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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

# Regulation of stomata closure by COP1 in Arabidopsis

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FEBRURY, 2018

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# Regulation of stomatal closure by COP1 in Arabidopsis

UNDER THE DIRECTION OF PROFESSOR HAK-SOO SEO

SUBMITTED TO THE FACULTY OF THE GRADUATE

SCHOOL OF SEOUL NATIONAL UNIVERSITY

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# Regulation of stomatal closure by COP1 in Arabidopsis

**WOOSUB KIM**

## ABSTRACT

In higher plants, stomata are paths for gas exchange. Therefore, stomata are important for the growth of plants (Weyers and Meidner, 1990). It is known that stomata of wild type plants are closed in darkness while stomata of cop1 mutants are open. Furthermore, stomata apertures of *cop1* mutants are wider than those of wild type plants under various light conditions (Mao et al., 2005 CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) is ubiquitin E3 ligase. COP1 is located nucleus in darkness and degrades proteins for photomorphogenesis (Lau and Deng, 2012). To know if COP1 controls not only darkness-related stomata closure but also stress-related stomata closure, we investigated the effect of various stress conditions on stomata closure of wild type plants and *cop1* mutants. Furthermore, we tried to investigate how COP1 controls stomata closure. All of these data demonstrate that stomata of *cop1* mutants are not closed under heat stress or ABA treatment while these of wild type plants are not. However, under various stress or not, there is not special difference in RNA expressional levels. Stomata closure-related proteins which interact with

COP1 are not identified through Yeast two-hybrid assay. These results indicate that COP1 controls stress-related stomata closure and subsequent studies are needed to investigate the mechanism.

Keywords: E3 ubiquitin ligase, COP1, Interaction, Regulation, Stomata, Stomata closure

Student number: 2016-21351

# CONTENTS

ABSTRACT…….... i

CONTENTS…….....iii

LIST OF TABLES AND FIGURES……iv

INTRODUCTION……....1

MATERIALS AND METHODS……..3

RESULTS……....8

DISCUSSIONS……..18

REFFERENCES……20

ABSTRACT IN KOREAN……23

# LIST OF TABLES AND FIGURES

Table 1. Primer sequences for Real-Time PCR

Figure 1. Stomata apertures of ABA-treated plants

Figure 2. Stomata closure-related RNA transcription levels of ABA-treated plants

Figure 3. Stomatal apertures of heat stress-treated plants

Figure 4. Stomata closure-related RNA transcription levels of heat stress-treated plants

Figure 5. Investigation of stomata closure-related proteins which interact with COP1

# INTRODUCTION

Higher plants exchange gas with external environment through stomata; stomata is essential for plants as passages of respiration, photosynthesis and transpiration. Therefore, stomata are important for plant growth (Weyers and Meidner, 1990). It is known that stomata of *Arabidopsis* wild type plants are closed in darkness while stomata of *Arabidopsis cop1* mutants are not. In addition, stomata apertures of *Arabidopsis cop1* mutants are wider than those of wild type plants under various light conditions (Mao et al., 2005).

COP1 is ubiquitin E3 ligase. Ubiquitin E3 ligases attach ubiquitin, a regulatory small peptide, to target proteins and the process is called ubiquitination. Ubiquitin is found in most tissue of eukaryotic organisms, or it occurs ubiquitously. Ubiquitin itself can be attached by other ubiquitin molecules. Thus, polyubiquitin chains are also attached to substrate proteins and it is called polyubiquitination. Polyubiquitinated proteins are degraded by 26s proteasome in plants (Pickart and Eddins, 2004).

COP1 is located nucleus in darkness and degrades proteins for photomorphogenesis. Thus, in *Arabidopsis cop1* mutants, photomorphogenesis-related proteins are not degraded by COP1 and *cop1* mutants do not show skotomorphogenic phenotypes (Lau and Deng, 2012).

COP1 regulates photomorphogenesis and skotomorphogenesis. However,

we tried to investigate the relationship between COP1 and stomata closure. To know if COP1 controls not only darkness-related stomata closure but also stress-related stomata closure, we investigated the effect of various stress conditions on stomata closure of wild type plants and *cop1* mutants. Furthermore, we tried to investigate the mechanism how COP1 controls stomata closure.

Here, we suggest that COP1 regulates stomata closure related to darkness and stress. Wild type plants close their stomata under stress and ABA treatment while *cop1* mutants do not.

# MATERIALS AND METHODS

## Plants material and growth conditions

The plants used for this study were Arabidopsis thaliana Columbia-0 ecotype as wild type and cop1-4 and cop1-6, ubiquitin E3 ligase COP1 T-DNA insertion mutants. We used two-week-old seedlings grown on MS medium (Murashige and Skoog) at 25℃ under 18hr light and 6hr dark condition for ABA treatment. We used three-week-old plants grown on soli at 25℃ under 18hr light and 6hr dark condition for ABA treatment.

## Stomata aperture measurement

Rosette leaves of *Arabidopsis* are collected for microscope observation. Stomata aperture (the ratio of width to length) was quantified using 15 stomata from each sample.

## Analysis of RNA transcription

To know whether COP1 controls stomata closure, we examine expression of several stomata closure-related genes by Real-Time PCR. Total RNAs were extracted from whole plants of WT, *cop1-4* and *cop1-6* by FavorPrep™ PaInts RNA total purification kit (Favorgen) and 1μg of total RNA was used for cDNA synthesis by using Superiorscript Ⅲ reverse transcriptase (Enzynomics) in a volume of 25μl. Real Time PCR assays were conducted with KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Kapa). Each amplification was independently repeated three times. To normalize the values of analyzed genes, *ACTIN2 (ACT2)* was used as an expression control. The primers used to examine expression are listed in Table 1.

**Table 1.** Primer sequences for Real-Time PCR

| Gene | Name | Sequence (5' to 3') |
|---|---|---|
| ACT2 | RTPCR-ACT2-F | TTC TCA GGT ATC GCT GAC CG |
| | RTPCR-ACT2-R | TCT GTG AAC GAT TCC TGG ACC |
| OST1 | RTPCR-OST1-F | GAA TAA GAT TAA GAT GTT TT |
| | RTPCR-OST1-R | TTT GTT TAG AAA TTT AGT CT |
| SLAC1 | RTPCR-SLAC1-F | CGT GAT CAT GTC CTC TG GTC |
| | RTPCR-SLAC1-R | TGG TTG ATC TTC CAA CTG AA |
| ABI1 | RTPCR-ABI1-F | TGT GGC ACA AGA AAA ACG CG |
| | RTPCR-ABI1-R | TCC TCT CTG TAT CGC CAG CT |
| ABI2 | RTPCR-ABI2-F | TCT TCC CAC CAT TAT TTA AT |
| | RTPCR-ABI2-R | ACA AGG GGG CTT TTT GTT TG |
| HSP101 | RTPCR-HSP101-F | GTT GTG CGT GAG GAA ATC GA |
| | RTPCR-HSP101-R | TTC TTG CCT GTT GAA GCG TC |
| HSP70b | RTPCR-HSP70b-F | TGT TGT TCT GGT TGG AGG GT |
| | RTPCR-HSP70B-R | CAC CTG TAA GAA TCG CAG CC |

## Yeast two-hybrid assay

Transformation of yeast was conducted through Lithium acetate yeast transformation method (Gietz et al., 2002). Plasmids used for yeast transformation were pGBT9 and pGBT9-*COP1* as DNA binding domain plasmids; and pGAD424, pGAD424-*SIZ1*, pGAD424-*ABI1*, pGAD424-*ABI2*, pGAD424-*ABI3*, pGAD424-*ABI5*, pGAD424-*PYL1*, pGAD424-*PYR1*, pGAD424-*OST1* and pGAD424-*SLAC1* as activation domain plasmids. We used yeast which transformed with pGBT9-*COP1* and pGAD424-*SIZ1* as the control because interaction between COP1 and SIZ1 has been already known (Kim. 2017).

# RESULTS

## Stomata of cop1-4 and cop1-6 mutants are not closed under ABA treatment.

To know the effect of COP1 on ABA-related stomata closure, we treated *Arabidopsis* wild type, *cop1-4* and *cop1-6* plants with ABA. The *Arabidopsis* plants were two-week-old seedlings. Seedlings were removed to ABA treated MS liquid medium. The concentration of ABA was 50μM. To measured apertures of *Arabidopsis* seedlings, we observed rosette leaves of ABA treated or control seedlings of wild type, *cop1-4* and *cop1-6* with microscope and calculated the ratio of width to length using 15 stomata for each case. Before seedlings are treated with ABA, stomata apertures of wild type and *cop1* mutants are similar. In the case of wild type, stomata apertures had decreased for the first 6 hours and the value was maintained after the first 6 hours. In the case of *cop1* mutants, stomata apertures did not change at all. The data are shown in Figure 1.

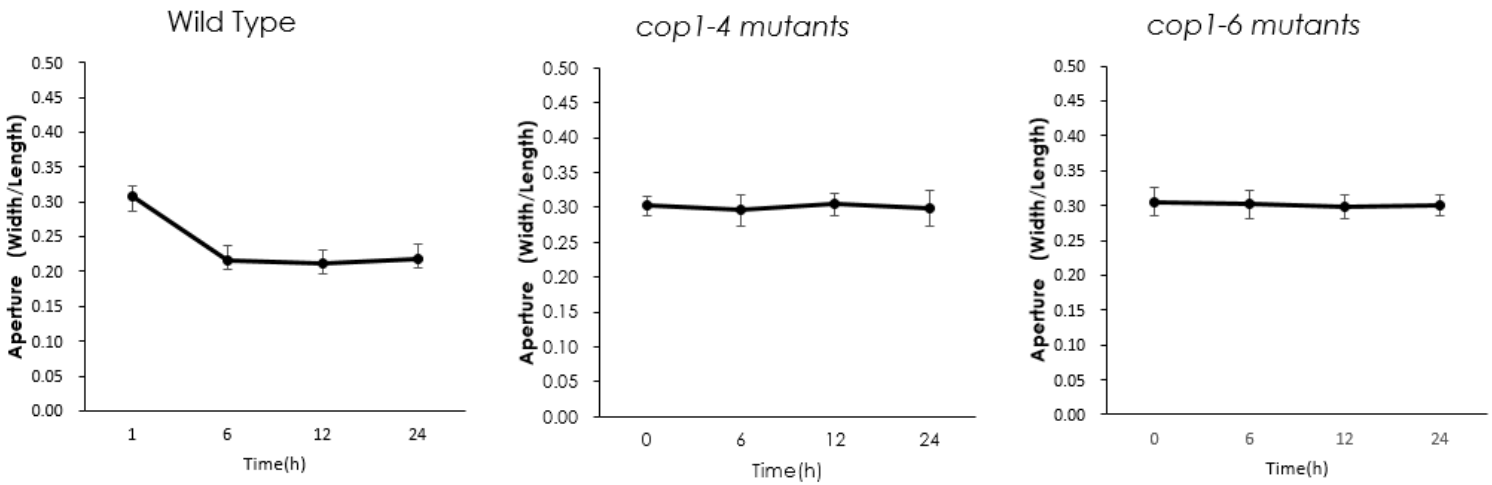


**Figure 1.** Stomata apertures of ABA-treated plants. Two-week-old seedlings of wild type (Col-0), *cop1-4* and *cop1-6* treated with ABA (50μM) for indicated time. Samples were collected for microscope observation. Stomata aperture (the ratio of width to length) was quantified using 15 stomata from each sample.

## ABA treatment does not affect stomata closure-related gene expression of cop1-4 and cop1-6 mutants.

After measurement of stomata apertures of ABA treated *Arabidopsis*, we investigate expression patterns of stomata closure-related genes to know if COP1 regulates stomata closure-related genes or not. There are many genes which regulate stomata closure of *Arabidopsis*, we chose *OPEN STOMATA1 (OST1)* and *S-TYPE ANION CHANNEL1 (SLAC1)* for the assay. Both genes work in terminal mechanism of stomata closure (Mustilli et al., 2002; Vahisalu et al., 2008). To confirm if ABA worked in plants, we also chose *ABA INSENSITIVE1 (ABI1)* and *ABA INSESITIVE 2 (ABI2)*. We used *ACT2* as an expression control. In the case of *ABI1* and *ABI2*, relative expression levels of wild type and *cop1* mutants increase as ABA treated. In the case of *OST1*, relative expression levels of wild type and *cop1* mutants increase as ABA treated like *ABI1* and *ABI2* cases. In the case of *SLAC1*, relative levels of wild type increase and patterns of *cop1* mutants are not clear. The data are shown in Figure 2.

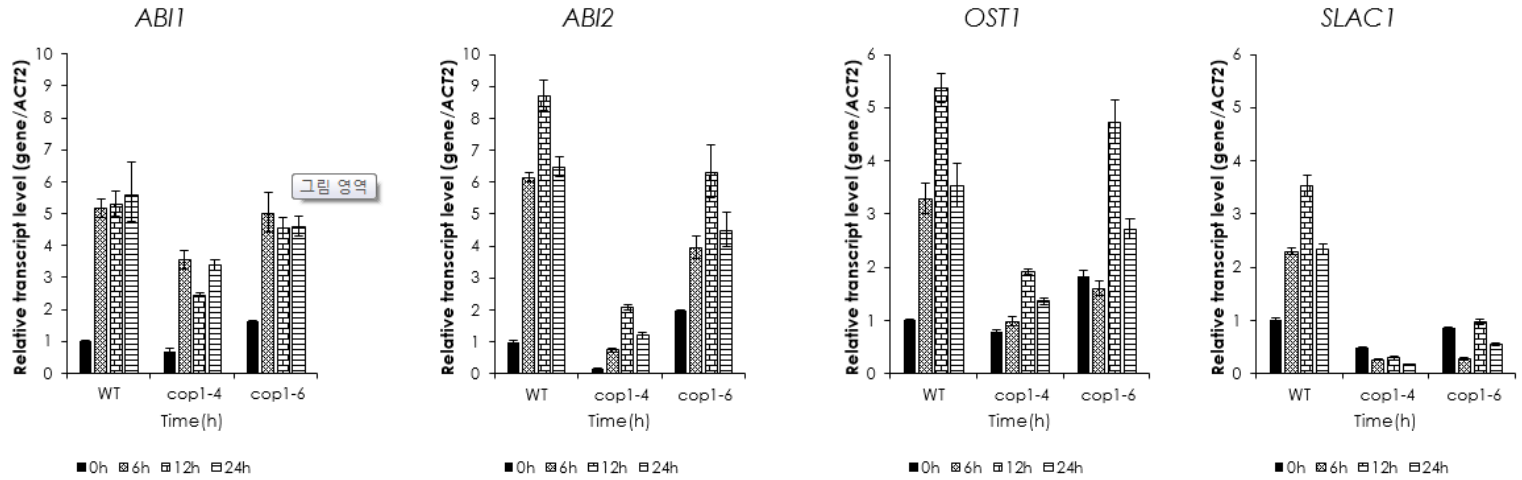


**Figure 2.** Stomata closure-related RNA transcription levels of ABA-treated plants. Two-week-old seedlings of wild type (Col-0), *cop1-4* and *cop1-6* treated with ABA (50μM) for indicated time. Samples were collected for Real-Time PCR analysis.

## Stomata of cop1-4 and cop1-6 mutants are not closed under heat stress treatment.

To know the effect of COP1 on heat stress-related stomata closure, we treated *Arabidopsis* wild type, *cop1-4* and *cop1-6* plants with heat stress. The *Arabidopsis* plants were three-week-old plants. Plants which had been grown on soil removed to a 37℃-set incubator, being potted. To measured apertures of *Arabidopsis* seedlings, we observed rosette leaves of heat stress-treated or control plants of wild type, *cop1-4* and *cop1-6* with microscope and calculated the ratio of width to length using 15 stomata for each case. Before plants are treated with ABA, stomata apertures of wild type and *cop1* mutants are similar. In the case of wild type, stomata apertures had decreased for the first 6 hours and the value was maintained after the first 6 hours. In the case of *cop1* mutants, stomata apertures did not change at all. The data are shown in Figure 3.

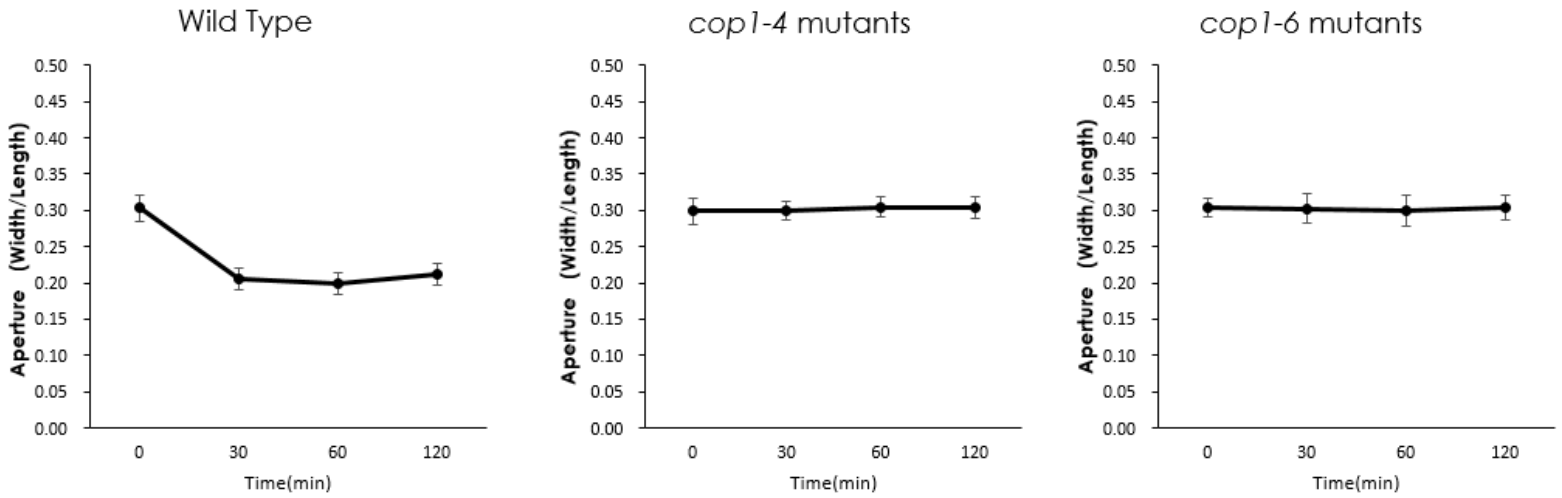


**Figure 3.** Stomatal apertures of heat stress-treated plants. Three-week-old plants of wild type (Col-0), *cop1-4* and *cop1-6* treated with heat stress (37℃) for indicated time. Samples were collected for microscope observation. Stomata aperture (the ratio of width to length) was quantified using 15 stomata from each sample.

## Heat stress does not affect stomata closure-related gene expression of cop1-4 and cop1-6 mutants.

After measurement of stomata apertures of heat stress treated *Arabidopsis*, we investigate expression patterns of stomata closure-related genes to know if COP1 regulates stomata closure-related genes or not. To confirm if heat stress treatment worked well, we also chose *HEAT SHOT PROTEIN101 (HSP101)* HEAT SHOT PROTEIN70b *(HSP70b)*. We used *ACT2* as an expression control. In the case of *HSP101* and *HSP70b*, relative expression levels of wild type and *cop1* mutants increase dramatically as heat stress treated. In the case of *OST1* and *SLAC1*, relative expression levels of wild type and *cop1* mutants decrease. The data are shown in Figure 4.

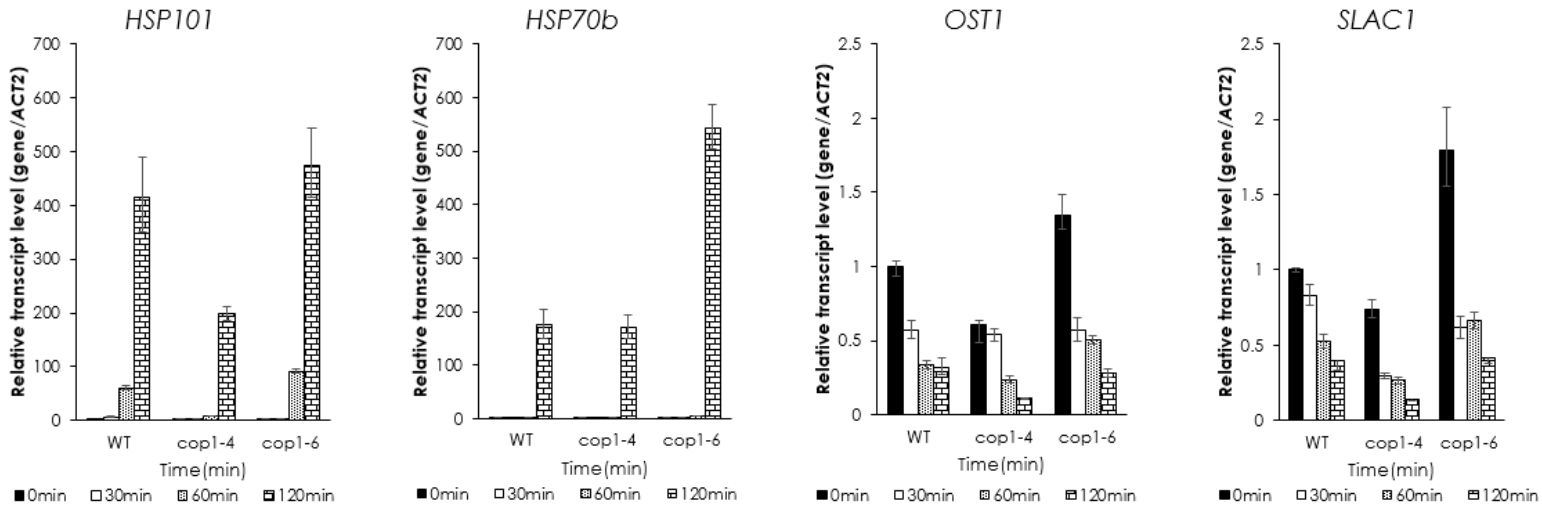


**Figure 4.** Stomata closure-related RNA transcription levels of heat stress-treated plants. Three-week-old plants of wild type (Col-0), *cop1-4* and *cop1-6* treated with heat stress (37℃) for indicated time. Samples were collected for Real-Time PCR analysis.

## Stomata closure-related proteins which interact with COP1 are not identified.

To investigate the mechanism how COP1 regulates stomata closure, we tried to identify stomata closure-related proteins which interact with COP1. We chose eight proteins for the assay: ABI1, ABI2, ABA INSENSITIVE3 (ABI3), ABA INSENSITIVE5 (ABI5), PYR1-LIKE1 (PYL1), PYRABACTIN RESISTANCE1 (PYR1), OST1 and SLAC1. We used pGAD424 plasmid as the activation domain plasmid and made pGAD424-*ABI1*, pGAD424-*ABI2*, pGAD424-*ABI3*, pGAD424-*ABI5*, pGAD424-*PYL1*, pGAD424-*PYR1*, pGAD424-*OST1* and pGAD424-*SLAC1* by molecular cloning. We used pGBT9 and pGBT9-*COP1* as DNA binding domain plasmids. We used yeast which transformed with pGBT9-*COP1* and pGAD424-*SIZ1* as the control. We used SD -2 (SD–Trp - Leu) media as control media and SD-3 (SD-Trp-Leu-His) media to investigate protein interactions. Any transformant except the control cannot survive on SD-3 media. All data are shown in Figure 5.

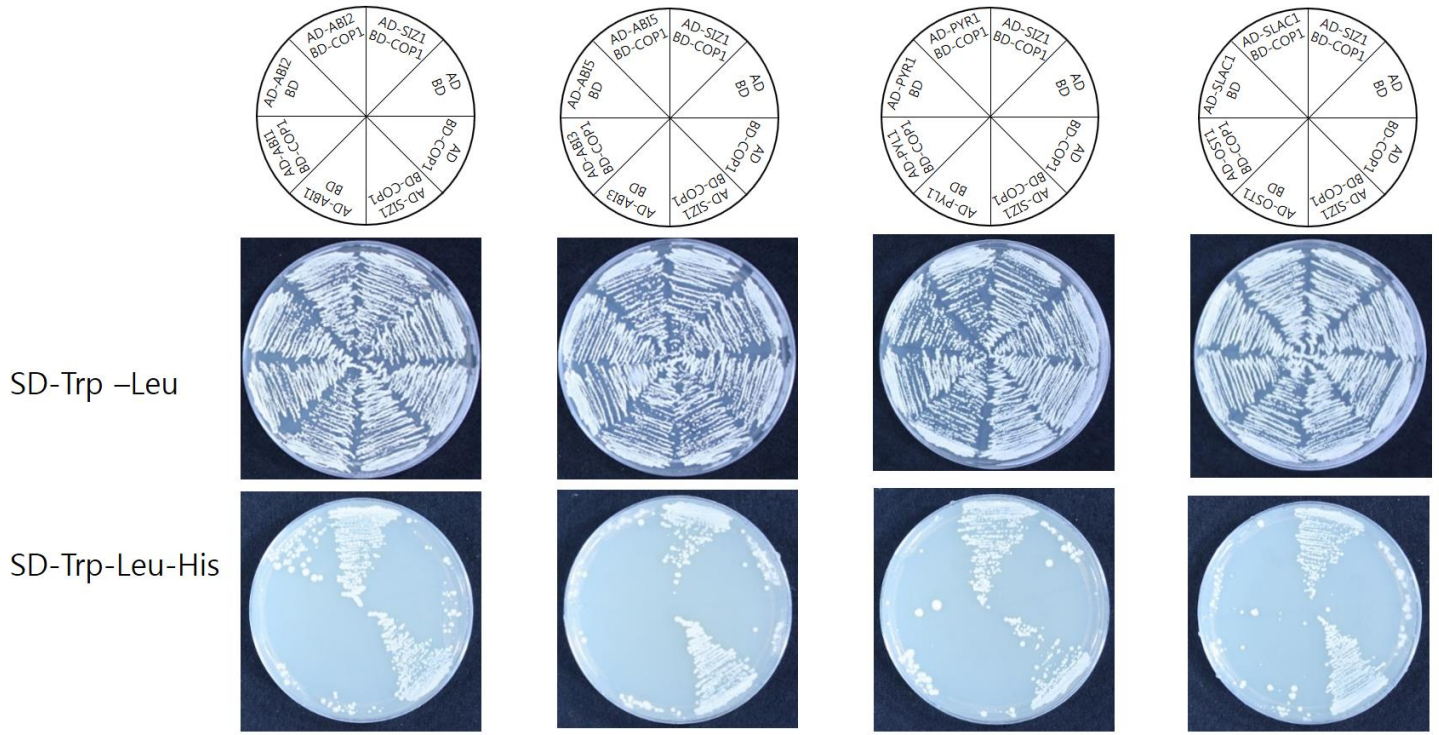


**Figure 5.** Investigation of stomata closure-related proteins which interact with COP1. Yeast transformants were assayed on SD-2 and SD-3 media. Transformants on SD-2 media are grown for 3 days and yeast transformants on SD-3 media are grown for 7 days.

# DISCUSSIONS

In this study, we checked patterns of stomata apertures when ABA or heat stress was treated. in *cop1* mutants, stomata were insensitive to ABA and heat stress; however, in wild type stomata were closed when ABA or heat stress was treated. We suggest that COP1 regulates stomata closure by not only darkness but also stress.

To know the mechanism how COP1 controls stomata closure, we checked patterns of stomata closure related genes when ABA or heat stress was treated. However, we failed to identify the difference among patterns of wild type and *cop1* mutants. Maybe, COP1 does not regulate stomata closure by changing relatively expression patterns of OST1 and SLAC1. However, other stomata closure related genes of which expression patterns are controlled by COP1 may exist. To know the mechanism, further studies on genes expression levels and COP1 should be conducted.

To identify stomata closure-related proteins which interact with COP1, we conducted yeast two-hybrid assy. However, proteins we chose were not proteins which interact with COP1. COP1 is a ubiquitin E3 ligase. To ubiquitinate substrates, COP1 interacts with substrates. Even though, we failed to identify stomata closure-related proteins which interact with COP1, further studies are required.

In short, stomata of *cop1* mutants are not closed under heat stress or ABA

treatment while these of wild type plants are not. However, under various stress or not, there is not special difference in RNA expressional levels. Stomata closure-related proteins which interact with COP1 are not identified through Yeast two-hybrid assay. These results indicate that COP1 controls stress-related stomata closure and subsequent studies are needed to investigate the mechanism.

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# 초록

기공은 호흡과 광합성을 위한 기체교환이 일어나는 공간으로 식물의 생장에 매우 중요한 역할을 한다. 그런데, cop1 돌연변이체가 야생형에 비해 다양한 파장의 빛 아래에서 기공의 aperture가 큰 것과, 암조건 하에서도 cop1 돌연변이체의 기공은 야생형과 달리 열려있다는 사실은 이미 선행연구를 통해 알려져 있다. COP1은 a ubiquitin E3 ligase이며, 암조건에서는 핵에 위치하여 광형성에 관여하는 단백질들의 분해에 관여한다는 것이 알려져 있다. 따라서 cop1 돌연변이체는 암조건에서도 광형성을 유지한다. 제 학위논문 연구에서는 어둠뿐 아니라 다양한 스트레스 조건 등을 포괄하여 다양한 야생형 애기장대가 기공을 닫는 조건하에서 cop1 돌연변이체가 기공을 닫는 지 여부를 알아보고, 더 나아가 COP1 단백질이 기공개폐를 조절하는 기작을 알아보는 것을 목적으로 하였다. 연구결과 Heat stress 및 ABA처리 하에서 cop1 돌연변이체는 야생형과 다르게 기공을 닫지 않았다. 스트레스 처리 하에서도 cop1 돌연변이체의 RNA

발현 level은 야생형과 뚜렷하게 다른 패턴을 보이지 않았다. Yeast two-hybrid로써 COP1과 interaction하는 기공개폐 관련 단백질을 찾지 못했다. 따라서 COP1은 명암뿐 아니라 ABA나 스트레스에 의한 기공개폐에도 관여한다는 것을 알 수 있었다. 다만 그 기작에 대해서는 추가적인 연구가 필요하다.

주요어: E3 ubiquitin ligase, COP1, Interaction, Regulation, Stomata, Stomata closure

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