THESIS FOR DEGREE OF MASTER OF SCIENCE

CONSTITUTIVE PHOTOMORPHOGENIC 1 is involved in gibberellic acid-mediated germination by repressing *RGA-LIKE 2* in *Arabidopsis thaliana*

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ABSTRACT

In flowering plants, germination is a sophisticated process, which is regulated by the cross-talk between endogenous signals and environmental cues such as hormones, light, water and temperature. GA hormone is a key regulator of seed germination. Many genes are involved in GA-mediated seed germination pathway. Among them, *RGL2* has been considered to be the major negative regulator by repressing germination associated genes. Here, we showed that *COP1* is closely involved in regulating GA-mediated seed germination. We found that the germination rate of *cop1* mutants were strongly decreased by paclobutrazol (PAC) treatments, that is inhibitor of GA biosynthesis, compared with Wild-type. However, germination of *COP1* overexpressed-transgenic plants is insensitive to PAC. Analysis of western blot using antibody against COP1 recombinant proteins demonstrated that GA affected on COP1 protein stability. While imbibed wild-type seeds under GA present condition significantly increased COP1 protein than that in mock

(distilled water) condition, COP1 protein was decreased by PAC treatment. The genetic study of *cop1-4 rgl2* double mutants provided strong evidence that *COP1* act as upstream negative regulator of *RGL2*. Further analysis by BiFC and in vitro pull-down assay indicated that COP1 physically interacts with RGL2. RGL2 protein was degraded in COP1 overexpressed plants. These results suggested that COP1 is partially involved negatively regulates in RGL2 protein stability. *COP1* regulates the transcript levels of the genes associated with germination such as *GASA6, EXPA1, EXPA2, EXPA8,* and *XTH33*. Taken together, our results suggested that COP1 regulates GA-mediated seed germination through degradation of RGL2 proteins.

Keywords: seed germination, gibberellic acid, *RGA-LIKE 2 (RGL2)*, *CONSTITUTIVE- PHOTOMORPHOGENIC 1 (COP1)*

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ABBREVIATION

COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
RGL2	RGA-LIKE 2
ACT2	ACTIN 2
GID1	GIBBERELLIN INSENSITIVE DWARF 1
SLY1	SLEEPY 1
PIF1	PHYTOCHROME INTERACTING FACTOR 1
GA	Gibberellic Acid
ABA	Abscisic Acid
PAC	Paclobutrazol
cDNA	Complementary Deoxyribosenucleic Acid
BiFC	Bimolecular Fluorescence Complementation
YFP	Yellow Fluorescent Protein
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription PCR
GST	Glutathione S-transferase
DIC	Differential Interference Contrast
LD	Long-day
DW	distilled water

INTRODUCTION

Seed germination is an immense importance stage initiating the life cycle of a plant, is therefore tightly regulated by external factors such as light, water, temperature and the major internal factor, phytohormones such as gibberellic acid (GA), abscisic acid (ABA), auxin, and ethylene [1, 2]. Among them, two classes of phytohormones, ABA and GA, antagonistically regulate seed germination. ABA functions to maintain seed dormancy, whereas GA releases dormancy and promotes germination [3-8].

The GA hormone concentration is important in regulating seed germination. At low endogenous GA levels, a group of GA signaling repressor protein, DELLA, represses GA responses, such as seed germination, stem elongation, leaf expansion, flowering and other GA-mediated processes [9, 10]. Whereas, in the presence of bioactive GA, the hormone binds to the *GIBBERELLIN INSENSITIVE DWARF 1* (*GID1*) receptor [11]. GA-GID1 complex is trans-located from the cytoplasm to the nucleus. In the nucleus, the GA-*GID1* complex enhances the interaction between *GID1* and DELLA-protein. Then, the F-box protein *SLEEPY 1* (*SLY1*;a subunit of SCF^{SLY1} complex) binds to the DELLA-GA-*GID1* complex, resulting in DELLA protein is rapidly degraded by ubiquitin-proteasome pathway, followed by expressing GA-response genes [9, 11-15]. Taken together, the present of GA hormone reduces DELLA protein stability for expression of germination-associated genes [16].

DELLA was first identified as the key repressor in GA-dependent manner, including seed germination, stem elongation, and transition to flowering [16-18]. The DELLA protein is a sub-family of the GRAS family, which contains negative regulators of GA signaling [17, 19]. DELLA family consists 5 kinds of genes as *GA INSENSITIVE* (*GAI*), *REPRESSOR OF ga1-3* (*RGA*), *RGA-LIKE 1* (*RGL1*), *RGA-LIKE 2* (*RGL2*), and *RGA-LIKE 3* (*RGL3*) [17, 20-23]. Based on previous genetic studies, *GAI*, *RGA* and *RGL1* are involved in stem elongation, and *RGL3* is involved in jasmonic acid and ethylene-associated defense response [16, 24] and *RGL2* has a key role in regulating GA-mediated seed germination [21]. In the defect of GA, *ga1-3* mutants or PAC (GA synthesis inhibitor) treatment condition, only *rgl2* mutation can rescue the seed germination rate in the presence of light, but in the dark, GAI and RGA are necessary for germination [21, 25, 26].

Light is also well-known for a major environmental factor that regulates seed germination, in which germination rate is affected by the present or absent of light in Arabidopsis. In dark condition, germination rate is less than light condition [26]. In previous studies, Phytochromes (photo-receptor) and *PHYTOCHROME INTERACTING FACTOR 1* (*PIF1/PIL5*) have a critical role in seed germination [1]. The Phytochrome, PHYA and PHYB, play a role in perceiving red (R) and far-red (FR) light. Under light condition, Pr changes to Pfr, in which PIF1 interacts with the Pfr form of phytochrome [27]. This interaction induces degradation of PIF1 protein through the ubiquitin-

dependent proteolysis [27-29]. PIF1 negatively regulates seed germination through inducing the transcription levels of *GAI*, *RGA* and *SOMNUS* (*SOM*) [30], which are negative and positive regulator of GA and ABA biosynthesis, respectively, leading to changes in amount of endogenous ABA and GA balances [31].

CONSTITUTIVE PHOTOMORPHOGENIC 1 (*COP1*) is tightly involved in light signaling pathway. *COP1* is multi-functional gene which is involved in plant developmental process including seed germination, skoto-/photo-morphogenesis, stress response, circadian clock, flowering, and seed maturation [32]. COP1 protein has three domains; the zinc-binding motif RING domain, coiled-coil region, and the multiple domain WD-40 repeat. Acts as RING type E3 ligase [33-36], in which *COP1* normally degrades target proteins by ubiquitin-proteasome-dependent proteolysis system, such as *GI* and *CO* for flowering*, HY5*, *HYH, LAF* for photomorphogenesis [37-41], and others. During seed germination, *COP1* negatively regulates *PIF1* protein stability in the light-dependent [42, 43]. However, the function of *COP1* in regulating seed germination is not clearly understood yet.

Therefore, this study uncovers a new GA-mediated germination pathway, which *COP1* acts as a critical negative regulator of *RGL2* in light-independently

MATERIALS AND METHODS

Plant materials and growth conditions

All Arabidopsis thaliana plants used in this study were Columbia-0 ecotype, with the exception of the *cop1-5* and *fus9-1* mutants in Ws background. Arabidopsis mutant lines used in this study; *cop1-4* [44] , *gai-t6* [45], *rga-28* (SALK_089146), *rgl1-SK62* (SALK_136162), *rgl2-SK54* (SALK_027654), *rgl3-3* (CS16355), *hy5-205* [46], *pif1-1* [47]. We generated the *cop1-4 rgl2-SK54* double mutant by genetic cross between *cop1-4* and *rgl2-SK54*, in which *cop1 rgl2* double mutant were selected by the Derived Cleaved Amplified Polymorphic Sequence (dCAPS) method for *cop1-4* mutant allele, and by genotyping with the specific primers for *rgl2-SK54* (Table1). To generate the *35S::COP1-GFP* transgenic plant, the full length COP1 cDNA was amplified from the first-strand cDNA of WT (Col-0) using gene-specific primers. The PCR-amplified *COP1* cDNA was ligated into the pDONR221 vector (Invitrogen), and then introduced into the pMDC85 vector for the expression of GFP-tagged *COP1* constructs. For the *COP1* overexpressing transgenic plants, cloned binary vector was transformed into Col-0, cop1-4, and *rgl2-SK54* mutant plants, using the floral dip transformation method [48]. Plants were grown on Murashige-Skoog (MS) medium containing 1% sucrose and 2mM MES (pH 5.7) buffer or on soil in the growth chambers at constant 22°C under cool white fluorescent light (100μmol m^{-2} s^{-1}) under long days (LD; 16-h

light/ 8-h dark) condition.

Germination assay

Fresh seeds of all genotypes were harvested within a month and stored at 4℃ before testing. Seeds were sterilized with 70% (v/v) ethanol containing 0.1% Triton X-100 for 20min, and rinsed three times with 100% ethanol. After air-dried on auto-cleaved 3M filter paper and seeds were plated on MS solid medium (Duchefa) containing 1% sucrose with or without Paclobutrazol (PAC) and GA_3. The plates were kept at 4^{o}C in darkness for 72h for stratification and then transferred to a growth chamber set at 22^{o}C under LD condition. The radicle tip emergence was defined as the first sign of seed germination. Seeds were scored at indicated time until seed germination rate reached over 98%. The average germination rate was calculated based on at least three independent replicates.

Yeast two-hybrid assays

The yeast two-hybrid assay was performed according to the instructions provided with the Matchmaker GAL4 two hybrid system (Clontech). The full and partial (RING; aa 1~104, CC; aa 121~213, WD-40 repeat; aa 371~675) cDNAs of *COP1* were cloned into the pGBK vector (as bait) [49], and *RGL2* full length cDNA was cloned into pGAD vector (as prey). Corresponding pairs of plasmids were transformed into the yeast strain AH109 as described in the

Yeast protocols Handbook (Clontech). Yeast- transformants were then plated on minimal SD/-Trp,-Leu agar plates for 3d at 30 ^{o}C. Finally, well-grown colonies were plated onto minimal SD/Gal/Raf/-Trp-Leu-His-Ade agar plates containing 5-bromo-4-chloro-3-indolylb-D-galactopyranoside (X-gal) for our interaction test. Liquid culture was used for Chlorophenol red-β-D-galactopyranoside (CPRG) assay to measure *β*-galactosidase activity according to the manufacturer's protocol.

Biomolecular Fluorescence Complementation (BiFC) assay

The full length cDNAs of COP1 and RGL2 were amplified and cloned into the pCR8/GW/TOPO vector (Invitrogen), followed by subcloning into the pCR8/GW/TOPO vector (Invitrogen, USA). LR recombinants using the Lambda integrase/excisionase (Elpis-Biotech) were introduced into the BiFC plasmid sets: pSAT5-DEST-cEYFP(175-end)-C1 (pE3130), pSAT5(A)-DEST-cEYFP(175-end)-N1 (pE3132), pSAT4(A)-DEST-nEYFP(1-174)-N1 (pE3134) and pSAT4-DEST-nEYFP(1-174)-C1 (pE3136). Each pair of recombinant plasmids encoding nEYFP or cEYFP fusion proteins was co-bombarded into onion epidermal cell layers using a DNA particle delivery system (Biolistic PDS-1000/He, Bio-Rad), and incubated with 50μM MG132 in MS phytoagar medium for 16 h at 22°C under darkness, followed by image analysis using confocal laser scanning microscopy (SP8 X STED, Leica, Germany). pEarleyGate104 (YFP vector), pEarleyGate104-COP1 (COP1-YFP) and

pEarleyGate104-RGL2 (RGL2-YFP) clones were used as positive controls.

Western-Blot Analysis

Total soluble protein from imbibed seeds was extracted using UREA buffer [50mM Tris-HCl pH 6.8, 150mM NaCl, 1mM DTT, 1Mm EDTA, 50μM MG132, protease inhibitor cocktail]. 100μg of total protein was sperated on 12% SDS-PAGE gels, and transferred to an Immobilon-P PVDF transfer membrane (Millipore). The membrane was blocked with 2% bovine serum albumin in phosphate-buffered saline (pH 7.5), and incubated overnight with primary antibody. After washing three times for 10 min each, the membrane was incubated with secondary antibody.

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In vitro GST pull-down assays

Full length cDNA of *RGL2* was cloned into pGEX-4T-1 vector (Pharmacia) for GST-RGL2 fusion protein, and transformed into the BL21-CodonPlus (Stratagene) *E. coli* strain. GST and GST-RGL2 were induced by IPTG method, and purified using Glutathione Sepharose resin beads (ELPIS biotech, Korea) according to the manufacturers' instruction. MBP and MBP-COP1 fusion protein were induced in BL21-CodonPlus (Stratagene) *E. coli* strain [50], and purified using Amylose resin beads (ELPIS bioteth, Korea). For pull down assay, 2ug of GST and GST-RGL2 protein were incubated with immobilized MBP and MBP-COP1 protein in the binding buffer (50mM Tris-

HCl [pH 8.0], 150mM NaCl and 1mM EDTA), and incubated at 4°C for 2h. After washing three times with binding buffer, protein retained beads were resolved by SDS sample buffer, and immunoblotted using anti-GST and anti-MBP antibodies (Santacruz).

Cell free degradation assay

GST-tagged RGL2 protein was prepared from BL21-CodonPlus (Stratagene), and purified using Glutathione Sepharose resin beads (ELPIS biotech, Korea) according to the manufacturers' instruction. GST-RGL2 protein was incubated at 30°C with 100ug total soluble protein in assay buffer [50mM Tris-HCl (pH7.5), 100mM NaCl, 10mM MgCl2, 5mM DTT, and 5mM ATP] from Col-0, cop1-4 and 35S::COP1-GFP seedling. The plants were grown in LD for 10days and harvested at ZT 0. The reaction was stopped by adding of SDS sample buffer at each indicated time.

Quantitative RT-PCR

Seeds were grown under 3days dark condition and 1day under light condition, 22℃ with soaking. Total RNA was extracted from germinating seeds using the Fruit-mate™ (Takara, Japan) and MG™ RNAsol (Macrogen, korea) according to the Takara's instructions. First strand cDNA was synthesized from 2ug total RNA using oligo $(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA), and diluted with water to 70ul.

Total 20ul of mixture included 1ul of 0.5uM primer, 3ul of cDNA mixture and 10ul of 2X QuantiTect LightCycler 480 SYBR Green I Master mix (Roche). PCR was performed by Light Cycler 480 Real-Time PCR System (Roche, Basal, Switzerland), using the following program : 95℃ for 2 min, 50 cycles of 95℃ for 10 sec, 59℃ for 10 sec and 72℃ for 10 sec. Relative expression levels of each genes were measured by RT-qPCR using gene-specific primers and *Actin2 (ACT2)* was used for an internal control. All real-time PCR were repeated at least three times (biological replicates). Primer sequences used were listed in Table 1.

Table1. Primers information used for this experiment

Gene	Forward primer(5'→3')	Reverse primer(5'→3')
A. dCAPS primers		
cop1-4	AGAAGGATGCGCTGAGTGGGTCAGACTAG	TGCCATTGTCCTTTTACCATTTCAGC
B. Genotyping primers		
RGL2	CTGCGTTTCCAAAGGAAGAG	GTCGGATCCTCTTGCTGCTA
C. quantitative real-time PCR primers		
UBQ1	CGCCAAGATCCAAGACAAAG	GTTGACAGCTCTTGGGTGAA
ACT2	TGGGATGAACCAGAAGGATG	AAGAATACCTCTCTTGGATTGTGC
COP1	TTCAGCCAACATTGTATCAAGC	AAACACCAGCAGTGGCAAA
RGL2	GGTAGAGATGACTCGCCTGA	CAAAGATACGCACAAGGTCC
GASA6	AGAAACCCCAATCTGTTTCC	GAAGGTCCATACACATTTCG
ABI5	ATGAGGAACCCGAGTTGTCC	CAGATGGTGTTCCTCCTACC
EXPA1	GTCCTTTCTTTCAATTGAGG	CAACTCAATACCTCTGC
EXPA2	TCGTTCCTGTCGCATTCAG	GATTCCCGTTTATCGTAAACCTT
EXPA8	GACGTGGCTCCTTCTAATTG	GGCACAATGAAAATACAACC
XERICO	CTATTGGAACATCACTTGCC	ATCTGCTCGAGAATCAACCG

RESULTS

COP1 is involved in GA-mediated seed germination.

The strong allele mutant of *COP1*, *cop1-5*, has a dark purple color seed, very late or failed germination, abnormal seedling and lethal phenotype [1]. To investigate a cause of late germination phenotype, we measured germination rate of wild-type (Ws), *cop1-5*, and *fus9-1* seeds imbibed in untreated (1/2MS) (Figure 1A) or 10μM GA treated medium (Figure 1B). *fus9-1* is used as a seed color negative control of cop1-5. The seeds were incubated in chilling condition for 3days after plating, and transferred to LD condition. After-ripening of seeds for 20 days resulted in the late germination phenotype of *cop1-5* was partially rescued and total germination rate was increased under GA treatment condition. However, the *fus9-1* seeds were not showed different germination phenotype with wild-type under both 1/2MS and GA treatment conditions, indicating that *COP1* may involve in GA-mediated germination pathway.

We then wondered whether different allele *cop1* mutant also shows same phenotype in response to GA. We measured germination rate of the *COP1* weak allele mutant, *cop1-4* (Columbia background), and *COP1* overexpression *35S::COP1-GFP* seeds comparing with Wild-type (Col-0) imbibed in supplementation 10μM GA (Figure 2B) or 10μM PAC (Figure 2C)

and untreated condition (Figure 2A). The *cop1-4* mutant has a slightly late germination phenotype. Consistently, we found that the *cop1-4* germination rate also recovered to WT levels in response to GA. Furthermore, the *cop1-4* mutant showed PAC hyper-sensitive phenotype. In contrast, *35S::COP1-GFP* plants showed early germination phenotype in 1/2MS condition, and exhibited PAC insensitive phenotype. These results show that COP1 is involved in GA-mediated germination.

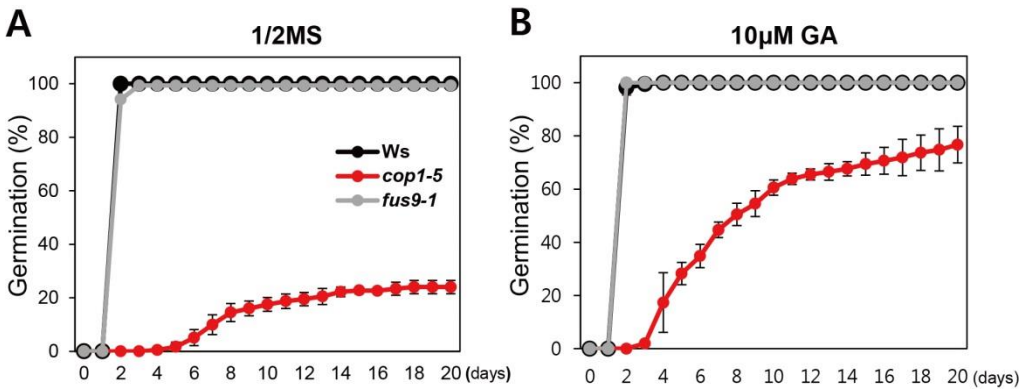

Figure 1. Germination phenotype of *cop1* strong allele mutant, *cop1-5*, under GA and PAC treatment condition.

Time-course seed germination analysis of the wild-type (Ws), *cop1-5* mutant and *fus9-1* mutant in **(A)** control (1/2MS) or **(B)** 10μM GA treatment condition. The data show the rate of germinated seed compared to the corresponding controls on the same day of germination. Each value represents the mean ±SD of three independent experiments. Error bars represent the standard deviation.

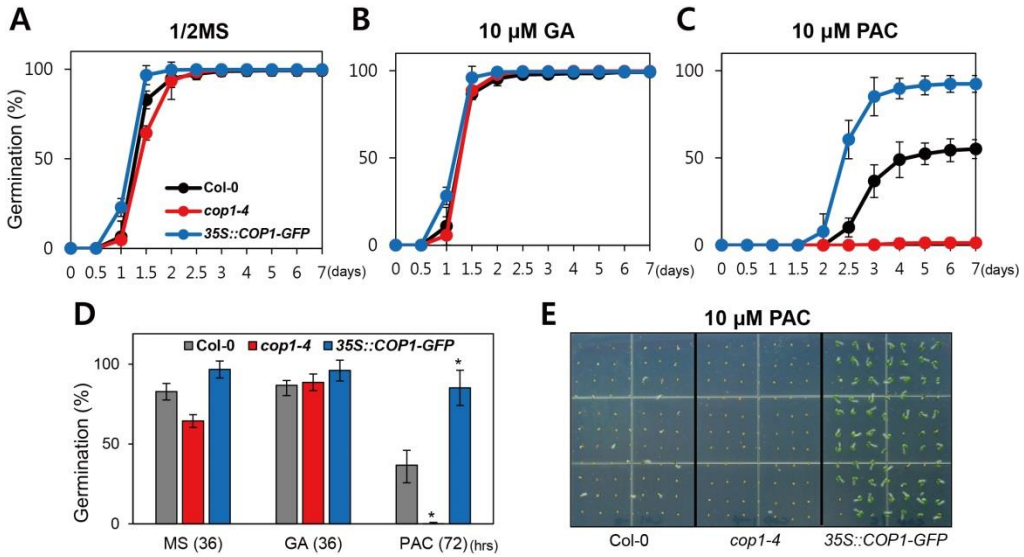

Figure 2. Germination phenotype of *cop1* weak allele mutant, *cop1-4* and *COP1* over-expression transgenic line, *35S::COP1-GFP*, under GA and PAC treatment condition.

Time-course seed germination analysis of the wild-type (Col-0), *cop1-4* mutant and *35S::COP1-GFP* in **(A)** control (1/2MS) or with **(B)** 10μM GA and **(C)** 10μM PAC treatment. **(D)** Statistical analysis of seed germination and **(E)** morphological observations of seed germination after 3days of incubation in MS medium supplemented with 10μM PAC. *P*-values were determined with a two-tailed student's *t*-test assuming equal variances (**p*<0.05). The data show the rate of germinated seed compared to the corresponding controls on the same day of germination. Each value shown represents the mean ±SD of three biological experiments. Error bars represent the standard deviation.

PIL5/PIF1 and *HY5* are not involved in the regulation of GA-mediated germination.

Previous studies have suggested that GA regulates seed germination through PIL5/PIF1, is involved in light-dependent manner. PIL5/PIF1 acts as negative regulator of DELLA, DAG1, and SOM proteins, which modulates GA responsiveness [1, 51]. Moreover, a recent study revealed that the COP1 negatively regulates PIL5/PIF1 protein stability for photomorphogenesis, including seed germination [43]. HY5 also a target protein of COP1 is involved in ABA signaling pathway [52] and possibly involved in seed germination. Thus, we hypothesized that COP1 acts as a positive regulator of seed germination by inhibiting PIL5/PIF1 protein stability.

To test whether *COP1* regulates seed germination through *PIL5/PIF1* or *HY5*, we examined seed germination assays in response to PAC. If they act in same pathway, *PIF1/PIL5* and *HY5* will show PAC insensitive phenotype like *35S::COP1-GFP*. The seed germination assays was performed using the *pif1* and *hy5-205* mutant under no-treatment condition (1/2MS) (Figure 3A) or PAC treatment condition (Figure 3B). Unexpectedly, *pif1* and *hy5-205* mutant seeds did not show PAC insensitive phenotype as *35S::COP1-GFP* plants, in which these mutants showed normal germination phenotype in MS and PAC conditions as Wild-type (Col-0) plants. These results indicate that PAC-insensitive germination phenotype of *COP1* is not due to *PIL5/PIF1* and *HY5*. Thus, those genes are not involved in GA-mediated germination

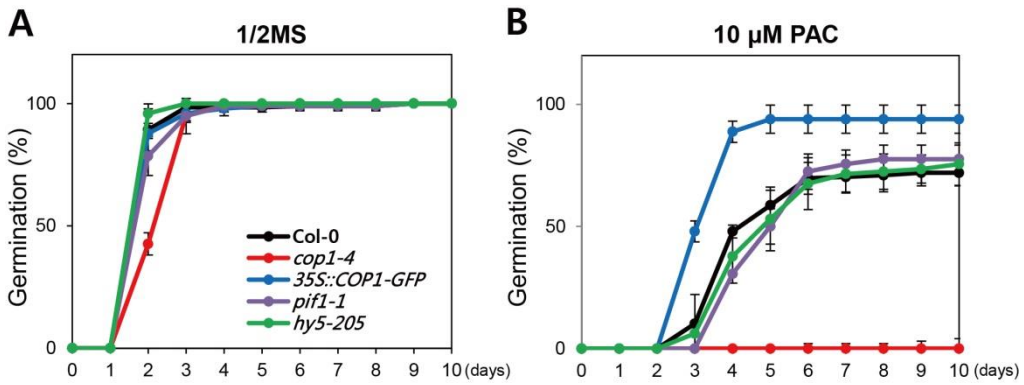

Figure 3. *PIL5/PIF1* and *HY5* are not involved in GA-mediated germination.

Time-course seed germination analysis of the wild type (Col-0), cop1-4 mutant, *35S::COP1-GFP, pif1-1* mutant and *hy5-205* mutant in **(A)** control (1/2MS) or with **(B)** 10μM PAC treatment. The data show the rate of germinated seed compared to the corresponding controls on the same day of germination. Each value shown represents the mean ±SD of three independent biological experiments. Error bars represent the standard deviation.

COP1 protein is stabilized by GA hormone.

The above results suggest that *COP1* is involved in GA-mediated seed germination, which is light-independent manner. We therefore next asked how *COP1* acts in GA-mediated germination. At first, we measured the transcript accumulation of *COP1* during seed germination from Wild-type (Col-0) seeds treated with 10μM GA and 10μM PAC or without (control). However, the transcript level of *COP1* was not altered by GA (Figure 4). We next examined whether the COP1 protein expression level is regulated by GA. To this end, seeds were imbibed in distilled water (DW), 10μM GA and 10μM PAC solution. Protein extracts were prepared from the time-course harvested seeds. The protein concentration was normalized, and the levels of COP1 protein were assayed by Western blot using COP1 specific antibody. The result shows that COP1 protein is early accumulated under GA treated condition than DW condition. Oppositely, PAC treatment leads to late accumulation of COP1. These results indicate that GA induces COP1 protein stability and then COP1 negatively regulates RGL2 protein stability in the regulation of seed germination (Figure 5).

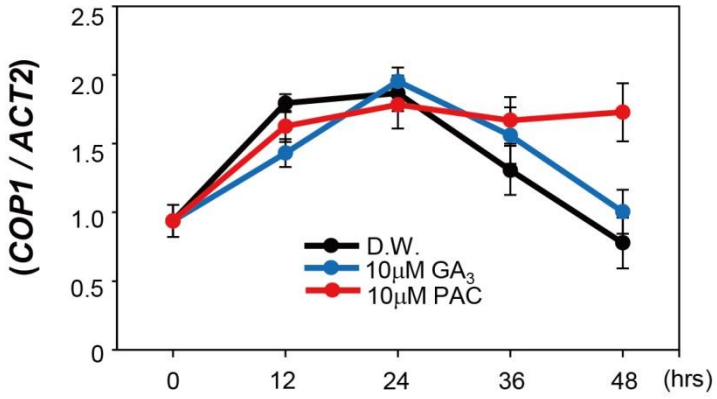

Figure 4. GA does not regulate *COP1* transcription.

Time-course mRNA expression levels of *COP1*. Col-0 seeds were treated with or without GA and PAC under LD condition, and were harvested at each time point. The expression levels of *COP1* were measured by qRT-PCR, and determined by *ACT2*. Each value shown is the mean ±SD of three independent biological replicates. Error bars represent the standard deviation.

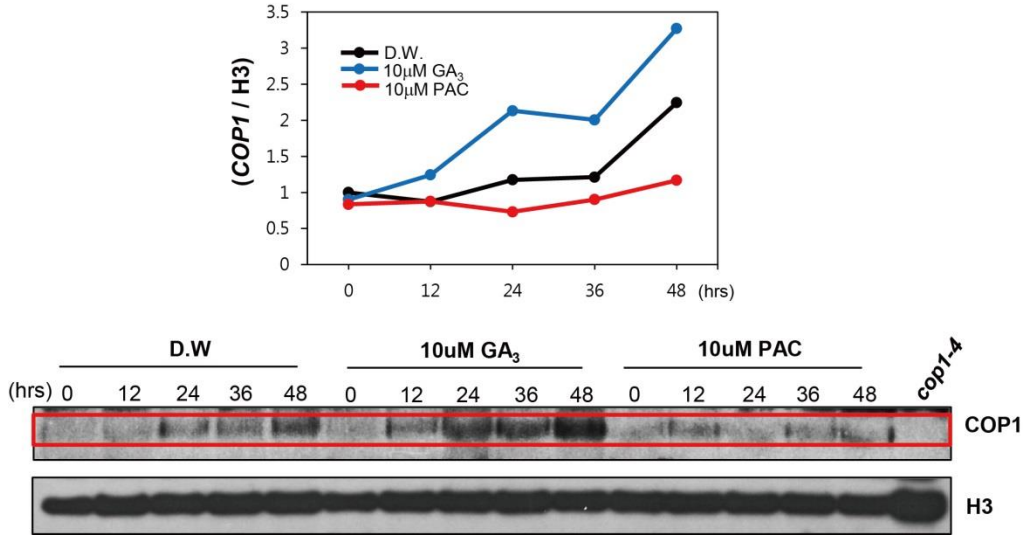

Figure 5. GA modulates the stabilization of COP1 protein

Time-course protein levels of COP1. Col-0 seeds were treated with or without GA or PAC under LD condition. Total proteins were extracted at each time point, and COP1 accumulation was analyzed by western blotting using Anti-COP1 antibody [44]. The levels of histone H3 were used as a loading control. cop1-4 used as a negative control for Anti-COP1

COP1 Functions upstream of RGL2 to regulate seed germination in GA-mediated pathway.

To understand germination phenotype of *COP1* overexpression plant, we searched PAC-insensitive phenotype in GA-mediated germination pathway. Previous studies have demonstrated that the *rgl2* mutant shows insensitive germination phenotype under PAC treatment condition [21]. It prompted us to examine whether *COP1* might be genetically related with *RGL2* in GA-mediated germination. To do this, we first confirmed germination phenotype of 5 kinds of *della* mutants, and we found that only *rgl2* mutant showed PAC-insensitive phenotype (Figure 6A and 6B). Thus, we hypothesized that *COP1* may be associated with *RGL2* in the regulation of seed germination. To investigate the hypothesis, we generated the *cop1-4 rgl2* double mutant by crossing two homozygous, and we compared the germination rate among Col-0, *cop1-4*, *rgl2*, and *cop1-4 rgl2* mutant seeds. *cop1-4* mutant showed PAC-sensitive phenotype (Figure 2C), and the *rgl2* mutant showed PAC-insensitive phenotype (Figure 6B). And interestingly, PAC-hypersensitive phenotype of *cop1-4* was rescued by *rgl2* mutant in the *cop1-4 rgl2* double mutant (Figure 7A and 7B). Furthermore, we generated *35S::COP1-GFP/rgl2* plant which also crossed the *35S::COP1-GFP* transgene into *rgl2* mutant background and investigated seed germination analysis. Germination rate of *35S::COP1-GFP/rgl2* seeds were similar to both *35S::COP1-GFP* and *rgl2* mutant seeds under PAC treatment condition (Figure 8A and 8B). Taken

together, these data suggest that *RGL2* is epistatic to *COP1*, and these genes act together in GA-mediated pathway.

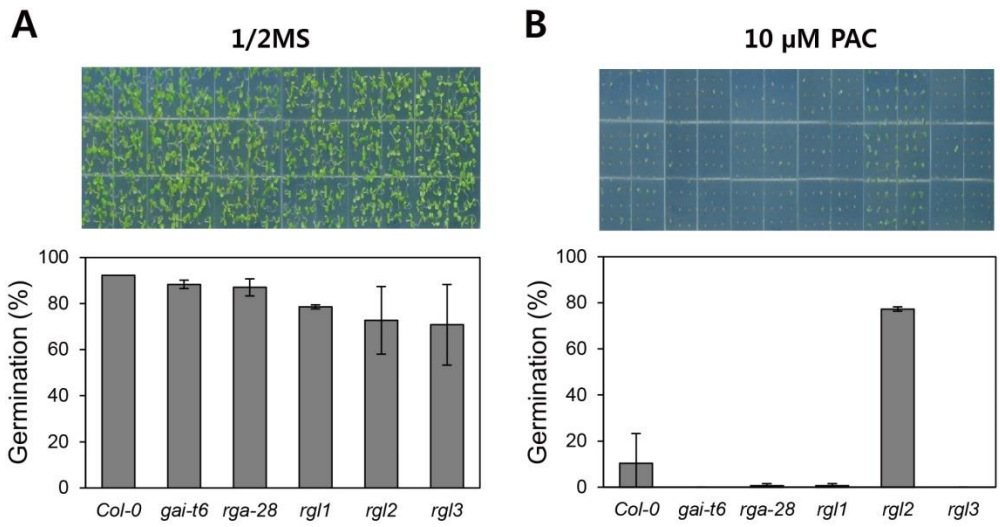

Figure 6. Germination rate of 5 kinds of *della* mutants under PAC treatment condition.

Germination analysis of the 5 kinds of *della* mutants comparing with wild-type (Col-0). All of seeds were imbibed in untreated (1/2MS) or 10μM PAC treatment medium. Morphological observations of seed germination after 5days of incubation in MS medium supplemented with 10μM PAC. Each value represents the mean $\pm$SD of three independent experiments. Error bars represent the standard deviation.

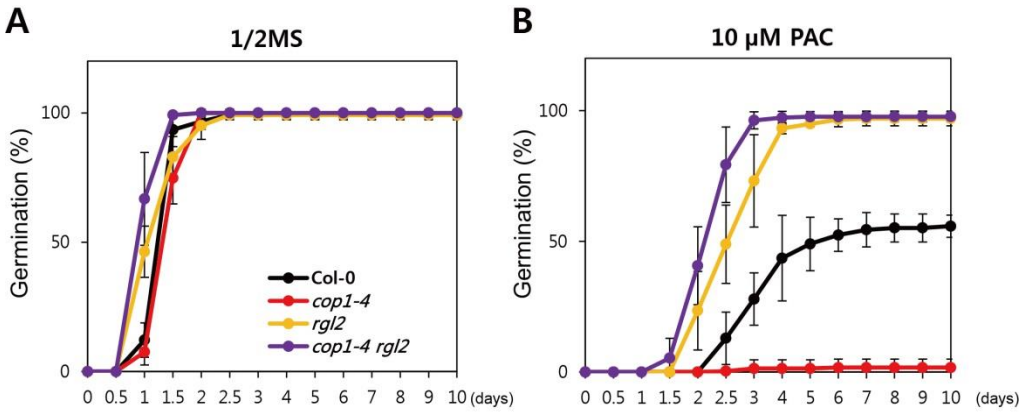

Figure 7. *RGL2* is epistatic to *COP1* in GA-mediated germination.

Time-course seed germination analysis of the Wild-type (Col-0), *cop1-4, rgl2* and *cop1-4 rgl2* seeds in **(A)** control (1/2MS) or with **(B)** 10μM PAC treatment. The data show the rate of germinated seed compared to the corresponding controls on the same day of germination. Each value shown is the mean $\pm$SD of three independent biological replicates. Error bars represent the standard deviation.

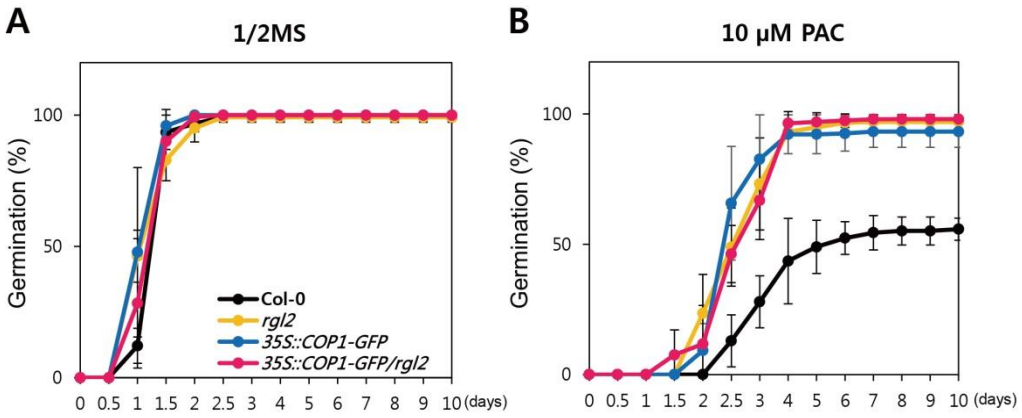

Figure 8. Germination phenotype of *rgl2*, *35S::COP1-GFP*, and *35S::COP1-GFP/rgl2* seeds under PAC treatment condition.

Time-course seed germination analysis of the Wild-type (Col-0), *rgl2, 35S::COP1-GFP* and *35S::COP1-GFP/rgl2* seeds in **(A)** control (1/2MS) or with **(B)** 10μM PAC treatment. The data show the rate of germinated seed compared to the corresponding controls on the same day of germination. Each value shown is the mean ±SD of three independent biological replicates. Error bars represent the standard deviation.

COP1 does not regulate mRNA expression level of *RGL2*

The genetic analysis showed that *COP1* acts as an upstream negative regulator of *RGL2*, in which it is possible that *COP1* negatively regulates *RGL2* in transcription or post-translation-step. To examine whether COP1 regulates RGL2 transcript expression level, we measured the mRNA expression levels of *RGL2* in *cop1-4* and *35S::COP1-GFP* plants compared with WT (Col-0) using qRT-PCR (Figure 9). The transcription levels of the *RGL2* were not affected by COP1. These observations raise the possibility that COP1 may negatively regulate the protein levels of RGL2.

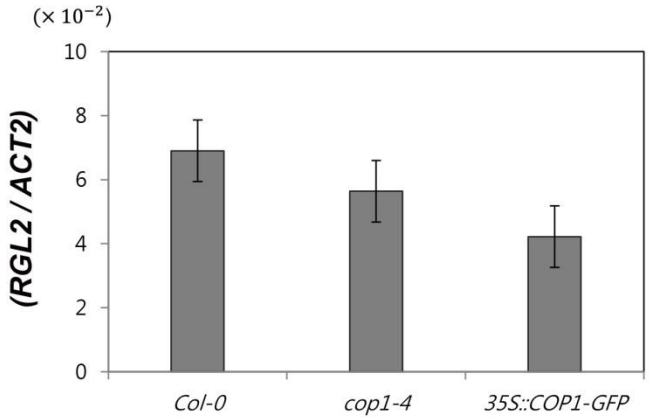

Figure 9. *COP1* does not regulate *RGL2* expression.

Expression level of *RGL2* in Col-0, *cop1-4*, and *35::COP1-GFP* seeds. All of the imbibed seeds were incubated at 4℃ for 2 days, and transferred to LD condition growth chamber for 1day, and harvested for RNA extraction. Expression level of *RGL2* was measures by qRT-PCR, and calculated by *ACT2*. Each value shown is the mean ±SD of three independent biological replicates. Error bars represent the standard deviation.

COP1 physically interacts with RGL2

Since *COP1* and *RGL2* does not regulate in transcription level. We next investigate whether COP1 regulates RGL2 in post-translation step. To examine this idea, we first used yeast two-hybrid assays to identify protein-protein interactions. To this end, we cloned the full length (aa1-2028) and partial (RING; aa 1~104, CC; aa 121~213, WD-40 repeat; aa 371~675) cDNAs of *COP1* into the pGBK vector (as bait), and full length of *RGL2* also cloned into the pGAD vector (as prey). The results show that the RGL2 strongly interact with RING domain of COP1 and also weakly binds to WD-40 repeat domain (Figure 10A).

To further confirm interaction between COP1 and RGL2 *in-vivo*. We conducted bimolecular fluorescence complementation (BiFC) assays (Figure 10B). For this experiment, we generated constructs of COP1 fused with C-terminal of YFP (cYFP-COP1) and RGL2 fused with N-terminal of YFP (nYFP-COP1). YFP vector and YFP-COP1, YFP-RGL2 were used as positive controls. When cYFP-COP1 and nYFP-RGL2 were bombarded into onion epidermal cells, we observed strong YFP fluorescence signals in the nucleus, indicating that COP1 interact with RGL2 in nucleus.

Moreover, we wondered whether the interaction of COP1 and RGL2 is direct or indirect To examine that, *in-vitro* pull-down assay were performed using MBP-COP1 and GST-RGL2 recombinant proteins. In this experiment, we used MBP and GST as negative control. MBP-COP1 was shown to interact

with GST-RGL2 (Figure 10C). Taken together, these results demonstrate that COP1 is genetically and physically interacts with RGL2, and this interaction is direct.

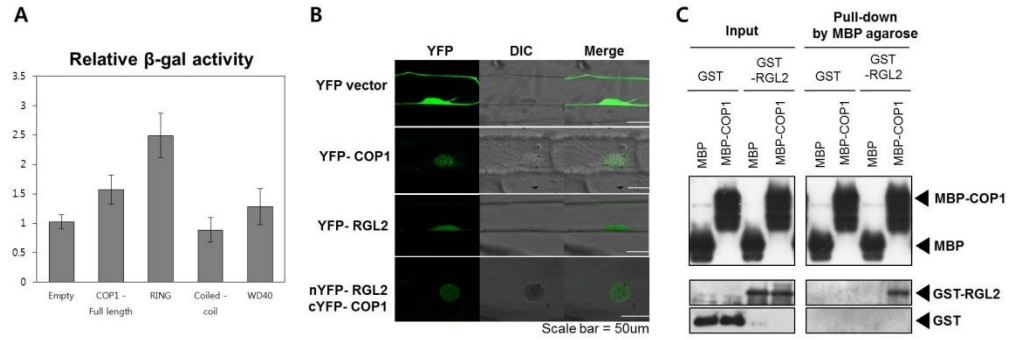

Figure 10. COP1 directly interacts with RGL2

(A) Relative β-galactosidase (β-gal) activity was assayed for each strain and is presented relative to that obtained for the COP1-RGL2 interaction. The empty vector was employed in the negative control. Error bars represent the standard deviation.

(B) BiFC assays show the interaction between COP1 and RGL2 in onion epidermal cells. Full-length RGL2 and COP1 were fused to the split N- or C-terminal (YN or CN) fragment of YFP. Dic, differential interference contrast in microscope mode; Merge, merged imaged of YFP channel and DIC, scale bar=50μm.

(C) Pull-down assays show direct interaction between COP1 and RGL2 *in-vitro.* GST and GST-RGL2 protein were incubated with immobilized MBP and MBP-COP1 proteins, and fractions were detected by anti-GST and anti-MBP antibodies.

COP1 negatively regulates RGL2 protein stability.

Our genetic and physical results strongly suggest that COP1 may negatively regulate RGL2 protein stability. Because it has been reported that RGL2 is inactivated by GA hormone [26] and protein stability also affected by GA signaling component, including SLY1 [53]. In addition, COP1 downregulates target genes to regulate phtomorphogenesis [37-41]. Thus, we examined whether COP1 negatively regulates RGL2 protein stability. To this end, Cell-free degradation assays was performed to illustrate the changing of RGL2 protein stability. GST-RGL2 proteins were incubated with total soluble proteins from WT, *cop1-4* and *35S::COP1-GFP* plants, and detected GST-RGL2 protein at indicated time point (Figure. 11). GST-RGL2 proteins were rapidly degraded in *35S::COP1-GFP* condition, GST-RGL2 were more stabilized in *cop1-4* condition. These results suggest that COP1 is partially involved in regulating RGL2 protein stability during seed germination.

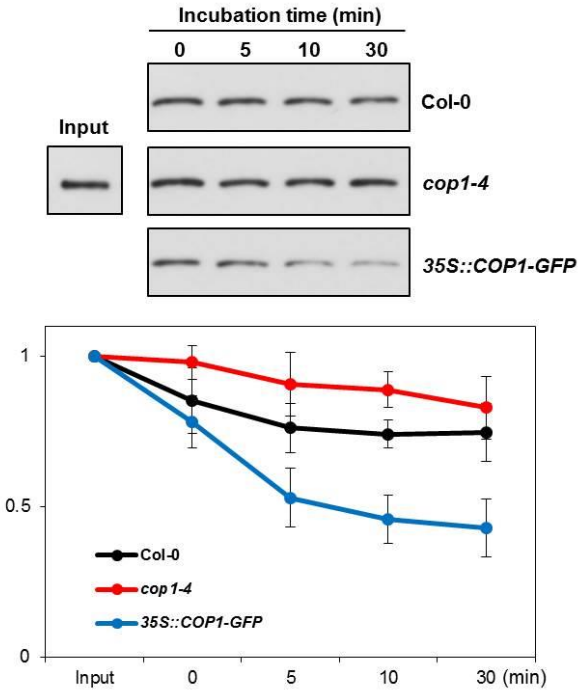

Figure 11. COP1 regulates RGL2 protein stability.

Cell free degradation assay of RGL2 recombinant proteins. Purified GST-RGL2 proteins were incubated with each soluble fractions from Col-0, cop1-4, and 35S::COP1-GFP plants. Samples were harvested at each time point, and detected by anti-GST antibody. Error bars represent the standard deviation.

COP1 regulates the expression levels of germination-associated genes during seed germination

In previous studies, it had been reported that RGL2 negatively regulates expression levels of germination-associated genes in GA-mediated manner [54]. Therefore, we examined whether COP1 also regulates the transcript expression of down-stream genes, which are regulated by RGL2 such as *GASA6, EXPA1, EXPA2, EXPA8,* and *XTH33*. The mRNA levels of those genes in *cop1-4*, *35S::COP1-GFP,* and *rgl2* imbibed seeds were measured by qRT-PCR (Figure 12). The results show that transcript levels of germination-associated genes were down-regulated in *rgl2* mutant seeds, consistent with the observation in previous reports [54], whereas those were up-regulated in *cop1-4* mutant seeds. Interestingly, the expression level were down-regulated in *cop1-4 rgl2* double mutant same as that of *rgl2* mutant. The expression pattern in *cop1-4 rgl2* is similar to *rgl2*. These data indicate that *COP1* positively regulates germination-associated genes by repressing *RGL2*.

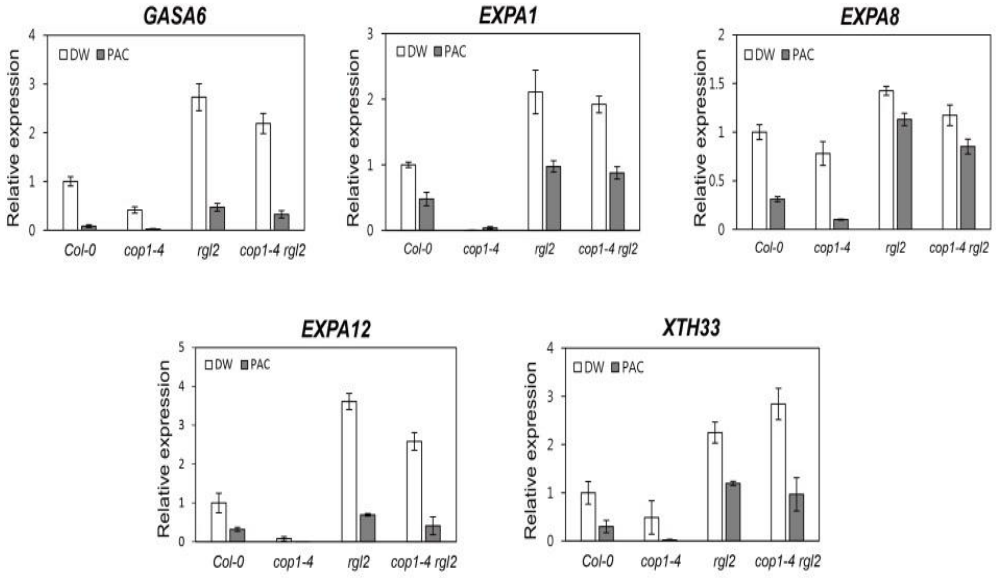

Figure 12. Transcript expression analysis of germination associated genes by qRT-PCR.

Seeds of wild-type, *cop1-4, rgl2* and *cop1-4 rgl2* double mutant were imbibed in distilled water or 10μM PAC treatment. Total RNA was extracted from germinating seeds and transcript levels of *GASA6* **(A)**, *EXPA1* **(B)**, *EXPA2* **(C)**, *EXPA8* **(D)**, and *XTH33* **(E)** were quantified by qRT-PCR relative to *ACT2*. Each value shown is the mean ±SD of three independent biological replicates. Error bars represent the standard deviation.

DISCUSSION

GA is a pivotal phytohormone, which regulates a wide range of plant life cycle including seed germination, stem elongation and flowering. Among GA signaling components, *RGL2* plays a major role in repressing GA-mediated seed germination. Under low GA level, RGL2 protein is degraded by 26S proteasome [55], causing enhanced expression of germination-associated genes including GASA6 and EXPA1 [54].

Light signal is also a key external factor of seed germination. Among the light-mediated seed germination pathway, the stability of PIF1/PIL5, which acts as negative regulator in seed germination can be determined by light [45]. In previous study reported that light-signal affects to the reversible localization of COP1 from nucleus to cytoplasm [35]. In darkness, COP1 is translocated to the nucleus and COP1 degrades target proteins by ubiquitin-proteasome-dependent proteolysis system [41].

Various mechanisms of seed germination have been reported, but the regulatory mechanism by both COP1 and RGL2 has not been revealed in light-independent/GA-mediated germination pathway.

In this study, we provide several pieces of evidence that GA regulates seed germination through COP1 in light-independently. First, GA induces COP1 protein stability during seed germination. The germination phenotype of *cop1* null-mutant, *cop1-5*, is extremely low and lethal germination. We wonder

whether the low germination rate is related to GA hormone. To determine this, we treated GA and GA synthesis inhibitor PAC to each *cop1-5* and *cop1-4* mutant seeds. The result showed that the germination phenotype of *cop1* mutant is recovered by GA treatment (Figure 1 and Figure 2). Thus, we postulate that *COP1* is related to GA hormone in regulating seed germination. Next we investigated whether GA affects COP1 transcription or translation level, and we found that GA induces COP1 protein level (Figure 5) without changes in transcript expression (Figure 4). Second, this mechanism is light-independent manner. Previously, On Sun Lau (Plant hormone signaling lightens up: integrators of Light and hormones) [51] suggested that seed germination regulated by GA hormone under light condition, in which PIF1/PIL5 protein stability is decreased. These reactions result in inducing GA synthesis genes and promoting seed germination. In addition, COP1 acts as negative regulator of PIF1/PIL5 We then wondered whether COP1 regulates seed germination via PIF1/PIL5 pathway. We performed seed germination assays using PIF1 and HY5 mutants (Figure 3). We found that germination regulate pathway of COP1 is not associated with PIF1 or HY5. These results indicate that COP1 controls GA-mediated germination, light independently. Third, COP1 directly interacts with RGL2 (Figure 10) through reducing its protein stability. Our results indicated that this mechanism is light-independent and GA-mediated manner. We wonder how COP1 regulates germination in GA pathway, and the result showed that COP1 is negatively regulates RGL2

protein stability (Figure 11). Additionally, many germination-associated genes are changed by COP1 expression (Figure 12), and we found that this mechanism is regulated by COP1 and RGL2 respectively. Various genetic experiments showed COP1 acts upstream of RGL2 and they are directly regulates seed germination via degrades RGL2 protein.

In conclusion, we suggest that the simple scheme of new germination pathway regulated by GA hormone (Figure 13). Our results provide an expanded understanding for regulatory mechanism of seed germination.

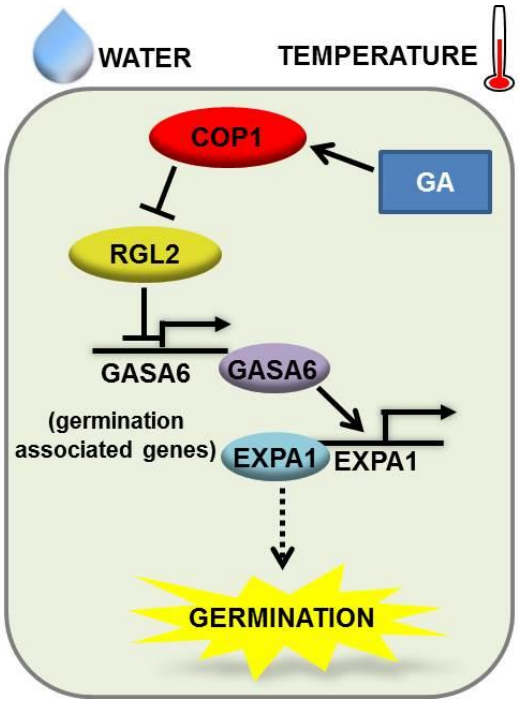

Figure 13. The proposed model of COP1 for seed germination.

In the presence of GA, GA promotes COP1 protein expression. The COP1 interact with the RGL2 protein. Then, COP1 repress the protein levels of RGL2. Subsequently, the germination is achieved by inducing downstream genes. This consecutive process is occurred light independently.

REFERENCES

1. Seo M, Nambara E, Choi G, Yamaguchi S: **Interaction of light and hormone signals in germinating seeds**. *Plant Mol Biol* 2009, **69**(4):463-472.
2. Gazzarrini S, Tsai AY: **Hormone cross-talk during seed germination**. *Essays in biochemistry* 2015, **58**:151-164.
3. M. Koornneef CMK: **Seed dormancy and germination**. *Cold Spring Harbor Laboratory Press* 1994:313-334.
4. McCarty DR: **Genetic Control and Integration of Maturation and Germination Pathways in Seed Development**. *Annual Review of Plant Physiology and Plant Molecular Biology* 1995, **46**:71-93.
5. Bewley JD: **Seed Germination and Dormancy**. *Plant Cell* 1997, **9**(7):1055-1066.
6. Steber CM, Cooney SE, McCourt P: **Isolation of the GA-response mutant sly1 as a suppressor of ABI1-1 in Arabidopsis thaliana**. *Genetics* 1998, **149**(2):509-521.
7. Holdsworth MJ, Bentsink L, Soppe WJ: **Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination**. *The New phytologist* 2008, **179**(1):33-54.
8. Eiji Nambara MO: **Abscisic acid and the control of seed dormancy and germination**. *Seed Science Research* 2010, **20**(2):55-67.
9. Sun TP, Gubler F: **Molecular mechanism of gibberellin signaling in plants**. *Annu Rev Plant Biol* 2004, **55**:197-223.
10. Yamaguchi S: **Gibberellin metabolism and its regulation**. *Annu Rev Plant Biol* 2008, **59**:225-251.

11. Ueguchi-Tanaka M, Ashikari M, Nakajima M, Itoh H, Katoh E, Kobayashi M, Chow TY, Hsing YI, Kitano H, Yamaguchi I *et al*: **GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin**. *Nature* 2005, **437**(7059):693-698.

12. Thomas SG, Sun TP: **Update on gibberellin signaling. A tale of the tall and the short**. *Plant Physiol* 2004, **135**(2):668-676.

13. Voss U, Bishopp A, Farcot E, Bennett MJ: **Modelling hormonal response and development**. *Trends Plant Sci* 2014, **19**(5):311-319.

14. Sasaki A, Itoh H, Gomi K, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Jeong DH, An G, Kitano H, Ashikari M *et al*: **Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant**. *Science* 2003, **299**(5614):1896-1898.

15. Gomi K, Sasaki A, Itoh H, Ueguchi-Tanaka M, Ashikari M, Kitano H, Matsuoka M: **GID2, an F-box subunit of the SCF E3 complex, specifically interacts with phosphorylated SLR1 protein and regulates the gibberellin-dependent degradation of SLR1 in rice**. *Plant J* 2004, **37**(4):626-634.

16. Thomas SG, Rieu I, Steber CM: **Gibberellin metabolism and signaling**. *Vitamins and hormones* 2005, **72**:289-338.

17. J P: **'Green revolution' genes encode mutant gibberellin response modulators.** *Nature* 1999, **400**:256-261.

18. Silverstone AL, Ciampaglio CN, Sun T: **The Arabidopsis RGA gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway**. *Plant Cell* 1998, **10**(2):155-169.

19. Pysh LD, Wysocka-Diller JW, Camilleri C, Bouchez D, Benfey PN: **The GRAS gene family in Arabidopsis: sequence characterization and basic**

expression analysis of the SCARECROW-LIKE genes. *Plant J* 1999, **18**(1):111-119.

20. Dill A, Sun T: **Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana**. *Genetics* 2001, **159**(2):777-785.

21. Lee S, Cheng H, King KE, Wang W, He Y, Hussain A, Lo J, Harberd NP, Peng J: **Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition**. *Genes Dev* 2002, **16**(5):646-658.

22. Richards DE, Peng J, Harberd NP: **Plant GRAS and metazoan STATs: one family?** *BioEssays : news and reviews in molecular, cellular and developmental biology* 2000, **22**(6):573-577.

23. Wen CK, Chang C: **Arabidopsis RGL1 encodes a negative regulator of gibberellin responses**. *Plant Cell* 2002, **14**(1):87-100.

24. Wild M, Daviere JM, Cheminant S, Regnault T, Baumberger N, Heintz D, Baltz R, Genschik P, Achard P: **The Arabidopsis DELLA RGA-LIKE3 is a direct target of MYC2 and modulates jasmonate signaling responses**. *Plant Cell* 2012, **24**(8):3307-3319.

25. Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun TP: **Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis**. *Plant Physiol* 2004, **135**(2):1008-1019.

26. Cao D, Hussain A, Cheng H, Peng J: **Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in Arabidopsis**. *Planta* 2005, **223**(1):105-113.

27. Castillon A, Shen H, Huq E: **Phytochrome Interacting Factors: central**

players in phytochrome-mediated light signaling networks. *Trends Plant Sci* 2007, **12**(11):514-521.

28. Monte E, Al-Sady B, Leivar P, Quail PH: **Out of the dark: how the PIFs are unmasking a dual temporal mechanism of phytochrome signalling**. *Journal of experimental botany* 2007, **58**(12):3125-3133.

29. Henriques R, Jang IC, Chua NH: **Regulated proteolysis in light-related signaling pathways**. *Curr Opin Plant Biol* 2009, **12**(1):49-56.

30. Kim DH, Yamaguchi S, Lim S, Oh E, Park J, Hanada A, Kamiya Y, Choi G: **SOMNUS, a CCCH-type zinc finger protein in Arabidopsis, negatively regulates light-dependent seed germination downstream of PIL5**. *Plant Cell* 2008, **20**(5):1260-1277.

31. Seo M, Hanada A, Kuwahara A, Endo A, Okamoto M, Yamauchi Y, North H, Marion-Poll A, Sun TP, Koshiba T *et al*: **Regulation of hormone metabolism in Arabidopsis seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism**. *Plant J* 2006, **48**(3):354-366.

32. Lau OS, Deng XW: **The photomorphogenic repressors COP1 and DET1: 20 years later**. *Trends Plant Sci* 2012, **17**(10):584-593.

33. Ang LH, Chattopadhyay S, Wei N, Oyama T, Okada K, Batschauer A, Deng XW: **Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of Arabidopsis development**. *Molecular cell* 1998, **1**(2):213-222.

34. Jang IC, Henriques R, Seo HS, Nagatani A, Chua NH: **Arabidopsis PHYTOCHROME INTERACTING FACTOR proteins promote phytochrome B polyubiquitination by COP1 E3 ligase in the nucleus**.

Plant Cell 2010, **22**(7):2370-2383.

35. Torii KU, McNellis TW, Deng XW: **Functional dissection of Arabidopsis COP1 reveals specific roles of its three structural modules in light control of seedling development**. *Embo j* 1998, **17**(19):5577-5587.

36. Yi C, Deng XW: **COP1 - from plant photomorphogenesis to mammalian tumorigenesis**. *Trends in cell biology* 2005, **15**(11):618-625.

37. Oyama T, Shimura Y, Okada K: **The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl**. *Genes Dev* 1997, **11**(22):2983-2995.

38. Zhang H, He H, Wang X, Wang X, Yang X, Li L, Deng XW: **Genome-wide mapping of the HY5-mediated gene networks in Arabidopsis that involve both transcriptional and post-transcriptional regulation**. *Plant J* 2011, **65**(3):346-358.

39. Ballesteros ML, Bolle C, Lois LM, Moore JM, Vielle-Calzada JP, Grossniklaus U, Chua NH: **LAF1, a MYB transcription activator for phytochrome A signaling**. *Genes Dev* 2001, **15**(19):2613-2625.

40. Duek PD, Fankhauser C: **HFR1, a putative bHLH transcription factor, mediates both phytochrome A and cryptochrome signalling**. *Plant J* 2003, **34**(6):827-836.

41. Holm M, Ma LG, Qu LJ, Deng XW: **Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in Arabidopsis**. *Genes Dev* 2002, **16**(10):1247-1259.

42. Xu X, Paik I, Zhu L, Bu Q, Huang X, Deng XW, Huq E: **PHYTOCHROME INTERACTING FACTOR1 Enhances the E3 Ligase Activity of CONSTITUTIVE PHOTOMORPHOGENIC1 to Synergistically Repress**

Photomorphogenesis in Arabidopsis. *Plant Cell* 2014, **26**(5):1992-2006.

43. Zhu L, Bu Q, Xu X, Paik I, Huang X, Hoecker U, Deng XW, Huq E: **CUL4 forms an E3 ligase with COP1 and SPA to promote light-induced degradation of PIF1**. *Nat Commun* 2015, **6**:7245.

44. McNellis TW, von Arnim AG, Araki T, Komeda Y, Misera S, Deng XW: **Genetic and molecular analysis of an allelic series of cop1 mutants suggests functional roles for the multiple protein domains**. *Plant Cell* 1994, **6**(4):487-500.

45. Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee HS, Sun TP, Kamiya Y, Choi G: **PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the GAI and RGA promoters in Arabidopsis seeds**. *Plant Cell* 2007, **19**(4):1192-1208.

46. Osterlund MT, Hardtke CS, Wei N, Deng XW: **Targeted destabilization of HY5 during light-regulated development of Arabidopsis**. *Nature* 2000, **405**(6785):462-466.

47. Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung WI, Choi G: **Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis**. *Plant J* 2006, **47**(1):124-139.

48. Clough SJ, Bent AF: **Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana**. *Plant J* 1998, **16**(6):735-743.

49. Yu JW, Rubio V, Lee NY, Bai S, Lee SY, Kim SS, Liu L, Zhang Y, Irigoyen ML, Sullivan JA *et al*: **COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability**. *Molecular cell* 2008, **32**(5):617-630.

50. Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Ma L, Hoecker U, Deng XW: **The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity**. *Genes Dev* 2003, **17**(21):2642-2647.

51. Lau OS, Deng XW: **Plant hormone signaling lightens up: integrators of light and hormones**. *Curr Opin Plant Biol* 2010, **13**(5):571-577.

52. Li Z, Zhang L, Yu Y, Quan R, Zhang Z, Zhang H, Huang R: **The ethylene response factor AtERF11 that is transcriptionally modulated by the bZIP transcription factor HY5 is a crucial repressor for ethylene biosynthesis in Arabidopsis**. *Plant J* 2011, **68**(1):88-99.

53. Ariizumi T, Steber CM: **Seed germination of GA-insensitive sleepy1 mutants does not require RGL2 protein disappearance in Arabidopsis**. *Plant Cell* 2007, **19**(3):791-804.

54. Zhong C, Xu H, Ye S, Wang S, Li L, Zhang S, Wang X: **Gibberellic Acid-Stimulated Arabidopsis6 Serves as an Integrator of Gibberellin, Abscisic Acid, and Glucose Signaling during Seed Germination in Arabidopsis**. *Plant Physiol* 2015, **169**(3):2288-2303.

55. Itoh H, Matsuoka M, Steber CM: **A role for the ubiquitin-26S-proteasome pathway in gibberellin signaling**. *Trends Plant Sci* 2003, **8**(10):492-497.

국 문 초 록

식물의 발아과정에는 여러 요인이 관여한다. 크게 외부적 요인으로는, 빛, 온도 그리고 물이 작용하며, 내재적 요인으로는 GA (Gibberellic acid), ABA (Abscisic acid), ethylene과 같은 식물호르몬이 작용한다. 이 중에서, GA는 종자의 발아를 촉진시키는 대표적인 호르몬으로 알려져 있다. GA에 의한 발아 조절 메커니즘 연구는 오랜 기간 진행되고 있으나, 아직도 많은 부분 밝혀지지 않았다. 암 조건에서 식물의 광 형태형성 (photomorphogenesis)을 억제시키는 대표적인 유전자로 알려진 COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1)은 유비퀴틴 기작을 통해 표적 유전자들의 기능을 억제한다. 본 연구에서는 GA에 의한 발아 조절 메커니즘에 COP1 유전자가 관여한다는 것을 규명하였다. 먼저, COP1 돌연변이체가 야생형에 비해 발아율이 현저히 낮은 것을 발견하였고, 발아관련 호르몬인 GA호르몬과 GA생합성 억제제인 PAC (PACLOBUTRAZOL)을 처리한 후 COP1 돌연변이체와 과다발현체의 발아율을 측정하였다. 결과, GA처리시 COP1 돌연변이체의 발아율이 야생형 수준으로 회복되는 것을 확인하였다. 또한 PAC처리시 돌연변이체는 sensitive한 표현형을 보였고, 과다발현체는 insensitive한 표현형을

보였다. 이 결과를 통해 발아과정에서 COP1과 GA호르몬의 연관성을 보았다. GA호르몬이 COP1에 어떤 영향을 끼치는지 보기 위해, GA와 PAC을 처리하여 COP1의 RNA와 단백질 함량 변화를 보았다. 결과, GA호르몬이 COP1의 단백질 발현을 촉진시킴을 확인하였다. 또한 기존의 연구 결과를 토대로, GA와 COP1 모두 빛에 의한 발아조절과정에서 PIF1/PIL5 유전자를 매개하므로 이 메커니즘 또한 빛 신호에 의한 GA메커니즘일 가능성을 확인해보았다. PIF1과 COP1의 표적 유전자인 HY5 돌연변이체에 PAC을 처리한 후의 그 표현형의 변화를 관찰하였다. 결과, 두 유전자 모두 PAC에서 COP1 과다발현체와 같은 표현형을 보이지 않았고, 이로써 빛에 의한 조절 가능성을 배제하였다. RGL2는 GA의 발아조절과정에 관여하는 대표적인 유전자이며, PAC에 insensitive한 표현형을 보인다. 여러 단백질 상호작용 실험을 통해, COP1과 RGL2가 GA에 의한 발아과정에 함께 작용함을 확인하였다. 마지막으로 COP1이 RGL2의 단백질의 발현을 억제함으로써, 발아관련 하부유전자들의 발현을 조절하여 발아를 유도시킨다는 결과를 얻었다. 이로써 COP1이 빛 신호와는 무관하게 GA호르몬에 의한 발아 조절과정에 관여한다는 사실을 확인하였다.