2, it is 110 aa in length. It used to be proposed that the blue light signal is perceived in the PHR domain and transduced to the CCE domain, leading to the downstream signal transduction, because the overexpression of CCE domain of CRY1 and CRY2 could result in the constitutive photomorphogenic phenotype without blue light signal (Yang *et al*. 2000). The CCE domain conducts protein-protein interaction, including the interaction of CRY/COP1 and CRY1/SPAs (Wang *et al*. 2001; Yang *et al*. 2001; Liu *et al*. 2011).

CRYs are phosphoproteins. *Arabidopsis* CRY1 and CRY2 undergo phosphorylation in etiolated seedlings exposed to blue light, and the cryptochrome phosphorylation is required for its photoactivation. There are multiple phosphorylation sites located in the cryptochrome CCE domain (Shalitin *et al*. 2002; Shalitin *et al*. 2003). The CCE domain appears intrinsically unstructured, but it may change conformation upon photoexcitation by light-induced unfolding. It has been proposed that blue light-dependent

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phosphorylation of cryptochromes causes electrostatic repelling of the CCE domain from the surface of the negatively charged PHR domain of cryptochrome, resulting in separation of the two domains, which enables CRYs to interact with their signaling partners. CRY1 has been found to bind with an ATP analogue, AMP-PNP, in the FAD binding pocket in PHR domain, which is supposed to auto-phosphorylate its CCE domain in response to blue light (Liu *et al*. 2016b). On the other hand, CRY2 has not been detected to have auto-phosphorylation activity, and the fusion protein GUS-CCT2 that contains only the CCE domain of CRY2 without the PHR domain is constitutively phosphorylated. CRY2 has been reported to be phosphorylated at S587 and T603 by CK1.3 and CK1.4 *in vitro* (Tan *et al*. 2013). Since there are multiple phosphorylation sites, more protein kinases are likely to be involved in cryptochrome phosphorylation.

After phosphorylation, activated CRY2 undergoes ubiquitination and degradation in response to blue light. Both the PHR domain, CCE domain and flavin chromophore, are required for the degradation. The multifunctional E3 ubiquitin ligase COP1 has been reported to be partly required for CRY2 degradation, and the SPA proteins, which are closely related to COP1 activity, act redundantly in the blue light-dependent CRY2 degradation, since CRY2 still appears to be degraded in the *cop1* null allele (*cop1-5*) or *spa1spa2spa3spa4* quadruple mutant. Recent report shows that a CUL1-based E3 ligase is involved in blue light-dependent degradation of CRY2, in addition to the CUL4-based

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E3 ligase COP1 (Liu *et al*. 2016b).

CRYs induce plant responses to blue light primarily through two mechanisms. One mechanism includes the COP1-mediated proteolytic regulation of transcription factors. The other involves the direct binding of CRY2 to the basic helix-loop-helix transcription factor proteins. For the first mechanism, COP1 and the SPA proteins form an E3 ubiquitin ligase complex, which controls the protein level of multiple transcription factors such as HY5 and CO, which are key regulators of hypocotyl elongation and flowering time, by targeting them for ubiquitination and degradation. CRY1 binds directly to SPA1 under blue light condition, inhibiting the interaction between COP1 and SPA1 in order to suppress COP1/SPA complex activity. Activated CRY2 also binds to SPA1, but it appears to increase the affinity between CRY2 and COP1, thought to inactivate COP1/SPA complex activity. For the second mechanism, CRY2 can directly bind with CIB proteins to promote photoperiodic flowering. CIBs (CIB1/2/3/5) form heterodimers and bind directly to the non-canonical E-box DNA sequences on the FT promoter to initiate FT expression and promote early flowering phenotype (App. 2, Liu *et al*. 2016b).

Additionally, phytochromes have also been reported to be CRY binding proteins, and the interaction between CRYs and phytochromes in various light responses might modify their functions, including anthocyanin accumulation, photoperiodic flowering, and root greening, etc (Mockler *et al*. 2003; Usami *et al*. 2004; Más *et al*. 2000).

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5. FIN219 and JA signaling

Jasmonic acid has been known to play important roles in plant stress responses and development. The Jasmonates are synthesized from α-linolenic acid (α-LeA) of plant chloroplast membranes by oxidative processes through lipoxygenase (LOX), Alene oxide synthase (AOS) and Alene oxide cyclase (AOC) to become JA intermediate oxo-phytodienoic acid (OPDA) after physically wounding. OPDA is transported into the peroxisome to undergo β-oxidation and finally forms JA. Then JA would be transported to the cytoplasm, and be modified into various forms (App.1, Wasternack *et al*., 2016). The bio-active form of JA is JA-Ile, which is catalyzed by JAR1/FIN219 (Schaller and Stintzi, 2009).

FIN219 was discovered when searching for the suppressors of the *cop1-6* mutant phenotype. The *cop1-6* mutant shows constitutive photomorphogenic short hypocotyl phenotype in darkness, while the mutation at *FIN219* gene resulted in closed cotyledons and longer hypocotyl in *cop1-6* mutant background under the dark condition (Hsieh *et al*., 2000). The *fin219* mutant shows longer hypocotyl than the wild type Col-0 under FR and BL conditions. Further studies showed that FIN219 protein could physically interact with COP1 and modulate its subcellular location depending on light conditions, which enhanced HY5 protein stability under FR light, and therefore turns to be a positive regulator of photomorphogenesis (Wang *et al*., 2011).

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The FIN219 protein contains 575 amino acids in length and is composed of a dynamic C-terminal domain, a N-terminal domain, and a flexible hinge linker controlling both domains orientation for substrate binding. The N- terminal and C-terminal domains contain a coiled-coil domain, respectively, which conducts the interaction between these domains and other proteins (Chen *et al*., 2017). In addition to COP1, the interactions between FIN219 and cryptochromes were indicated in our previous work, suggesting that FIN219 might be involved in the blue light signaling. JAR1 was identified to be allelic to FIN219 (Staswick *et al*., 2002). Since the *fin219* mutant and *jar1-1* mutant showed FR insensitivity phenotype, and the level of JA-Ile was significantly lower in *fin219* mutant than that in wild type seedlings, we inferred that FIN219/JAR1 participates in both light and JA signaling (Chen *et al*., 2007).

The mechanism of JA signaling is similar to that of Auxin signaling. The bio-active JA-Ile could be perceived by COI1, an F-box protein that forms the SCF^{COI1} E3 ligase. At low JA concentration, the SCF^{COI1} complex could hardly target JAZ proteins for degradation, leading to JAZ protein accumulation and interaction with MYCs, a group of bHLH family transcription factors that regulate various JA responses, by repressing their transcriptional activity. JA-Ile could increase the binding affinity between JAZ proteins and COI1, resulting in the ubiquitination and degradation of JAZ proteins, so that the downstream genes could be induced and the JA signaling transduction could be turned on

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(Pérez *et al*., 2013; Carvalhais *et al*., 2017).

6. Crosstalks between light and JA signaling

FIN219/JAR1 is a key conjugation enzyme in JA signaling and positively regulates photomorphogenesis under far-red light, which establishes a crosslink between the FR light and JA signaling. Our previous data indicated that it was also involved in other light signaling such as blue light. The physical interaction between CRY2 and FIN219 had been observed by *in vitro* pull-down and yeast two-hybrid assays. *FIN219* overexpression seedling was more sensitive to BL, while *FIN219* overexpression in the *cry1 cry2* double mutant background did not show hypersensitive photomorphogenic phenotype than its background, which implies that FIN219 involved in the promotion of photomorphogenesis under BL requires functional CRYs.

On the other hand, in addition to the gibberellic acid, other plant hormones also participate in flowering regulation. Recently, JA has been reported to take part in flowering time regulation through COI1, in addition to the regulation of filament elongation, pollen maturation and anther dehiscence (Smyth *et al*., 1990; Zhai *et al* 2015). In our previous data, the *FIN219* overexpression plants showed early flowering phenotype under LD condition, suggesting that FIN219 may be involved in flowering regulation. Considering CRY2 has functions in regulating photoperiodic flowering, we wonder whether FIN219 participates in control of flowering through interaction with

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CRY2.

This study aims to find out the regulatory relationship between FIN219 and CRY2, and the possible role of FIN219 in CRY2-mediated blue light signaling.

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MATERIALS AND METHODS

Plant materials and Growth conditions

In this study, the Columbia-0 (Col-0) is used as the wild-type plant of *Arabidopsis thaliana*. The *fin219-2* (T-DNA insertion line SALK.059774 obtained from ABRC) and *cry2* (*cry2-1*) (Guo *et al*., 1999) mutant lines are in the Col-0 ecotype. The *pGR219* (*pGR::FIN219*) is homozygous transgenic plants overexpression *FIN219* gene with inducible pGR promoter in the *fin219-2* mutant background, and *HisCRY2* (CRY2 overexpression line) was made previously in the Col-0 background.

All seeds were surface sterilized in 30% bleach with 0.02% Triton 100, rinsed at least three times and sown on Petri dishes containing GM medium (1x MS, 0.3% sucrose, 0.5 g/L MES buffer, 0.7% agar gel, pH 5.7). Seeds would be moved into cold room at 4 ℃ and kept in dark condition as stratification. After 3 days of stratification, dishes would be air dried in the laminar flow for 1 hour and then incubated in continuous white light growth chamber at 22 ℃.

As for phenotypic analysis of hypocotyl elongation and sample collection, seeds would be incubated for 16 hr in the WL chamber and then moved to other light conditions. The wavelength of the blue LED light is 460 nm. The light wavelength and fluence rates are measured by LI-200SA pyranometer sensor (LI-COR).

For flowering phenotype analysis and sample collection, seeds would be incubated

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for 7 days in the WL chamber and transplanted to pots in long day (LD) or short day (SD) chamber, with the light fluence rates 60 and 40 $\mu molm^{-2}s^{-1}$, respectively. For *pGR* promoter induction, dexamethasone (Dex) was used at the concentration of 1 $\mu molL^{-1}$.

Protein isolation and Western Blotting

Arabidopsis total proteins were extracted with protein extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM phenylmethylsulfony fluoride, 0.1% NP-40, with 3x protease inhibitor (Invitrogen, Carlsbad, CA) The sample was ground thoroughly with liquid nitrogen and mixed with half-volume of protein extraction buffer. The mixture was centrifuged at 19,000 x g for 10 minutes at 4 ℃, transfer the supernatant to a new tube and repeat the centrifugal process once again, and collect the supernatant for protein analysis. The proteins were quantified by using Bradford reagent (Bio-rad Protein Assay, Bio-rad, Munich, Germany).

Protein samples were separated by SDS-PAGE after boiled with 6x protein sample buffer at least 90℃ for 10 minutes. The fully separated proteins were transferred to the PVDF membrane in Tris-glycine Buffer at 4℃. The membranes were washed with PBST and blocked with Blocking buffer (5% non-fat milk in PBST) for 5 minutes at RT, twice. After blocking, the membranes were incubated with specific antibodies with 5000-10000 dilution in new Blocking buffer at 4 ℃, for at least 8 hrs. The membranes were washed twice with Blocking buffer before incubation with the secondary antibody in new

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blocking buffer at RT, for at least 90 minutes. After washing with PBST and PBS twice, the protein signals were developed using T-pro LumiFast Plus ECL Kit, and the fluorescence images were taken by KETA CL Luminescent Imaging System.

Recombinant protein purification and Pull-down assays

The *Escherichia coli* (*E. coli)* containing recombinant plasmids were incubated firstly in 5 ml sterilized liquid LB medium with antibiotic selection at 37℃ for 16 hours. Then the culture was transferred to 200 ml fresh liquid LB medium and incubated at 37℃ for another 4-5 hours till OD600 = 0.5-0.6. Then add IPTG to the culture with a final concentration of 0.5 mM to induce the recombinant protein production for 4 hrs at 28℃. The samples were collected after spinning down at 4,000 g, and resuspended in lysis buffer and sonicated in short pulses (Chrom Tech). Different resins were incubated for over 12 hrs with the samples according to the tag of target recombinant protein (CBP: Calmodulin Affinity Resin; GST: Glutathione sepharose 4B). The proteins were purified by elution from the resins after washing.

In order to perform the *in vitro* interaction between the candidate proteins, their recombinant proteins were raised and mixed with the interaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM phenylmethylsulfony fluoride, 0.04% NP-40, 1x protease inhibitor) and incubated at 4℃ at 25 rpm for 1 h. Activated resins were added to the mixture and incubated under the same condition for another hour. The resins

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were spin down and washed for five times with the interaction buffer, and transferred to new tubes after the final washing. After centrifugation, the pellets were boiled with 4x protein sample buffer and subjected for SDS-PAGE. Western blot analysis was performed as mentioned above.

Co-immunoprecipitation Assay

Arabidopsis seedlings underwent cross link process before protein extraction. Seedlings were vacuum-infiltrated in 1% formaldehyde (FA) for 3 minutes. Fixation was stopped using 125 mM of glycine under vacuum for 5 minutes. Fixed seedlings were washed for three times using sterile water, dried, and ground with liquid nitrogen to fine powder for protein extraction.

Protein G Mag Sepharose Xtra (GE healthcare) was washed with 1x TBS (50mM Tris-HCl, 100 mM NaCl, pH 7.5) once. Beads were incubated with anti-FIN219 antibody at 4℃, 25 rpm overnight. Then beads were washed with 1x TBS once, and incubated with 1.5 mg *Arabidopsis* total protein at 4℃, 25 rpm overnight. The beads were washed with TBST (1x TBS with 0.01% Tween-20) for three times, and transferred to new tubes at the last time. Beads were boiled with 4x protein sample buffer and subjected for SDS-PAGE. The Co-immunoprecipitation result was showed by Western Blotting.

Protoplast transfection and BiFC Analysis

Arabidopsis mesophyll protoplasts were isolated and transfected as described

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previously (Wang *et al*. 2011). Leaves of 4~5-week-old plants grown in chambers with SD photoperiod were collected, shredded and immersed in enzyme solution (1% Cellulase R10, 0.25% Macerozyme R10, 0.4 M Mannitol, 20mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$, 5 mM β-ME, 0.1% BSA) and then incubated at 25 rpm for 4 hrs. Harvest the cells by spinning down the solution at 100 x g for 3 mins. Cells were washed for three times with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glucose, 2 mM MES, pH 5.7) and incubated on ice for 30 mins. The cells were centrifuged and resuspended with MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7) at a concentration of 3 x 10^5 cell/ml.

DNA fragments of *FIN219* and *CRY2* were cloned into *35S::YFP-N155/pRTL2* and *35S::YFP-C84/pRTL2*, respectively. For each combination, 3 μg of plasmid DNA was mixed with 200 μl of protoplasts. The nuclei of protoplasts were marked with mCherry (2 mg/ 200 μl protoplasts). The mixture was mixed with 200 μl of PEG solution gently but quickly, and incubated at RT for 10 mins. Add 2.5 ml W5 buffer to the mixture immediately after incubation, and wash twice. The protoplasts were resuspended with 1 ml W5 buffer and transferred to a new eppendorf. Protoplasts were incubated under different light conditions for over 16 hours, and fluorescence signals were observed with fluorescence microscope.

All fluorescence images were captured by digital camera (Nikon) and merged using

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ImageJ Software.

Hypocotyl length measurements

Three-day-old *Arabidopsis* seedlings of different treatments as described were laid on plates (1% agarose with black ink) and taken into photos. The hypocotyl lengths were measured by using ImageJ Software.

Flowering-time measurement

Flowering time was measured by counting the days from the seeds being exposed to light to flowering, and counting the rosette leaf number at flowering. For each genotype, at least 10 plants were measured, and average (AVG), standard error of mean (S.E.M), and Student's T test were calculated by using Microsoft Excel.

RNA isolation and Quantitative real-time PCR

Arabidopsis total RNA of each sample was isolated by LabPrep^{TM} RNA plus Mini Kit. Isolated RNA was quantified and concentrated by Nano Drop (Thermo Scientific), and diluted to appropriate concentration. RNA was converted to the first-strand cDNA by undergoing reverse transcription via mixing with Oligo-dT (Invitrogen), RNasin (Promega) and reverse transcriptase (Applied Biosystems). Quantitative real-time PCR was carried out by the use of SYBRR Green Supermix reagent (Bio-rad) in CFX96 Touch^{TM} (Bio-rad) device. The primers used for real-time PCR were listed as follows: FT-forward: 5'- CAA CCC TCA CCT CCG AGA ATA T -3'; FT-reverse: 5'- TTG CCA

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AAG GTT GTT CCA GTT-3’; UBQ10-forward: 5’- TCC GGA TCA GCA GAG GCT TA-3’; UBQ10-reverse: 5’- TCA GAA CTC TCC ACC TCA AG-3’; FIN219-forward: 5’- CTC TGG AAC TAG TCA AGG CCG TCC AAA GTT-3’; FIN219-reverse: 5’- GGC AAT ACA AGG CTT GAT GGA CAT CAG G-3’; CRY2-forward: 5’- GGA TCC ATG TCA AGA ACC CGT GAA GCA CAG-3’; CRY2-reverse: 5’- CCC GGG TTT GCA ACC ATT TTT TCC CAA AC-3’ ; Actin2-forward: 5’- ATG AAG CAC AAT CCA AGA GAG GTA TTC TTA-3’; Actin2-reverse: 5’- GAG CTT CTC CTT GAT GTC TCT TAC AAT TTC-3’.

Data was analyzed by CFX Manager 1.6 (Bio-Rad), and presented by using Microsoft Excel.

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RESULTS

1 *Arabidopsis* FIN219 physically interacts with the C-terminal of CRY2.

Previous studies showed the interaction between CRY1, CRY2 and FIN219 protein by both pull-down and yeast two-hybrid assays (Du, 2005; Fu, 2008). This study investigated the interaction of both proteins in more detail. The GST-FIN219, CBP-CRY2, CBP-CNT2 and CBP-CCT2 recombinant proteins used in the pull-down assay were expressed in *E.coli*. CNT2 contained the N-terminal 485 amino acids of CRY2, and CCT2 contained the C-terminal 127 amino acids of CRY2. The recombinant GST-FIN219 protein interacted with CBP-CCT2, but did not interact with CBP-CRY2 nor CBP-CNT2 in pull-down assay (Fig. 1A, B). As mentioned in the introduction, CRY2 undergoes conformation change and CCT2 would be exposed and be able to interact with other proteins when activated by blue light (BL). While in pull-down assay, the recombinant proteins might fail to change their conformation. Therefore the BiFC assay was performed. Strong fluorescence was detected not only in the nuclei of protoplasts co-transformed with FIN219-YN and CCT2-YC (Fig. 2 D, E, F), but also in those co-transformed with FIN219-YN and CRY2-YC under BL and WL (Fig. 2A, B), indicating that FIN219 interacted with CRY2 under BL and WL conditions. On the other hand, little fluorescence was detected in the nuclei of protoplasts co-transformed with FIN219-YN and CRY2-YC in darkness (Fig. 2 C), which indicated little interaction between FIN219

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and the inactive form of CRY2. Thus, FIN219 is more likely to interact with the photoactive form of CRY2 in its exposed C-terminal region. The interaction of FIN219 and CRY2 was further confirmed by co-immunoprecipitation (Co-IP) assay. The *Arabidopsis* total proteins were isolated from the 3-day-old seedlings of Col-0, *fin219 mutant*, *cry2 mutant*, *pGR219* (the inducible *FIN219* overexpression line), and *HisCRY2* (the *CRY2* overexpression line). To obtain the largest amount of CRY2 protein, seedlings were grown in darkness for three days, and exposed to BL for 30 mins before protein extraction in order to activate CRY2. Although little CRY2 protein co-precipitated with FIN219 in the WT seedling, CRY2 could be perceived to be co-precipitated with FIN219 in the *pGR219* seedling, and more CRY2 protein was co-precipitated when treated with MeJA (Fig. 1C). These led to the conclusion that CRY2 undergoes BL-dependent physical interaction with FIN219.

2 CRY2 and FIN219 act synergistically in BL-mediated inhibition of hypocotyl elongation.

The inhibition of hypocotyl elongation is a central phenotype of photomorphogenesis. As mentioned above, CRYs play an important role in the inhibition of hypocotyl elongation in response to BL, and CRY2 primarily mediates the inhibition under low BL intensity. In our previous studies, FIN219 also inhibits hypocotyl elongation and acts as a positive regulator of photomorphogenesis under BL, in addition to FR (Fu, 2008). This

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study tried to elucidate how FIN219 and CRY2 participated in BL signaling. The hypocotyl phenotypes of the 3-day-old Col-0, *fin219-2*, *cry2*, *pGR219*, *HisCRY2*, *fin219cry2*, *fin219HisCRY2*, *pGR219cry2* and *pGR219HisCRY2* seedlings under three kinds of BL intensities and dark were examined (Fig. 3). The *cry2* mutant seedlings showed longer hypocotyl than Col-0 under 10 and 1 $\mu molm^{-2}s^{-1}$ BL (Fig. 3 A, B, C, D), and the *HisCRY2* seedlings showed shorter hypocotyl under 1 and 0.1 $\mu molm^{-2}s^{-1}$ BL (Fig. 3 C, D, E, F), indicating that CRY2 was functional under these light conditions. The *fin219-2* mutant seedlings showed longer hypocotyl under 1 $\mu molm^{-2}s^{-1}$ BL than WT seedlings (Fig. 3 C, D), but did not show significant difference under 10 or 0.1 $\mu molm^{-2}s^{-1}$ BL or in dark (Fig. 3 A, B, E, F, G, H). In contrast, *pGR219* seedlings exhibited shorter hypocotyl than Col-0 under all these light conditions (Fig. 3 A-H). In BL conditions, the *fin219cry2* double mutant showed longer hypocotyl than *fin219* and *cry2* single mutant (Fig. 3 A-F). CRY2 overexpression in the *fin219* mutant background could effectively increase their BL insensitivity and led to the hypocotyl length similar to HisCRY2 seedlings, and FIN219 overexpression in the *cry2* mutant background could also rescue its long hypocotyl phenotype under BL (Fig. 3 A-F), implying that FIN219 and CRY2 may function together under BL conditions.

3 CRY2 inhibits JA sensitivity depending on FIN219

The JAs also play a role in the inhibition of hypocotyl elongation (Robson *et al.*,

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2010). Applying MeJA treatments led to the inhibition of hypocotyl elongation, and since FIN219 is the major enzyme that mediates the formation of bio-active JA-Ile, the JA sensitivity was reduced in *fin219* mutant. The inhibition of hypocotyl elongation of the seedlings examined under MeJA treatment was calculated and displayed in percentage to represent JA sensitivity (Fig. 3 I). Calculation was done according to the equation: [(Mock-MeJA)/Mock x 100%]. As expected, the *fin219* mutant showed lower JA sensitivity under all light conditions. The JA sensitivity was weaker in WT Col-0 seedling along with the BL intensity increasing (Fig. 3 I). In contrast, the *cry2* mutant showed higher JA sensitivity under the same condition, and FIN219 overexpression in the *cry2* mutant background even led to high JA sensitivity in darkness (Fig. 3 I), which suggests that the mutation at *cry2* might increase JA sensitivity. However, the *cry2* mutant in the *fin219* mutant background failed to increase JA sensitivity (Fig. 3 I), suggesting that the limitation of JA sensitivity by CRY2 needs FIN219.

4 FIN219 and CRY2 protein level under blue light conditions and MeJA treatment

4.1 FIN219 level was not directly influenced by CRY2.

Our previous study showed that FIN219 protein level could be negatively regulated by CRY1 under BL (Fu, 2008). Since CRY2 function is redundant with CRY1, we speculate whether it would influence FIN219 protein level under BL or not, and therefore examined the level of FIN219 in seedlings mentioned above. Disappointedly, FIN219

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protein level was lower in *cry2* or *HisCRY2* seedlings than in Col-0 under 1 μmolm^{-2}s^{-1} BL (Fig. 4B), and higher under 10 μmolm^{-2}s^{-1} BL (Fig. 4 A). In darkness, FIN219 level in *cry2* or *HisCRY2* seedlings was similar to that in Col-0 (Fig. 4 C), which seems that CRY2 expression level is not directly related with FIN219 level. Though showing a short hypocotyl phenotype under blue light (Fig. 3 A-G), *pGR219* did not have increased FIN219 level under either high BL or darkness conditions (Fig. 4 A, B, C). As observed previously, FIN219 was induced by MeJA treatment, and it was well repeated in these seedlings regardless of genotype or light conditions (Fig. 4 A, B, C).

4.2 FIN219 increased CRY2 protein accumulation under blue light.

As mentioned in the introduction, CRY2 is BL-photoactived, and undergoes ubiquitination and degradation under high blue light. Less CRY2 protein was detected in *fin219* mutant seedlings than that in Col-0 seedlings under both 1 and 10 μmolm^{-2}s^{-1} BL, and more CRY2 protein was detected in *pGR219* seedlings (Fig. 4 A, B). Meanwhile, the *fin219HisCRY2* seedlings showed lower CRY2 protein level than *HisCRY2* seedlings under 1 μmolm^{-2}s^{-1} BL (Fig. 4 B). By contrast, more CRY2 protein was detected in *fin219* mutant and *pGR219* seedlings than in Col-0, and more CRY2 protein was detected in *fin219 HisCRY2* seedlings than in *HisCRY2* under darkness condition (Fig. 4 C). To further understand the effect of FIN219 on CRY2 protein, the light transition study was performed. Since CRY2 is a stable protein in etiolated seedlings and degraded rapidly

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after exposed to high blue light (Qing *et al*., 2016), 3-day-old etiolated seedlings of Col-0, *fin219*-2, *pGR219*, *HisCRY2*, *fin219HisCRY2* and *cry2* were transferred from the dark to blue light (1 $\mu molm^{-2}s^{-1}$), and FIN219 and CRY2 protein levels were examined at 15, 30, 60, 240 mins and 24 hours blue light exposure. After exposure to blue light, CRY2 protein in Col-0 seedlings was clearly detectable for at least 1 hour BL irradiation, while in *fin219* mutant seedlings, detection of CRY2 came to be only 15 mins (Fig. 5). In *pGR219* seedlings, CRY2 protein was still clearly detectable even after 24 hours BL exposure (Fig. 5). Moreover, CRY2 protein was degraded more rapidly in *fin219 HisCRY2* seedlings than in HisCRY2 (Fig. 5). Taken together, FIN219 might increase CRY2 protein accumulation under weak BL.

4.3 CRY2 protein stability was decreased by MeJA treatment under blue light.

Co-IP studies revealed that MeJA treatment seemed to enhance the affinity between FIN219 and CRY2 (Fig. 1C). Moreover, FIN219 might increase CRY2 protein stability under blue light. We expected to see more CRY2 protein with MeJA treatment. While in fact, less CRY2 protein was observed in almost all seedlings expressing *CRY2* gene examined after treatment with MeJA under 1 or 10 $\mu molm^{-2}s^{-1}$ BL (Fig. 4 A, B). The reduction of CRY2 level occurred substantially in *fin219* mutant seedlings under 1 $\mu molm^{-2}s^{-1}$ BL (Fig. 4 B) and *fin219 HisCRY2* seedlings under 10 $\mu molm^{-2}s^{-1}$ BL (Fig. 4 A), although it happened to increase in *fin219 HisCRY2* mutant seedlings under 1 $\mu molm^{-}$

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$^{2}s^{-1}$ BL (Fig. 4 B) and HisCRY2 seedlings under 10 μmol$m^{-2}s^{-1}$ BL (Fig. 4 A), in which the CRY2 level seemed extremely low without MeJA treatment. In *pGR219* and *pGR219HisCRY2* seedlings, CRY2 protein levels normally decreased (Fig. 4 A, B), which suggests that at least FIN219 would not repress the reduction of CRY2 protein level induced by MeJA treatment. On the other hand, more CRY2 protein was observed after treatment with MeJA in darkness, except in *pGR219* seedlings, which showed a reduced level of CRY2 protein (Fig. 4 C), indicating that the MeJA induced reduction of CRY2 protein depended on blue light.

5 Effect of FIN219 and CRY2 on flowering time under LD and SD conditions.

Our previous studies showed that FIN219 was a positive regulator of flowering. The *fin219* mutant slightly delayed flowering and FIN219 overexpression resulted in early flowering phenotype under long day condition (Fu, 2008). Further studies showed similar results under short-day conditions (Chang, 2015). CRY2 has been known for a positive regulator of flowering, and positively regulates flowering-time transcription factors, CONSTANS and CIBs.

This study tried to find out whether FIN219 functions in flowering control through CRY2-mediate signaling. Both the days to flowering and the leaf number did not show significant difference among the plants examined under LD condition (Fig. 6 A, B). Only *fin219HisCRY2* plants slightly delayed flowering compared with HisCRY2 (Fig. 6 A, B).

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Under short-day condition, *fin219* mutant plant surprisingly showed an early flowering phenotype with shorter day to flowering and less leaf numbers compared with Col-0 plants, and *pGR219* plants showed a delayed flowering phenotype (Fig. 6 C, D). The mutation or overexpression of FIN219 failed to make any significant changes in *HisCRY2* background (Fig. 6 C, D). Mutation of CIB1 delayed flowering as reported, and showed no significant difference from the *fin219cib1* double mutant (Fig. 6 C, D).

Considering the confusing results, we conducted the Quantitative real-time PCR to examine *FT* gene expression that represents the promotion of flowering. Under LD condition, *FT* gene expression was higher in *HisCRY2* than in Col-0 as expected, but was lower in *fin219HisCRY2* than in *fin219* or *HisCRY2* plants (Fig.7 A), which did not match with the day to flowering or leaf number phenotype (Fig.6 A, B). The *fin219* mutant and *pGR219* plants showed lower *FT* gene expression compared to Col-0 plants. Little *FT* gene expressed in *cry2* mutant, and both *fin219* mutation and overexpression in *cry2* mutant background would not rescue the expression (Fig.7 A). While under SD condition, *fin219* and *pGR219* plants showed higher FT gene expression compared to Col-0, and more *FT* gene expression in *fin219HisCRY2* than in *fin219* or *HisCRY2* plants, which indicates a nearly opposite effect as compared with that under LD condition (Fig.7 B). Since the real-time PCR results showed that the mRNA expressions of the plants examined were consistent with their genotypes (except pGR219HisCRY2) (Fig.7 C, D),

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further studies are necessary in order to uncover the function of FIN219 in flowering.

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DISCUSSION

1. FIN219 physically interacts with BL-activated CRY2.

The physical interaction between CRY2 and FIN219 *in vitro* had been observed in our previous studies. Du (2005) showed that the C-terminal domain of FIN219 interacted with CRY2 and CCT2 by yeast-two-hybrid assay, and Fu (2008) showed similar results by pull-down assays, while the interaction of the full-length FIN219 and CRY2 could not be observed in those assays. This work confirmed the observations by pull-down assay and found that although the full-length FIN219 could interact with CCT2, it could not interact with the full-length CRY2 *in vitro* (Fig.1 A, B). On contrary, the full-length FIN219 could interact with the full-length CRY2 at the presence of blue or white light by BiFC (Fig.2 A, B). Cryptochromes have been found to change their conformation in the presence of BL (Liu *et al*. 2016b), which makes the interaction between FIN219 and the separated C-terminal of CRY2 reasonable.

2. The regulation between FIN219 and CRY2 proteins in photomorphogenesis.

Although FIN219 was found to be negatively regulated by CRY1 (Fu, 2008), here, it seemed that the absence or overexpression of CRY2 fairly interfered with the level of FIN219. However, FIN219 was increased and decreased in *cry2* mutant and *CRY2* overexpression seedlings compared with the Col-0, respectively (Fig. 4 A, B), and these two genotypes of etiolated seedlings showed similar decreasing patterns of FIN219 after

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exposure to BL (Fig.5).

On the other hand, more CRY2 protein was detected in FIN219 overexpression seedlings and less CRY2 protein in *fin219* mutant seedlings compared with the Col-0 (Fig. 4). CRY2 protein level decreased more rapidly in *fin219* seedlings compared with Col-0 seedlings after exposure to BL, right in contrast with pGR219 seedlings (Fig. 5). Taken together, we supposed that FIN219 increased CRY2 protein accumulation. As mentioned in the introduction, CRY2 was photo-activated when exposed to BL, and then undergoes ubiquitination and degradation through COP1 E3 ubiquitin ligase (Liu *et al*. 2016a). Based on these information, two hypotheses were proposed.

One was that FIN219 might stabilize CRY2 protein by their direct interaction and prevent CRY2 from the interaction of COP1/SPA1 complex. Both FIN219 and COP1 interacted with CRY2 in its C-terminal region, which suggests that the interaction of FIN219 and CRY2 might interfere the binding of CRY2 with the COP1/SPA1 complex. Since CRY2 inhibits the COP1 complex and positively regulates photomorphogenesis by interacting with the complex, FIN219 might inhibit CRY2 activity, and thus the *fin219* mutation would lead to more active CRY2, which was consistent with the observation that the *fin219HisCRY2* seedling showed shorter hypocotyl than *HisCRY2* under 0.1 μ $molm^{-2}s^{-1}$ BL (Fig. 3 E, F).

The other hypothesis is that FIN219 might negatively regulate COP1, repressing the

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ubiquitination and degradation process of CRY2 under BL. FIN219 has been reported as a COP1 suppressor under FR, and overexpression of FIN219 could exclude COP1 from the nucleus and help the accumulation of its substrate HY5 in the nucleus in darkness (Wang *et al*., 2011). FIN219 and COP1 had been found to be co-localized after 2 hours exposure to the BL (Fu, 2008), given highly potential that FIN219 also suppressed the function of COP1 under BL. In this case, the *fin219* mutation would result in more COP1 left in the nucleus and the degradation of CRY2 as well as other COP1 substrate, such as HY5. This was consistent with the observation that the *fin219HisCRY2* seedling showed longer hypocotyl than *HisCRY2* under 1 μ $molm^{-2}s^{-1}$ BL(Fig. 3 C, D).

After all, we did not have direct evidence to support these hypotheses. The regulation of FIN219 on COP1 under BL would be the most concerned that worth further study. At least, we could confirm that FIN219 and CRY2 worked synergistically to positively regulate photomorphogenesis, as for the *fin219cry2* double mutant showed longer hypocotyl than either *fin219* or *cry2* single mutant under various intensities of BL (Fig. 3 A-F).

3. The crosstalk between CRY2 and JA response in photomorphogenesis.

There were few reports about CRY2 directly involved in the JA response, but CRY2 could interact with COP1, and thus might take part in the MeJA-induced inhibition of hypocotyl elongation. MeJA treatment would lead to the inhibition of hypocotyl

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elongation, which indicates that JA sensitivity was reduced in the *fin219* or *cop1* mutated seedlings under various light conditions, including FR, BL, RL, WL and darkness. Further studies showed that MeJA could induce FIN219 protein expression and promote the exclusion of COP1 from the nucleus to cytoplasm even in the dark, leading to the HY5 accumulation in the nucleus and resulting in the inhibited hypocotyl phenotype (Lo, 2015).

In this study, *cry2* mutant seedling was more sensitive to MeJA in BL, but did not show similar high sensitivity in *fin219cry2* double mutant seedling, which indicated CRY2 acted upstream of FIN219 in MeJA-mediated inhibition of hypocotyl elongation (Fig. 3 I). However, the increased JA sensitivity in *cry2* mutant seedlings might not be caused by altering the protein quantity of FIN219, for the FIN219 protein level actually decreased in the *cry2* mutant seedling and *pGR219cry2* seedling under 1 μ $molm^{-2}s^{-1}$ BL (Fig. 3 I, Fig.5 B). Meanwhile, under 10 μ $molm^{-2}s^{-1}$ of BL, FIN219 level increased in these seedlings (Fig.5 A). Since CRY2 has been reported to interact with COP1 in the WD-40 domain (Wang *et al*., 2001), which is the same region as the binding surface of FIN219 and COP1 (Wang *et al*., 2011), we proposed that CRY2 could reduce the interaction between FIN219 and COP1, and impair the exclusion of COP1 from nucleus to cytoplasm. Under these circumstances, CRY2 would undergo ubiquitination and degradation by COP1 in the presence of BL, which well explained the decreased protein level of CRY2 after treatment with MeJA under BL, in contrast to its accumulation in

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darkness (Fig. 4 A, B, C).

4. Possible roles of FIN219 in flowering regulation.

Previous studies showed that FIN219 was also involved in flowering control and acted as a positive regulator (Fu, 2008). While in this work, no significant flowering time difference was detected between Col-0 and *fin219* mutant plant, or among other the plants examined under LD condition (Fig. 6 A, B). In order to perceive more significant changes, we performed flowering time tests in SD condition, in which an early flowering phenotype was detected in the *fin219* mutant plant, and the pGR219 plant showed a delayed flowering phenotype (Fig.6 C, D), which seems to be different from the previous observation. Then we tried to avoid the operation error that possibly existed, including the wounding during transplant seedling to the soil or the pGR promoter induction, by conducting the QPCR to examine the FT gene expression as the deputy of flowering promotion, and verifying the mRNA expression of indicated plants by real-time PCR (Fig. 7 C, D). As mentioned in the introduction, CRY2 can positively regulate flowering-time transcription factors CONSTANS and CIBs, which promote the transcription of FT. HisCRY2 plant showed higher FT gene expression, and cry2 mutant plant showed lower expression as expected in LD conditions (Fig.7 A). But both *fin219* mutant and *pGR219* plants showed lower FT gene expression compared to Col-0 plants (Fig.7 A), which failed to repeat the previous data. In SD condition, *pGR219* plants showed higher FT gene

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expression compared to Col-0 and even to *HisCRY2*, and more FT gene expressed in *fin219HisCRY2* than HisCRY2 plants, which differs from the observation in LD condition (Fig.7 B).

Compared with the previous experiments, currently the light source has been changed into LED lights that mainly provide Red and Blue light, while the traditional light source that fairly provides full spectrum of lights was used in the past. We doubt that increased RL ratio might enhance phyB-mediate negative regulation of flowering, which is reported to be inhibited by phyA and CRY2 in the presence of FR and BL (Mockler *et al*., 2003). Moreover, recent studies reported that the JA receptor COI1 negatively regulate flowering though the AP2 family transcription factors TOE1 and TOE2, which repress FT gene transcription (Zhai *et al*., 2015). Further studies are needed in order to uncover the function of FIN219 in flowering.

CONCLUSION

CRY2 is the blue light receptor that functions in mediating de-etiolation under weak blue light and control of photoperiodic flowering in *Arabidopsis*. Previous studies showed that FIN219, a crucial enzyme in JA signaling and a positive regulator of phyA-mediate FR light signaling, also participated in cryptochrome signaling, while the mechanism remained unclear. This study verified the physical interaction between CRY2 and FIN219 proteins under blue light (Fig.1, Fig. 2), and observed that the CRY2 protein accumulation

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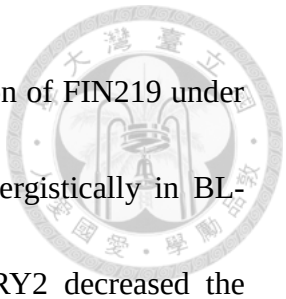

decreased with MeJA treatment but increased with the overexpression of FIN219 under blue light (Fig. 4 A, B, Fig5). CRY2 and FIN219 functioned synergistically in BL-mediated inhibition of hypocotyl elongation (Fig.3 A-H), and CRY2 decreased the inhibition of hypocotyl elongation caused by MeJA depending on FIN219 (Fig.3 I). However, more evidence was needed to elucidate the role of FIN219 in CRY2-mediate regulation of flowering in *Arabidopsis*.

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FIGURES

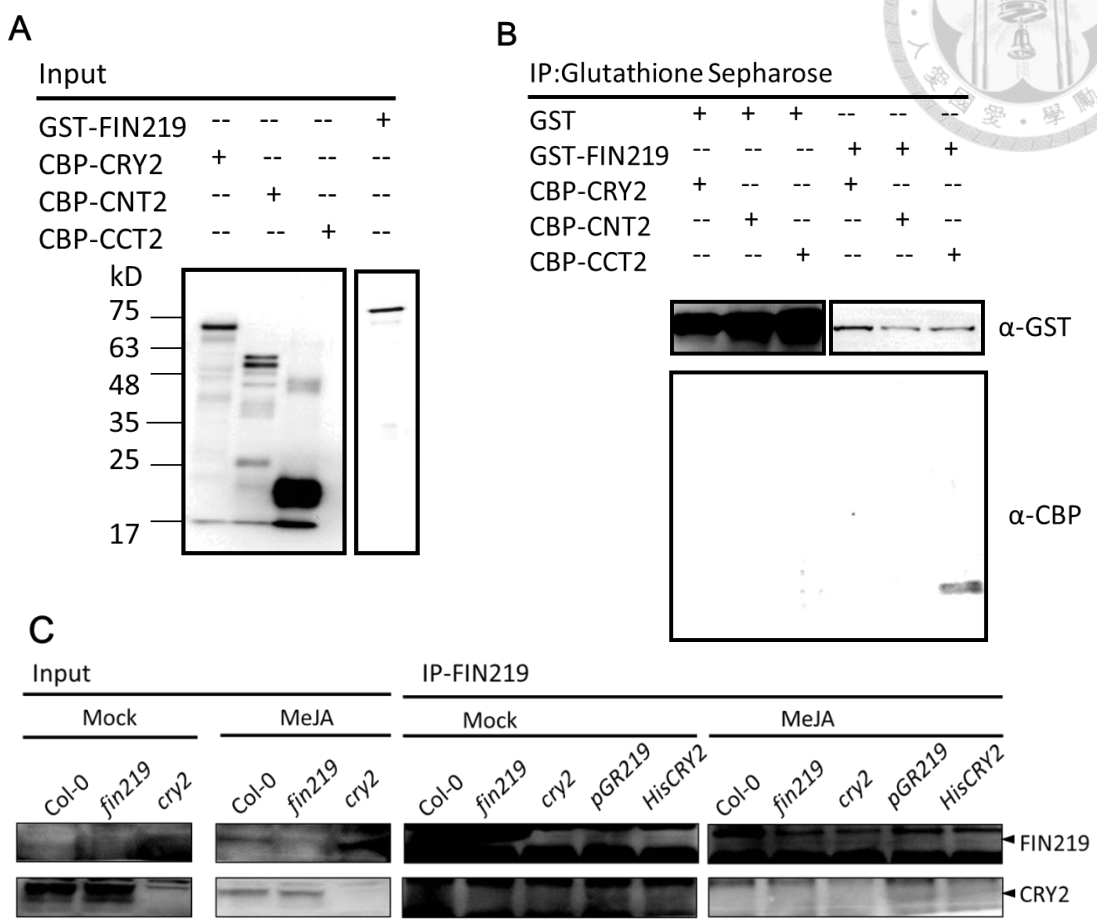

Fig.1 Physical interaction of *Arabidopsis* FIN219 and CRY2.

(A)Immunoblots showing the recombinant fusion proteins as the input, proteins were probed with anti-CBP or anti-GST antibodies. CNT2, the N-terminal 485 amino acids of CRY2; CCT2, the C-terminal 127 amino acids of CRY2. (B) Pull-down assays showing interaction between GST-FIN219 and CBP-CCT2, no interaction between GST-FIN219 and CBP-CRY2 nor CBP-CNT2, and no interaction between GST and CRY2 fragments. 5 μg of the indicated combination of recombinant proteins were mixed and immunoprecipitated with Glutathione Sephrose against GST tag. The beads were washed and underwent immunoblots, proteins were probed with anti-CBP or anti-GST antibodies as indicated. (C) *Arabidopsis* whole proteins were isolated from 3-day-old etiolated seedlings treated with 50 mM MeJA or Mock (EtOH), exposed to 1 $\mu molm^{-2}s^{-1}$ BL for 30 mins before collection. 100 μg of the proteins from the indicated seedlings were used as input, and 1.5 mg of the proteins were used to perform the co-immunoprecipitation. Co-immunoprecipitation was performed using the anti-FIN219 antibody. Proteins were probed with anti-FIN219 and anti-CRY2 antibodies. The arrowheads indicated the CRY2 and FIN219 proteins, respectively.

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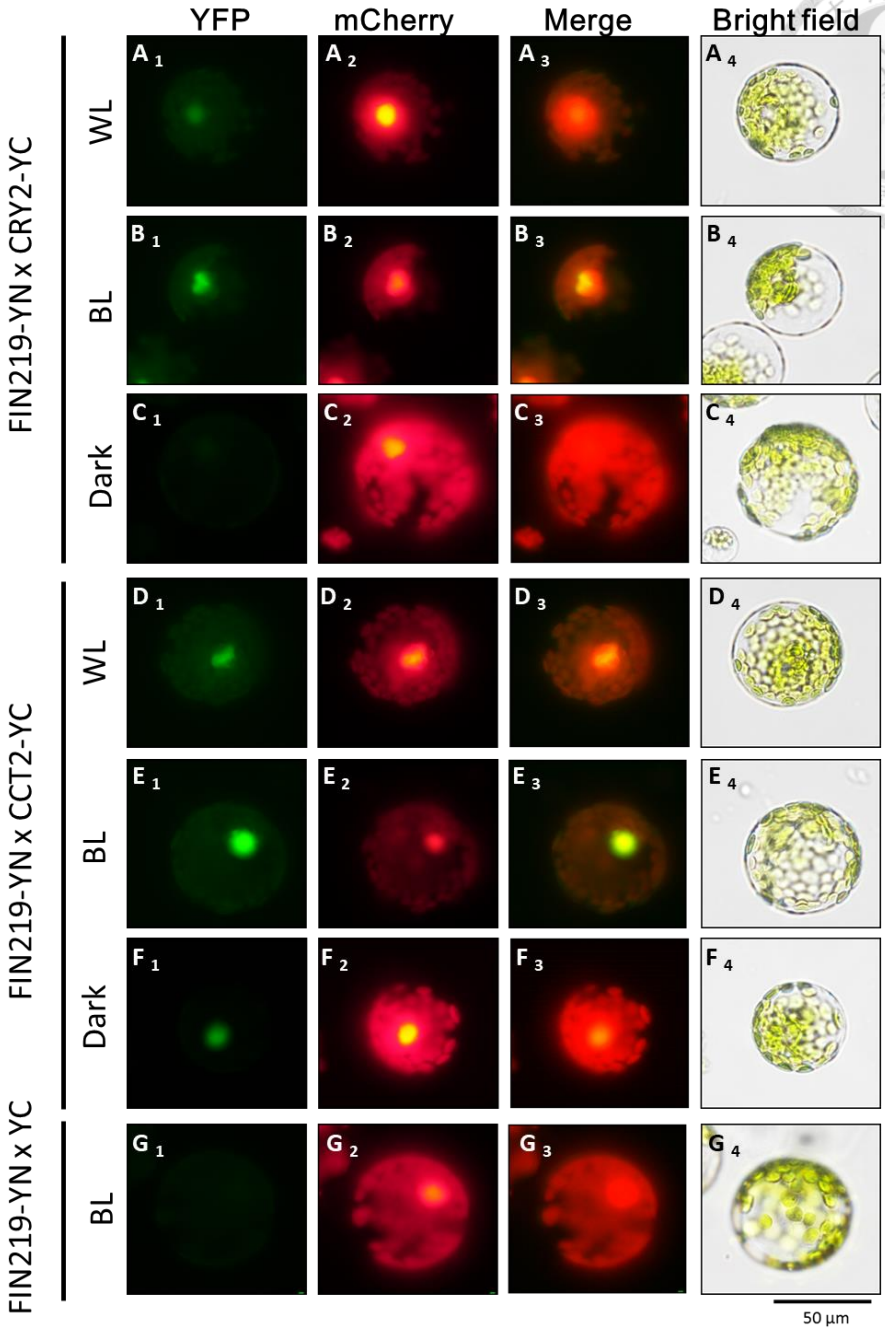

Fig.2 FIN219 interacts with CRY2 in a light dependent manner.

BiFC assays of the protein interaction *in vivo*. Protoplasts from *Arabidopsis* were co-transformed with FIN219-YN and CRY2-YC (A-C) or FIN219-YN and CCT2-YC (D-F). The FIN219-YN and YC (G) combination were showed as the negative control. The co-transformed protoplasts were cultured in W5 solution for 16 hours under 50 $\mu molm^{-2}s^{-1}$ white light (WL) (A, D), 1 $\mu molm^{-2}s^{-1}$ blue light (BL) (B, E, G) or in darkness (C, F). The nuclei were indicated by NLS-mCherry. Scale bar: 50 μm.

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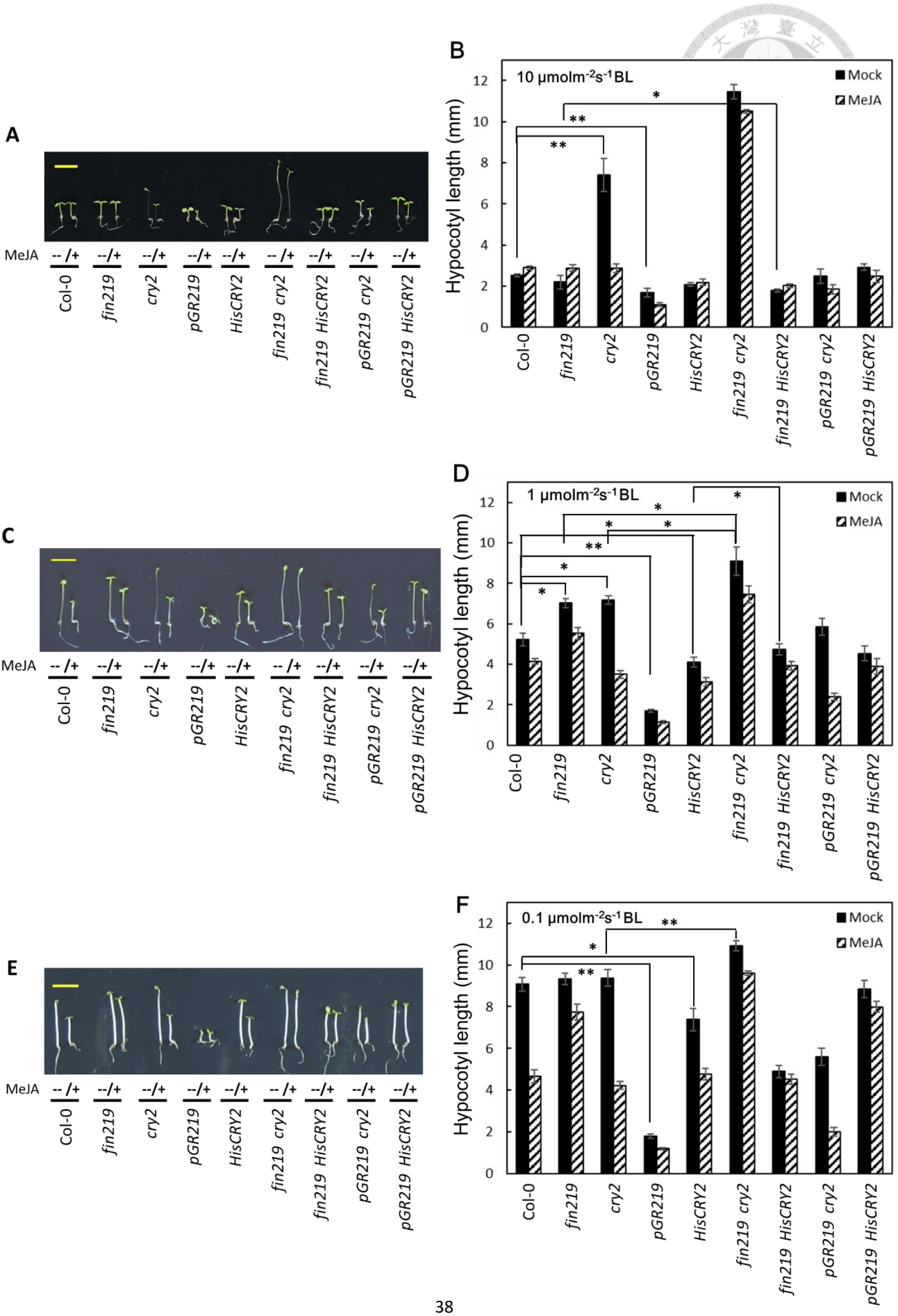

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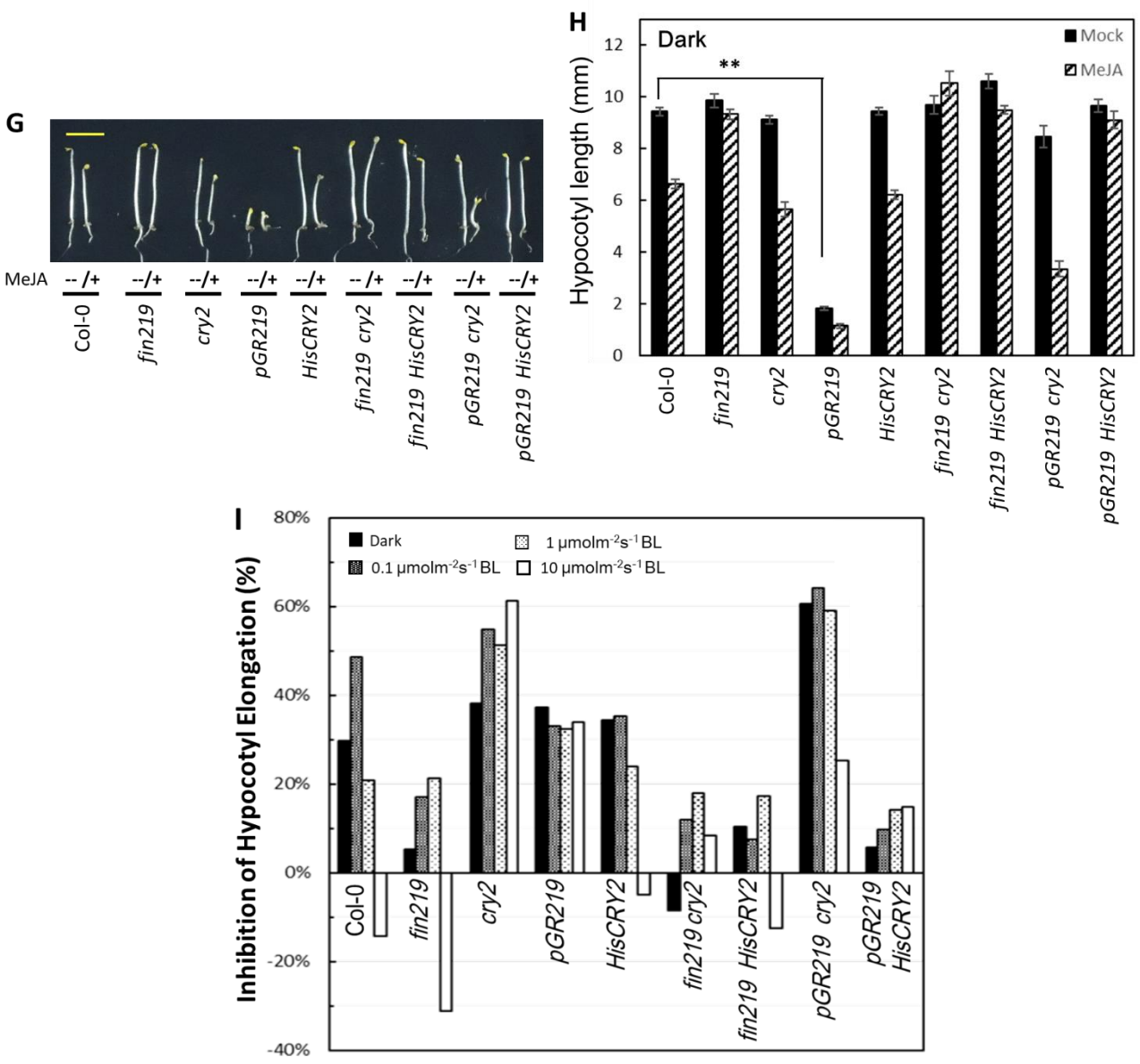

Fig.3 FIN219 and CRY2 mediate hypocotyl elongation in response to BL and exogenous MeJA.

Hypocotyl phenotype of the 3-day-old Col-0, *fin219*, *cry2*, *pGR219*, *HisCRY2*, *fin219cry2*, *fin219HisCRY2*, *pGR219cry2* and pGR219HisCRY2 seedlings under 10 μmolm^{-2}s^{-1} BL (A), 1 μmolm^{-2}s^{-1} BL (C), 0.1 μmolm^{-2}s^{-1} BL (E) or in darkness (G), with 50 μM MeJA treatment(+) or with 50 μM EtOH added as Mock(-). Scale bar: 5 mm. (B, D, F, H), the quantification of hypocotyl length of the seedlings showed in (A, C, E, G). Error bars indicated S.E.M. (n > 15). Significance shown by Student's t test, *: p<0.05, **: p<0.01. (I) Inhibition percentage of the hypocotyl elongation by MeJA was calculated according to the equation: [(Mock-MeJA)/Mock x 100%]. The value was from panel (B, D, F, H) under indicated BL intensity and in darkness.

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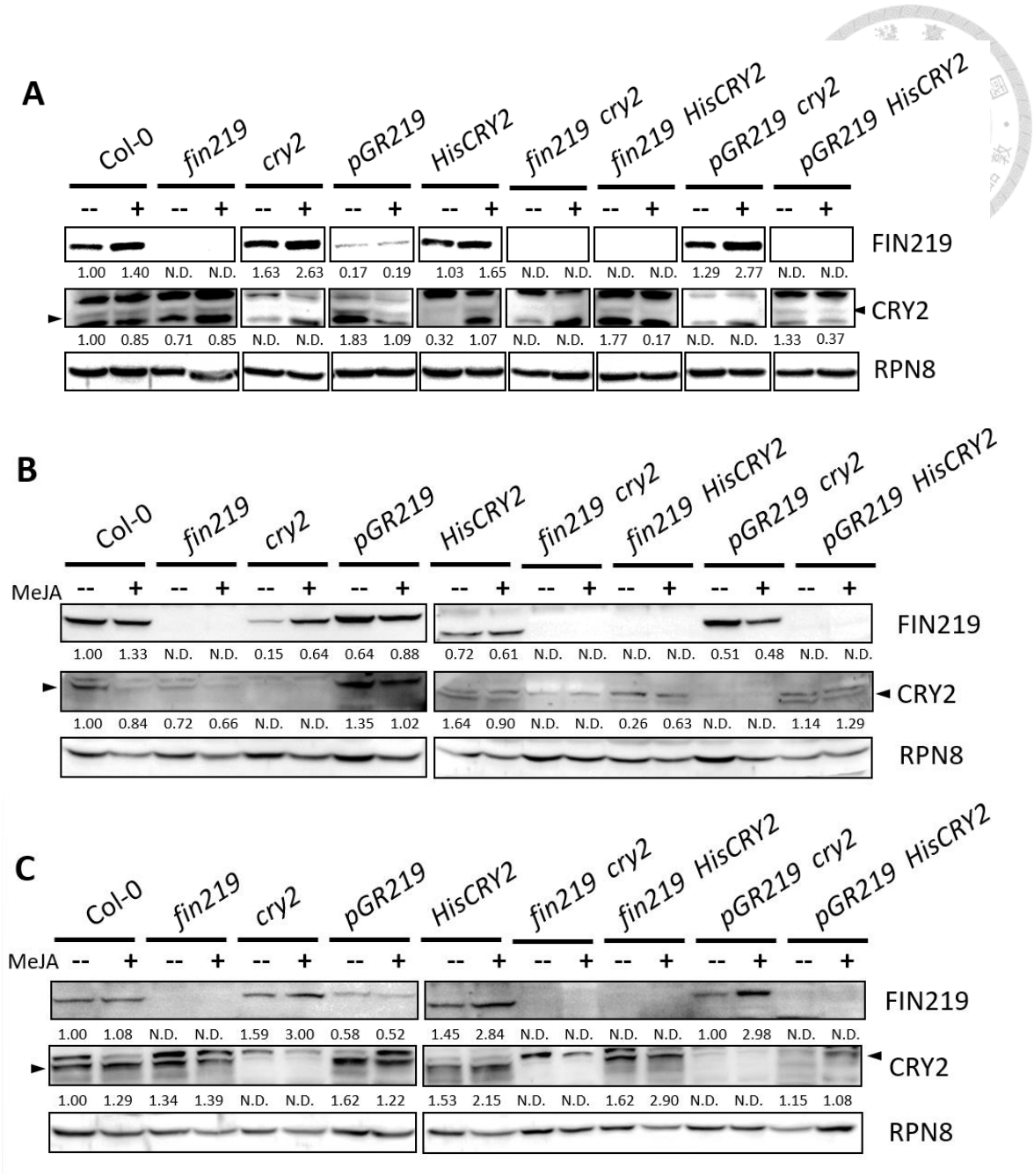

Fig. 4 The FIN219 and CRY2 protein levels under blue light and MeJA treatment.
Arabidopsis total protein of the 3-day-old indicated seedlings grown under 10 $\mu molm^{-2}s^{-1}$ BL (A), 1 $\mu molm^{-2}s^{-1}$ BL (B) or in darkness (C) with 50 μM MeJA treatment(+) or 50 μM EtOH added as Mock(-) were separated by SDS-PAGE and immunoblotted with antibodies. RPN8 was used as an internal control. The arrowheads indicated the CRY2 protein. Numbers indicated the protein levels standardized by RPN8.

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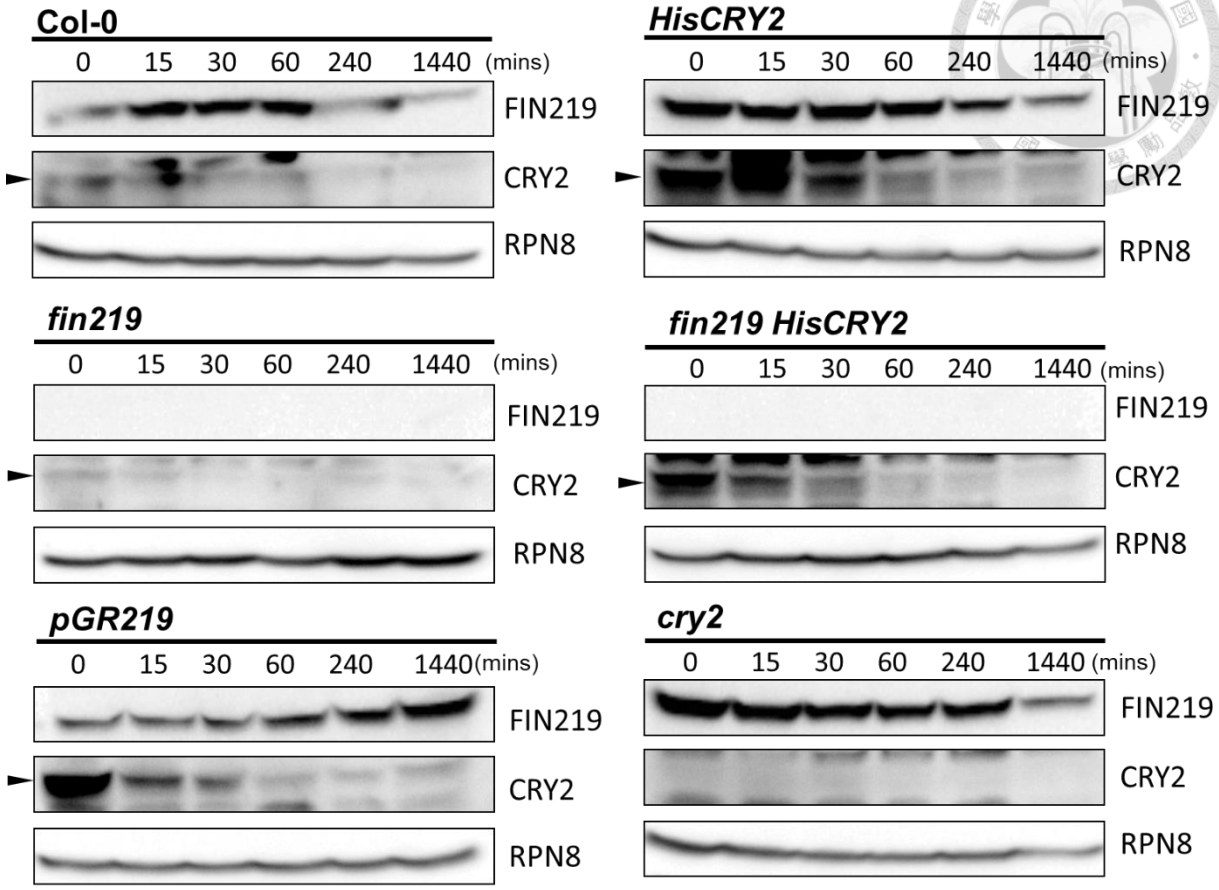

Fig.5 FIN219 protein enhances CRY2 protein accumulation under blue light.

Immunoblots showed FIN219 and CRY2 protein levels in the 3-day-old etiolated seedlings of *Col-0, fin219, pGR219, HisCRY2, fin219 HisCRY2* and *cry2* exposed to blue light (1 $\mu molm^{-2}s^{-1}$) for the indicated time. The FIN219 and CRY2 proteins were probed with antibodies. The RPN8 was used as an internal control. The arrowheads indicated the CRY2 protein.

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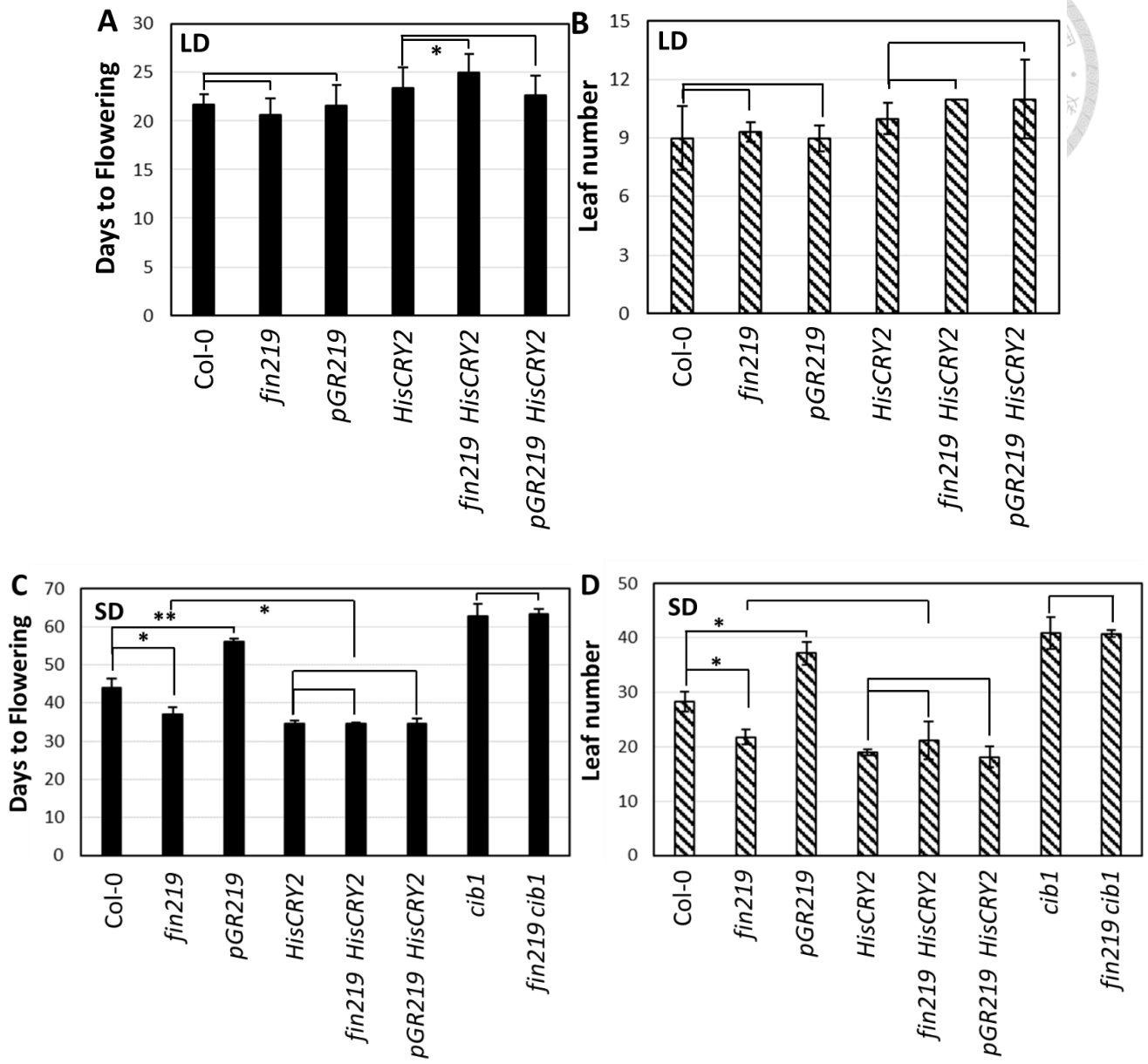

Fig. 6 Flowering phenotype under LD and SD conditions.

The quantitative flowering time measured as days to flowering (A, C) and the number of rosette leaves (B, D). Flowering time was determined at the day floral buds grown to 1 cm in height. Flowering phenotype of indicated genotypes grown in LD condition (A, B), and SD condition (C, D). Error bars indicated S.E.M. (n=10) Significance shown by Student's t test, *: $p<0.05$, **: $p<0.01$.

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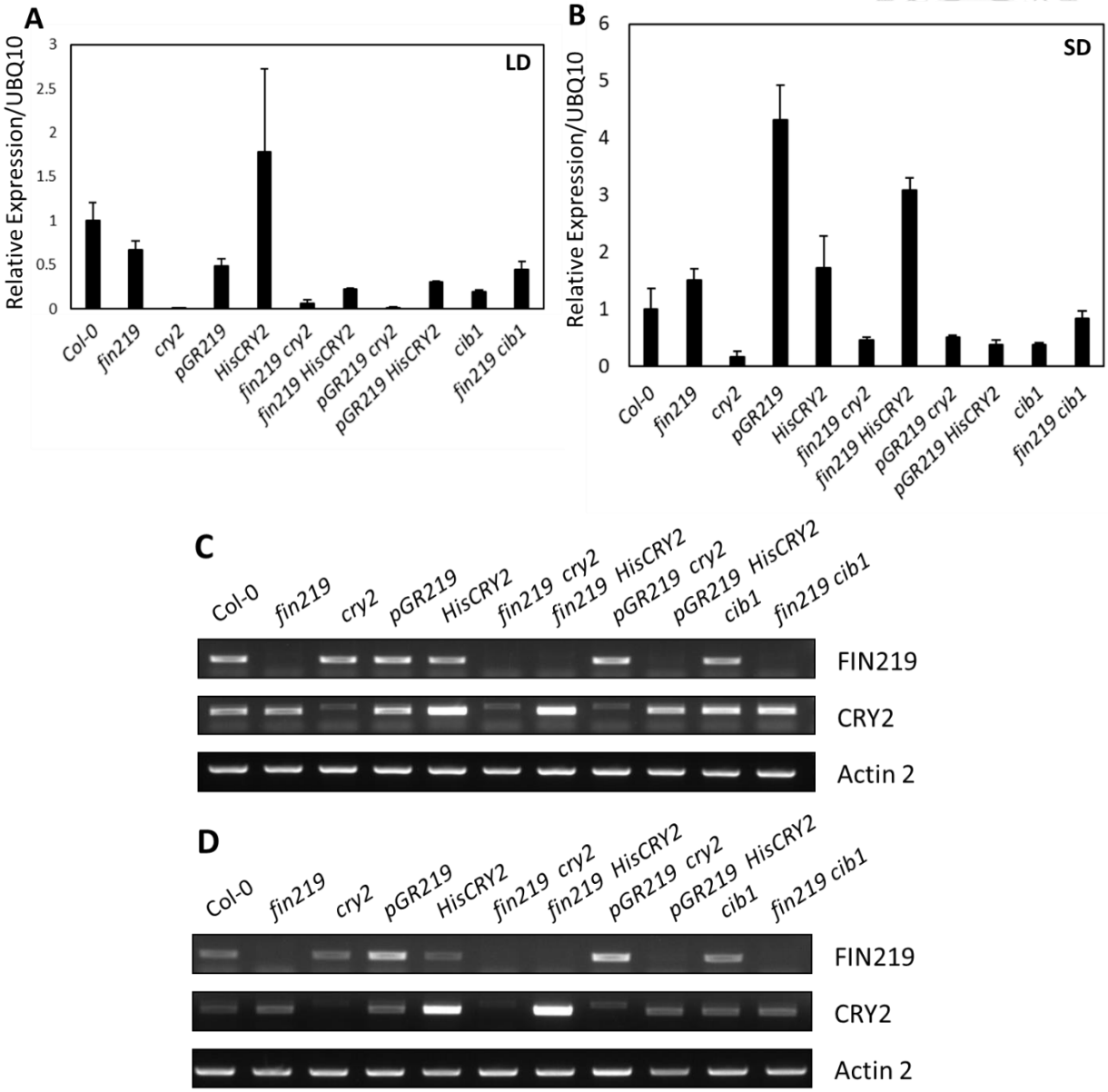


Fig. 7 Transcription of *FT* gene under LD and SD conditions.

Quantitative real-time PCR results showing mRNA expression of FT gene in 10-day-old light grown plants of indicated genotypes. The plants were grown in Petri dishes under LD (A) or SD (B) condition. Samples were collected at the 16 hrs of LD or at the 8 hour of SD. Expression levels were normalized to the UBQ10 mRNA level. Error bar represented S.E.M. (n=3). (C) and (D), the *FIN219* and *CRY2* mRNA expression levels detected by real-time PCR under LD (C) or SD (D), Actin2 was used as an internal control.

doi:10.6342/NTU201701965

REFERENCES

Ahmad, M., Jarillo, J., A., Cashmore, A., R. (1998a). Chimeric proteins between cry1 and cry2 *Arabidopsis* blue light photoreceptors indicate overlapping functions and varying protein stability. Plant Cell **10:** 197–208.

Ahmad, M., Jarillo, J., A., Smirnova, O., Cashmore, A., R. (1998b). The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A *in vitro*. Mol Cell **1:** 939–948.

Andrés, F., and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. Nature Reviews Genetics **13:** 627–639.

Arsovski, A. A., Galstyan, A., Guseman, J. M., and Nemhauser, J., L. (2012). Photomorphogenesis. The *Arabidopsis* Book e0147.

Banerjee, R., Schleicher, E., Meier, S., Viana, R., M., Pokorny, R., Ahmad, M., Bittl, R., Batschauer, A. (2007). The signaling state of *Arabidopsis* cryptochrome 2 contains flavin semiquinone. J Biol Chem **282:**14916–14922.

Bolouri Moghaddam, M., R., and Van den Ende, W. (2013). Sugars, the clock and transition to flowering. Front Plant Sci **4:** 22.

Briggs, W. R., and Christie, J. M. (2002). Phototropins 1 and 2: versatile plant blue-light receptors. Trends in Plant science **7:** 204-210.

Campos-Rivero, G., Osorio-Montalvo, P., Sánchez-Borges, R., Us-Camas, R., Duarte-Aké, F., and De-la-Peña, C. (2017). Plant hormone signaling in flowering: an epigenetic point of view. Journal of Plant Physiology **214:** 16.

Carvalhais, L. C., Schenk, P. M., and Dennis, P. G. (2017). Jasmonic acid signalling and the plant holobiont. Current Opinion in Microbiology **3:** 42-47.

doi:10.6342/NTU201701965

Chang, Tzu-Huang. (2015). Functional Studies of *Arabidopsis* FAR-RED INSENSITIVE219 and Cryptochrome2 interaction in the integration of blue light and JA signaling. National Taiwan University Master Thesis.

Chen, C., Huang, I. C., Liu, M. J., Wang, Z. G., Chung, S. S., and Hsieh, H. L. (2007). Glutathione S-transferase interacting with far-red insensitive 219 is involved in phytochrome A-mediated signaling in *Arabidopsis*. Plant Physiology **143:** 1189-1202.

Chen, C. Y., Ho, S. S., Kuo, T. Y., Hsieh, H. L., and Cheng, Y. S. (2017). Structural basis of jasmonate-amido synthetase FIN219 in complex with glutathione S-transferase FIP1 during the JA signal regulation. Proceedings of the National Academy of Sciences **114:** 1815-1824.

Deng, X. W., and Quail, P., H. (1999). Signalling in light-controlled development. In Seminars in Cell & Developmental Biology **10:** 121-129.

Du, Xin-Jie. (2005). Investigation of the Involvement of *Arabidopsis* FIN219 in the Integration of Blue Light and Ethylene Signaling Pathways. National Taiwan University Master Thesis.

Duek, P., D., Elmer, M., V., van Oosten, V.R., and Fankhauser, C. (2004). The degradation of HFR1, a putative bHLH class transcription factor involved in light signaling, is regulated by phosphorylation and requires COP1. Current Biology **14:** 2296-2301.

Fairchild, C. D., Schumaker, M.A., and Quail, P.H. (2000). HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction. Genes Dev **14:** 2377-2391.

Fornara, F., de Montaigu, A., and Coupland, G. (2010). SnapShot: Control of flowering in *Arabidopsis*. Cell **141:** 550–550.

doi:10.6342/NTU201701965

Fornara, F., Panigrahi, K. C., Gissot, L., Sauerbrunn, N., Rühl, M., Jarillo, J.A. and Coupland, G. (2009). *Arabidopsis* DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. Development Cell **17:** 75–86.

Fu, Tsu-Yu. (2008). Functional studies of *Arabidopsis* FIN219 in cross-talks between blue light and far-red light signaling. National Taiwan University Master Thesis.

Helliwell, C.A., Wood, C.C., Robertson, M., James Peacock, W., and Dennis, E.S. (2006). The *Arabidopsis* FLC protein interacts directly *in vivo* with SOC1 and FT chromatin and is part of a highmolecular- weight protein complex. Plant Journal **46:** 183–192.

He, S. B., Wang, W. X., Zhang, J. Y., Xu, F., Lian, H. L., Li, L., Yang, H. Q. (2015). The CNT1 domain of *Arabidopsis* CRY1 alone is sufficient to mediate blue light inhibition of hypocotyl elongation. Molecular Plant **8:**822–825.

Hoang, N., Bouly, J. P., Ahmad, M. (2008). Evidence of a light-sensing role for folate in *Arabidopsis* cryptochrome blue-light receptors. Mol Plant **1:**68–74

Hornitschek, P., Lorrain, S., Zoete, V., Michielin, O., and Fankhauser, C. (2009). Inhibition of the shade avoidance response by formation of non-DNA binding bHLH heterodimers. The EMBO Journal **28:** 3893-3902.

Hsieh, H. L., Okamoto, H., Wang, M., Ang, L. H., Matsui, M., Goodman, H., and Deng, X. W. (2000). FIN219, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. Genes & Development **14:** 1958-1970.

doi:10.6342/NTU201701965

Jang, I.C., Henriques, R., Seo, H.S., Nagatani, A. and Chua, N.H. (2010). *Arabidopsis* PHYTOCHROME INTERACTING FACTOR proteins promote phytochrome B polyubiquitination by COP1 E3 ligase in the nucleus. Plant Cell **22:** 2370-2383.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. and Weigel, D. (1999). Activation tagging of the floral inducer FT. Science **286:** 1962–1965.

Kennedy, M. J., Hughes, R. M., Peteya, L. A., Schwartz, J. W., Ehlers, M. D. and Tucker, C. L. (2010). Rapid blue-light-mediated induction of protein interactions in living cells. Nature Methods **7:** 973–975.

Kronenberg, G. H. and Kendrick, R. E. (1994). Photomorphogenesis in plants.

Leivar, P. and Quail, P.H. (2011). PIFs: pivotal components in a cellular signaling hub. Trends in Plant Science **16:** 19-28.

Lian, H. L., He, S. B., Zhang, Y.C., Zhu, D.M., Zhang, J.Y., Jia, K. P., Sun, S. X., Li, L., Yang, H. Q. (2011). Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. Genes & Development **25:**1023–1028.

Liu, B., Zuo, Z., Liu, H., Liu, X., Lin, C. (2011). *Arabidopsis* cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. Genes & Development **25:**1029–1034.

Liu, H., Liu, B., Zhao, C., Pepper, M., Lin, C. (2011). The action mechanisms of plant cryptochromes. Trends in Plant Science **16:** 684-691.

Liu, H., Yu, X., Li, K., Klejnot, J., Yang, H., Lisiero, D., Lin, C. (2008). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in *Arabidopsis*. Science **322:** 1535-1539.

doi:10.6342/NTU201701965

Liu, Y., Li, X., Li, K., Liu, H., Lin, C. (2013). Multiple bHLH proteins form heterodimers to mediate CRY2-dependent regulation of flowering- time in *Arabidopsis*. PLoS Genetics **9:** e1003861.

Liu, Q., Wang, Q., Liu, B., Wang, W., Wang, X., Park, J., and Lin, C. (2016a). The blue light-dependent polyubiquitination and degradation of *Arabidopsis* Cryptochrome2 requires multiple E3 ubiquitin ligases. Plant and Cell Physiology **57:** 2175-2186.

Liu, B., Yang, Z., Gomez, A., Liu, B., Lin, C., and Oka, Y. (2016b). Signaling mechanisms of plant cryptochromes in *Arabidopsis thaliana*. Journal of Plant Research **129:** 137.

Lo, Yi-Ying. (2015). Functional Studies of FIN219, COP1 and COI1 Proteins in the Integration of Far-red Light and Jasmonate Signaling in *Arabidopsis*. National Taiwan University Master Thesis.

Malhotra, K., Kim, S.T., Batschauer, A., Dawut, L., and Sancar, A. (1995). Putative blue-light photoreceptors from *Arabidopsis* thaliana and Sinapis alba with a high degree of sequence homology to DNA photolyase contain the two photolyase cofactors but lack DNA repair activity. Biochemistry **34:** 6892–6899.

Mathieu, J., Yant, L.J., Mürdter, F., Küttner, F., and Schmid, M. (2009). Repression of flowering by the miR172 target SMZ. PLoS Biology **7:** e1000148.

Más, P., Devlin, P. F., Panda, S., and Kay, S. A. (2000). Functional interaction of phytochrome B and cryptochrome 2. Nature **408:** 207–211

Mockler, T.C., Guo, H., Yang, H., Duong, H., and Lin, C. (1999). Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. Development **126(10):** 2073–2082

doi:10.6342/NTU201701965

Mockler, T., Yang, H., Yu, X., Parikh, D., Cheng, Y.C., Dolan, S., and Lin, C. (2003) Regulation of photoperiodic flowering by *Arabidopsis* photoreceptors. Proc Natl Acad Sci USA **100:** 2140–2145

Osterlund, M.T., Hardtke, C.S., Wei, N., and Deng, X.W. (2000). Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. Nature **405:** 462-466.

Oyama, T., Shimura, Y., and Okada, K. (1997). The *Arabidopsis* HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes Development **11:** 2983-2995.

Pérez, A. C., and Goossens, A. (2013). Jasmonate signalling: a copycat of auxin signalling?. Plant, cell & environment **36(12):** 2071-2084.

Pokorny, R., Klar, T., Hennecke, U., Carell, T., Batschauer, A., and Essen, L.O. (2008). Recognition and repair of UV lesions in loop structures of duplex DNA by DASH-type cryptochrome. Proc Natl Acad Sci USA **105:** 21023–21027.

Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The CONSTANS gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80:** 847–857.

Riboni, M., Robustelli Test, A., Galbiati, M., Tonelli, C., and Conti, L. (2014). Environmental stress and flowering time: The photoperiodic connection. Plant Signaling & Behavior **9:** e29036.

Ritz, T., Yoshii, T., Helfrich-Foerster, C., and Ahmad, M. (2010). Cryptochrome: a photoreceptor with the properties of a magnetoreceptor? Commun Integr Biol **3:** 24–27.

doi:10.6342/NTU201701965

Rizzini, L., Favory, J. J., Cloix, C., Faggionato, D., O'Hara, A., Kaiserli, E., and Ulm, R. (2011). Perception of UV-B by the *Arabidopsis* UVR8 protein. Science **332:** 103-106.

Rosenfeldt, G., Viana, R. M., Mootz, H. D., von Arnim, A. G., and Batschauer, A. (2008). Chemically induced and light-independent cryptochrome photoreceptor activation. Mol Plant **1:** 4–14.

Sang, Y., Li, Q. H., Rubio, V., Zhang, Y. C., Mao, J., Deng, X. W., and Yang, H. Q. (2005). N-terminal domain-mediated homodimerization is required for photoreceptor activity of *Arabidopsis* CRYPTOCHROME 1. Plant Cell **17:**1569–1584.

Schaller, A. and Stintzi, A. (2009). Enzymes in jasmonate biosynthesis–structure, function, regulation. Phytochemistry **70:** 1532-1538.

Shalitin, D., Yang, H., Mockler, T. C., Maymon, M., Guo, H., Whitelam, G. C., and Lin C. (2002). Regulation of *Arabidopsis* cryptochrome 2 by blue-light-dependent phosphorylation. Nature **417:** 763–767.

Shalitin, D., Yu, X., Maymon, M., Mockler, T., Lin, C. (2003). Blue lightdependent *in vivo* and *in vitro* phosphorylation of *Arabidopsis* cryptochrome 1. Plant Cell **15:**2421–2429.

Shin, J., Kim, K., Kang, H., Zulfugarov, I.S., Bae, G., Lee, C.H., Lee, D., and Choi, G. (2009). Phytochromes promote seedling light responses by inhibiting four negatively-acting phytochrome-interacting factors. Proc Natl Acad Sci USA **106:** 7660-7665.

Smyth, D.R., Bowman, J.L., Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell **2:** 755–767.

doi:10.6342/NTU201701965

Song, Y.H., Ito, S., and Imaizumi, T. (2013). Flowering time regulation: photoperiod- and temperature-sensing in leaves. Trends in Plant Science **18:**575–583.

Srikanth, A., and Schmid, M. (2011). Regulation of flowering time: all roads lead to Rome. Cellular and molecular life sciences **68:** 2013-2037.

Staswick, P. E., Tiryaki, I., and Rowe, M. L. (2002). Jasmonate response locus JAR1 and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell **14:** 1405-1415.

Sullivan, J. A. and Deng, X. W. (2003). From seed to seed: the role of photoreceptors in *Arabidopsis* development. Developmental biology **260:** 289-297.

Tan, S. T., Dai, C., Liu, H. T., and Xue, H. W. (2013). *Arabidopsis* casein kinase1 proteins CK1.3 and CK1.4 phosphorylate cryptochrome2 to regulate blue light signaling. Plant Cell **25:** 2618–2632.

Usami, T., Mochizuki, N., Kondo, M., Nishimura, M., and Nagatani, A. (2004). Cryptochromes and phytochromes synergistically regulate *Arabidopsis* root greening under blue light. Plant Cell Physiology **45:**1798–1808.

Wasternack, C. and Song, S. (2016). Jasmonates: biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. Journal of Experimental Botany **68:** 1303-1321.

Wang, J. G., Chen, C. H., Chien, C., T., and Hsieh, H. L. (2011). FAR-RED INSENSITIVE219 modulates CONSTITUTIVE PHOTOMORPHOGENIC1 activity via physical interaction to regulate hypocotyl elongation in *Arabidopsis*. Plant Physiology **156:** 631-646.

doi:10.6342/NTU201701965

Wang, H., Ma, L., G., Li, J., M., Zhao, H., Y., and Deng, X., W. (2001). Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. Science **294:**154–158.

Yang, H., Q., Tang, R., H., and Cashmore, A., R. (2001). The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. Plant Cell **13:**2573–2587.

Yang, H., Q., Wu, Y., J., Tang, R., H., Liu, D., Liu, Y., and Cashmore, A., R. (2000). The C termini of *Arabidopsis* cryptochromes mediate a constitutive light response. Cell **103:**815-827.

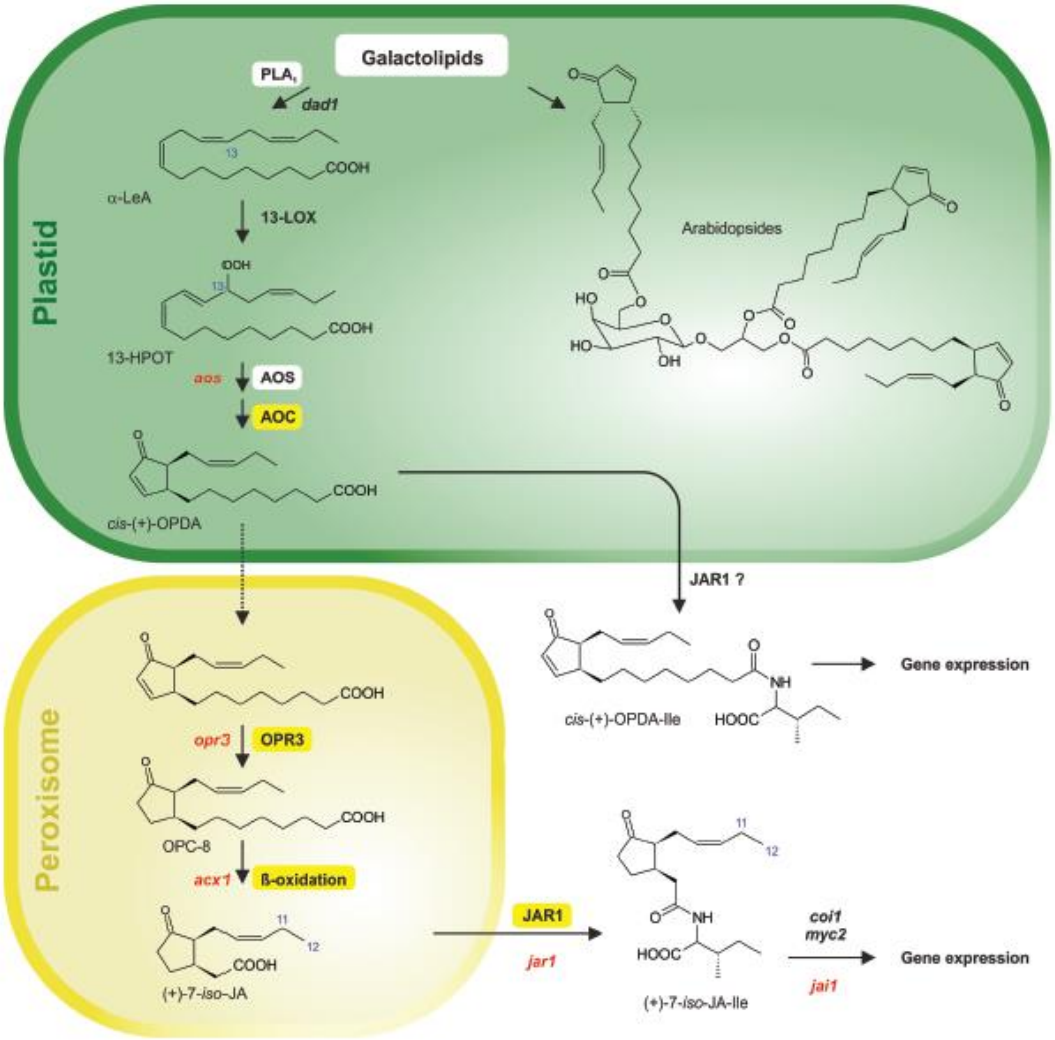
Yi, C. and Deng, X., W. (2005). COP1-from plant photomorphogenesis to mammalian tumorigenesis. Trends Cell Biol **15:** 618–625.

Zhai, Q., Zhang, X., Wu, F., Feng, H., Deng, L., Xu, L. and Li, C. (2015). Transcriptional mechanism of jasmonate receptor COI1-mediated delay of flowering time in *Arabidopsis*. Plant Cell **27:** 2814-2828.

Zuo, Z., Liu, H., Liu, B., Liu, X., and Lin, C. (2011). Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in *Arabidopsis*. Current Biology **21:**841–847.

doi:10.6342/NTU201701965

APPENDIX



App.1 Synthesis of JA/JA-Ile from α-linolenic acid generated from galactolipids. (Wasternack *et al*. 2016)

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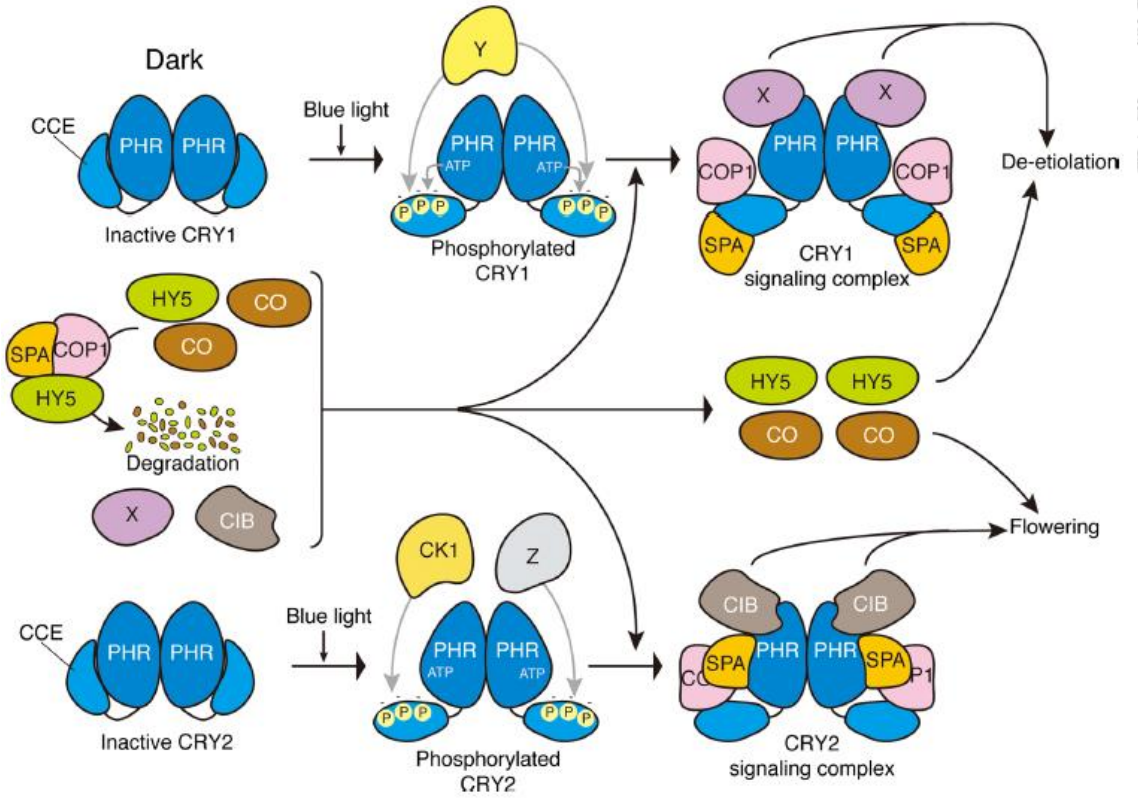

App.2 A model depicting cryptochrome signal transduction mechanisms in *Arabidopsis.* (Liu *et al*. 2016b)

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