ABSTRACT

A Genetic Analysis of the two potential BIN2 Interactors At5g62970 & At5g56690

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Ubiquitination is the mechanism used to remove proteins from cells, and plays a very important role in protein synthesis, function, and degradation. In Arabidopsis, brassinosteroid (BR) signaling leads to a direct gene response by ubiquitinating the BIN2 protein along with the receptor. BIN2 is known to be a constituent of the Skp, Cullin, F-box containing (SCF) complex,

and thus the F-box E3 Ligase protein binds to the target protein (BIN2) to carry out ubiquitination.

To confirm the role of the F-box protein in the BIN2 degradation process, we cloned two candidate genes: At5g62970 and At5g56690, which were confirmed by previous in vitro pull-down assay. The two candidate genes were cloned into an overexpression vector and transformed into the wild-type Col-0 and Pro35S:BIN2 transgenic lines. Selected plant individuals had a recovered mutant phenotype similar to wild types, but the phenotype was not consistent. In confirming biosynthesis and the expression of BR signaling marker genes through RT-PCR, the genes affecting biosynthesis did not change much, but the genes involved in the signaling pathway did change, confirming the effect of At5g62970. After treatment with the brassinosteroid biosynthesis inhibitor propiconazole, we confirmed that Col-0/Pro35S:At5g62970 did not activate signal transduction by continuously removing BIN2.

It can be inferred that At5g62970 is related to BIN2 expression because phenotype recovery of Pro35S:BIN2 occurred in Pro35S:BIN2/Pro35S:At5g62970. However, no significant phenotype changes were observed in some Pro35S:BIN2/Pro35S:At5g62970 individual plants, although these individuals expressed the At5g62970-FLAG protein. This implies that the At5g62970 is not the only E3 ligase to bind to BIN2; it can be assumed that there is another E3 ligase which binds to

BIN2 when At5g62970 does not function. Recently, it has been reported that the gene, KIB1, functions as an E3 ligase of BIN2, suggesting that there are many E3 ligases of BIN2. At5g62970 is suspected to be a transient or weak-binding E3 ligase in BIN2 degradation, which acts as a minor pathway.

Keywords : *BRASSINOSTEROID INSENSITIVE 1 (BIN2)*, Ubiquitination, GSK3β-Like Kinase(GLYCOGEN SYNTHASE KINASE3), F-Box Protein, Brassinosteroid, c*Arabidopsis*

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ABBREVIATIONS

BIN2	Brassinosteroid Insensitive 2
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
MS	Murashige-Skoog
BZR1	Brassinazole Resistant 1
CS	Castasterone
PTMs	Post-Translational modifications
GSK-3	Glycogen Synthase Kinase 3
BES1	Bri1 EMS Suppressor 1
BSU1	Bri1 Suppressor 1
BL	Brassinolide
BR	Brassinosteroid
RING	Really Interesting New Gene
HECT	Homologous to the E6-AP Carboxyl Terminus

Ⅰ. Introduction

1. Composition and mechanism of polyubiquitination in Cell

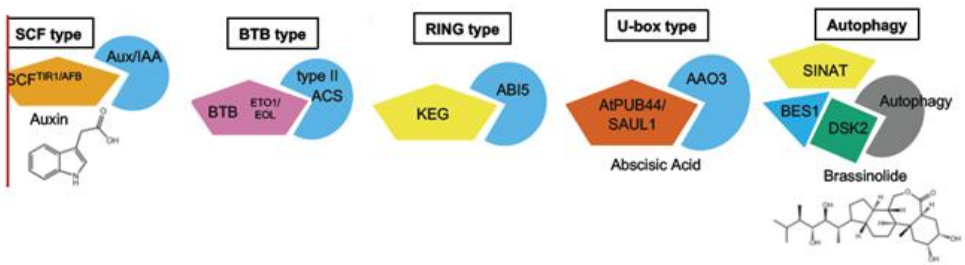
In eukaryotic Cells, proteins were modified in various ways. Ubiquitination is such an One of the post-translational modifications (PTMs), which generally regulate histone regulation, endocytosis, and when ubiquitylated as a chain (Cardozo & Pagano, 2004). This called polyubiquitylation and regulates protein degradation and DNA repair. Among these regulations, degradation process precisely related with gene expression. To ilustrate this polyubiquitylation process, After protein which modified to marked with degradation signal, called degrons, the ubiquitin ligase binds to this signal. This ubiquitin ligase called E3 ligase. This E3 Ligase bind with other accessory protein called E2 protein, the ubiquitin-conjugation proteins. This ubiquitin-conjugation protein attached ubiquitin on the substrate lysine side chain, and repeat of this process make polyubiquitin chain. This polyubiquitin chain is recognized by proteasome, and proteasome degraded polyubiquitylated protein substrate (Jeong et al., 2011).

Researchers first discovered F-box protein for candidate

genes at polycystic kidney disease (PCKD) gene locus, but it soon classified differently with other cyclins. And after the discovery of Cyclin F, this candidate which first noted in cyclin F bacame F-box and F-box Family (Kraus et al., 1994). The E3 ligase have 30 different constructal form in mammals (Carlos et al., 2014), and hundreds of E3 proteins were complexed with specific of E2 proteins. The E3 ligase classified into three groups. The single-subunit RING-finger type, the multi-subunit RING-finger Type and the HECT-domain type (Dior R. Kelley, 2018). Most of the multi-subunit RING-finger type of E3 ligases contain a cullin protein (Cul1), cRING-finger protein (Rbx1), crucial adaptor subunit Skp1 (S-phase-kinase-associated protein-1), specific E2 enzyme and these complexs were binds to one of many F-Box Protein (FBP) (Figure 1). In mammals, ubiquitylation process was further understanded while studying Skp1-Cul1-F-box protein (SCF) Ubiquitin ligase, which has an important role in many mammalian functuions like Cell division, development, and various of other cell metabolism (Iconomou & Saunders, 2016). There are more than 70 F-box proteins were found in mammalian cell and 326 of F-box protein were discovered in C.elegans (Cardozo & Pagano, 2004). In case of Plant, 1400 E3 ligase were discovered (Vierstra, 2009).

In plant, not many ubiquitination research were studied profoundly. Ubiquitination was known in plant for regulation of

cell proliferation, embryogenesis, abiotic stress, senescence, and hormone signaling pathway. studies in plant hormone signaling pathway were understaned. About 1600 genes are involved in Ubiquitin pathway in Arabidopsis, and this indicates approximate 6% of total genome (Vierstra, 2009). Therefore, this small number of related genes shows Ubiquitination process were greatly interwoven in plant life.



Dior R. Kelley, 2018

Figure 1. Various types of plant E3 ligase and F-box system

2. Arabidopsis BIN2 and Brassinosteroid Signaling Pathway

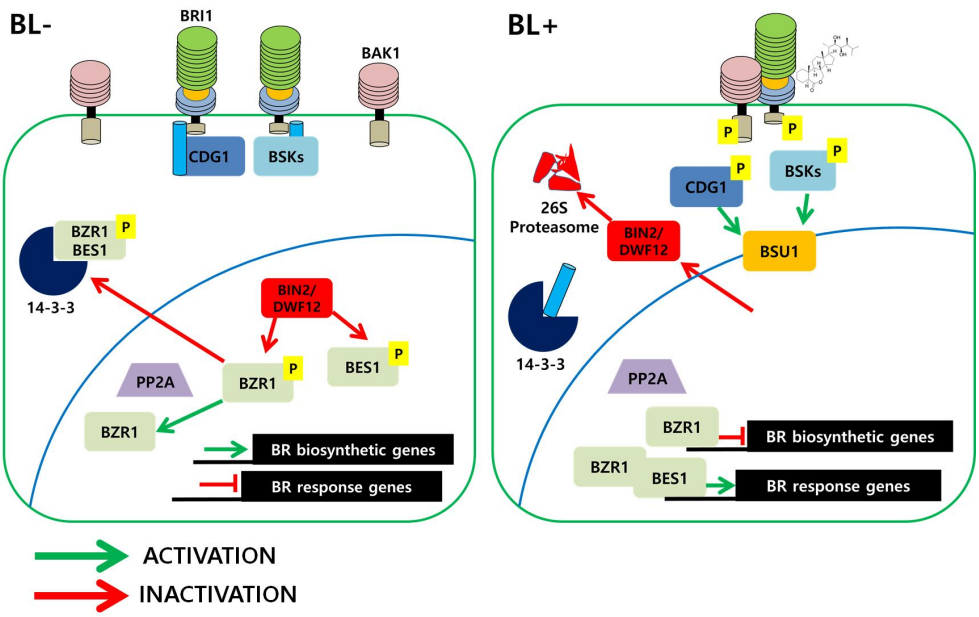
Generally, plants have growth hormones that act as regulators of entire growth process. Representatively, Auxin, Cytokinin, Abscisic acid, Gibberellin, Ethylene and Brassinosteroid are discovered to date. And Brassinosteroids are a group of about 50 structurally related steroid hormones that are involved in the overall life cycle of a plant, including growth, cell division, cell differentiation, stress response, and reproduction (Chung & Choe, 2013). On the top of that, BR had been researched for a decade, because it affect mainly on crop yield.

BR started its research with a breakthrough in the growth of pollen extracts stemmed from the stem of plants and then purified the extracts for the first time as brassinosteroids. Brassinolide (BL), the first brassinosteroid extracted from pollen, was found, and mutagenic studies have since been found to be similar to steroid hormones in other animals. Thereafter, defective mutants were found in BR synthesis, and biosynthetic pathways, delivery pathways, receptors, and signal transduction mechanisms of brassinosteroids. Through studying these mutants and other model plants, It is known that Castasetrone (CS), which is not the final product, is the active from of Brassinosteroid in rice (Kim et al., 2008). To date, research on BR biosynthesis and signal transduction has been carried out

through plants such as Arabidopsis, rice, tomatoes and soybeans. And BR has been applied to various fields such as cell growth and division, vascular differentiation, which is known to play an important role in the growth and development of plants in a variety of ways (Fridman & Goldstein, 2013).

Synthesized brassinosteroids react through genes through a variety of signaling pathways. In the absence of brassinosteroids, GSK3β-Like kinase BIN2 / DWF12 (Brassinosteroid Insensitive 2; BIN2) plays a particularly important role in the cell (Choe et al., 2002). In the absence of brassinosteroids, it plays a role in inhibiting the reaction by phosphorylating BZR1 and BES1 that bind to the brassinosteroid reaction gene in the cell (Wang et al., 2002; Yin et al., 2002). Particularly, BZR1 plays a role of binding to a promoter of a synthetic gene to inhibit the synthesis, and thus has a role of phosphorylating and promoting the synthesis (Vert and Chory, 2006). When the Brassinosteroid is conjugated to the receptor BRI1 receptor phosphorylase, BRI1 phosphorylates BKI1 and leaves it from BRI1. Subsequent BAK1 phosphorylates and binds BRI1, which initiates signaling by phosphorylating CDG1 and BSKs. These two enzymes then activate the phosphatase, BSU1, and the activated BSU1 deactivates BIN2 / DWF12 by removing the phosphate group of the Tyt200 residue of BIN2. When BIN2 is inactivated, phosphorylated BZR1 and BES1 become active, and bind to a BR reaction gene to synthesize a reactive gene and inhibit the

synthesis reaction by binding to a synthetic gene promoter. At this time, the inactivated BIN2 / DWF12 goes out of the nucleus and is degraded by the proteasome after ubiquitination (Chung & Choe, 2013).



Chung and Choe, 2014

Figure 2. Brassinosteroid signaling pathway

3. GSK3 (GLYCOGEN SYNTHASE KINASE 3) and BIN2 Ubiquitylation

In general, Glycogen Synthase Kinase 3 (GSK-3) in animals is a central enzyme that regulates glycation and regulates the oncogenesis mechanism. GSK3 (GLYCOGEN SYNTHASE KINASE 3) is a multifunctional phosphate enzyme that is highly conserved throughout eukaryotes (Bert De Rybel et al., 2009). It has been known that after the first gene is discovered in Drosophila, it has been shown to phosphorylate glycogen synthase in the muscle cells of rabbits and plays an important role in signal transduction. It is also known that more than 80 varieties are found in mammals, and that GSK3 participates in a variety of cellular responses, especially essential processes such as insulin (Kaidanovich & Woodgett, 2011). The GSK-3 protein has many families and BIN2/DWF12 is the most famous GSK3β-like kinase family in plants. BIN2 phosphorylates two BR-responsive transcription factors, BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (bri1 EMS SUPPRESSOR 1), in the process of BR signal transduction. In particular, the gene that plays a role of GSK3 in Arabidopsis plants has been identified as AtSKs, and 10 types have been identified (Casson & Hetherington, 2012). GSK3 increases the phosphorylation function by 1000-fold when the C-terminal Ser / Thr residue is phosphorylated in the animal, which is well known in plants, but

functions in plants without scaffold proteins binding the phosphorylation targets (Yan et al., 2009). Recently, it has been reported that GSK3 of plants can have different functions depending on the substrate without phosphorylation of the ends (Youn & Kim, 2015). The regulatory mechanism of the phosphorylation enzyme also depends on the autophosphorylation of the tyrosine group in the T-Loop region of the phosphorylated domain in plants if the phosphorylation of the N-terminal serine group in the animal. In particular, in the case of BIN2 in plants, the phosphorylation of the 200th tyrosine is reduced by the dephosphorylating enzyme BSU1 (bri1 SUPRESSOR 1), and phosphorylation is inhibited, and the pathway is taken into the degradation pathway (Youn & Kim, 2015).

Thus, the response to BR is regulated by a key protein called BIN2, which plays a role in growth regulation, flower pattern, stress response and signal transduction (Tong et al., 2012). In animals, this GSK is regulated by phosphorylation, and various types of GSKs are influenced each other. And the role of GSK3 β-like kinase in plants is expected to vary (Youn & Kim, 2015). However, specific signal transduction and degradation pathways are not yet known even in the case of representative BIN2, and it is presumed that there are many other factors controlling this function. Recent studies have shown that a protein called KIB1, which has the potential of this E3 ligase, is

involved in ubiquitination (Zhu et al., 2017). However, it is expected that E3 ligase, which is additionally regulated by the type of E3 Ligase.

4. Purpose of this study

Research on the ubiquitination of plants is one of the key tasks in not only plants but also in molecular cells and biochemistry, and there are a number of genetic research methods that can not be approached or revealed many.

The E3 ligase associated with brassinosteroids is not yet known, but since it is known that BIN2 is ubiquitinated, it has been researched through various information that it is degraded by ubiquitination, Will be the beginning of the next turn. In particular, it is expected to have a significant impact on animal and human cell molecular biology research as well, as it is directly related to BIN2, a GSK3β-like kinase that is important in many organisms (Cardozo & Pagano, 2004). There are about 1,500 E3 ligases present in plants. In Arabidopsis, they are HECT (Homologous to the E6AP carboxyl terminus) type, RING (Really Interesting New Gene) family, Kelch-type and U-box Containing ubiquitin protein ligase , And Cullin-RING Family. They are known to affect the hormonal regulation of ABA (Absisic Acid) or JA (Jasmonic Acid), and about 100 E3 ligases have been found to play a role in hormone-related processes (Vierstra, 2009).

However, only one of the most recently identified proteins has been identified in the signal transduction pathways of BR, and it is not yet known which additional E3 ligase is important.

Therefore, we investigate whether two candidate genes are directly involved in ubiquitination, and how to identify the function of two candidate genes and how they actually function as F-box proteins.

The aim of this study is to identify the E3 ligase in the ubiquitination process of BIN2, a key protein of brassinosteroid hormone, which plays a pivotal role in many plant growth regulators, Indeed, it is possible to identify the role of BIN2 in ubiquitination through phenotype and genotyping, and to predict the function of the gene.

Ⅱ. MATERIALS AND METHODS

1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type. At5g62970(SALK_057064) and At5g56690 (SALK_098699C) plants were ordered from the ABRC, Ohio State University. Arabidopsis plants were grown in the growth room or growth chamber under long day conditions (16h of light at 23℃ /8h of darkness at 22℃). Seeds were sown on the surface of soil (Sunshine #5, Sungro), then put in 4℃ cold chamber with darkness for 2~5 days before moved to growth room. For transgenic plant screening, surface of seeds were sterilized by treating with 70% ethanol for 10 minute, 75% ethanol containing 0.08% Triton X-100 (SIGMA) for 5min thrice, followed by washing briefly with 100% ethanol (MERCK). The seeds were dried on Whattman filter paper and plated on solidified MS agar (0.5X Murashige and Skoog salts including Gamborg' s B5 vitamins [DUCHEFA], 1% sucrose [DUCKSAN Reagent] and 0.07% plant agar [DUCHEFA], pH between 5.7 and 5.8 into 1L of distilled water was autoclaved in 121℃ for 15min) plates containing appropriate antibiotics. Antibiotics were used for transgenic line selection, 50㎍/ml of Kanamycin [DUCHEFA]

was used for Salk line selection, and 25㎍/ml of DL-Phosphinothricin [DUCHEFA] was used for overexpression transgenic line selection. Before transferring to the growth room, MS plates with seeds were also put in 4℃ for cold treatment for 2-5 days. Per construct, around 20 Columbia-0 (Col-0) wild-type plants and 200 BIN2 Overexpression plants were used for *Agrobacterium*-mediated transformation by the floral dipping method.

2. Characterization of the At5g62970 and At5g56690 allele

To confirm the T-DNA insertion, genotyping PCR was carried out by using genomic DNA as a template. According to Arabidopsis.org, T-DNA in At5g56690 was inserted into the exon, and T-DNA in At5g62970 was inserted into the intron, 364 nucleotides downstream of the start codon. The left border junction of At5g62970 and At5g56690 were determined using the T-DNA primer LBb1.3 and genomic primer At5g62970 RP (NR1). For amplification of the At5g62970 wild-type allele, gene specific primers At5g62970 LP(NR2) and At5g62970 RP (NR1) were used. After genotyping, phenotype of the heterozygous and homozygous At5g62970 and At5g56690 plants were observed, then harvested separately and stored in 4℃.

3. Recombinant plasmid construction

For analysis of candidate gene At5g56690 and At5g62970, two genes were isolated from BL treated Col-0 plant. Two candidate genes were not expressed dominantly when they are in normal status. On the other hand, when Brassinolide treated in arabidopsis, Arabidopsis plant cell started BR signaling pathway and BIN2 were started to degradate. In this degradation pathway, F-Box protein were expressed and binds with BIN2. So the F-box protein gene were expressed higher than untreated Arabidopsis. BL-treated Arabidopsis seedling were grinded into fine powder, and isolated RNA by using Trizol reagent (Invitrogen). Plant tissue was grinded into fine poweder, and 1ml of QIAZOL is added in the tube. Samples are incubated in Room temperature for 5 min, and centrifuge at 12,500 rpm for 15 minute, 4C° . After the centrifuge, clear solution was transferred into new tube, and 200ul of chloroform is added. Mixed sample were stayed for 3 minute, and centrifuged 13000rpm, 4C° . The upper layer were carefully transferred into new tube, and 200ul of isopropanol were added. After 30 minutes of incubation on room temperature, samples were centrifuged at 12,000rpm, 4C° . after the centrifugation, 75% of EtOH were added for washing pallet. and centrifuge at 7500rpm, 5min. Discard and evaporate all the EtOH, and RNA were diluted with pure water. Isolated RNA were synthesized into cDNA by using Reverse Transcriptase (ThermoFisher Scientific). 100ng of isolated RNA

and OligoDT were incubated at 65C° for 5 minutes. and added 5X reaction buffer, RNase Inhibitor, dNTP mix and Reverse Transcriptase were mixed and incubates at 42C° for 60 minutes. Using F primer (NR3) which start from start codon ATG, template was amplified by PCR. Amplified clone was attached CACC in front of the start codon, by using different Foward primer (NR4) for pENTR™ Directional TOPO Cloning Kit. These clones were inserted into pENTR™/SD/D-TOPO vector, and confirmed by using MluI and EcoRV restriction enzymes (New England Biolab).

4. *Agrobacterium tumefaciens* transformation and plant transformation by floral dipping

The cloned constructs were introduced into *Agrobacterium* GV3101 by electroporation for transforming Arabidopsis genome. For transformation, 50㎕ *Agrobacterium tumefaciens* (GV3101) cell stock in a 1.5ml tube was thawed on ice for 10min, and then 2㎕ of DNA of interest was added to the competent cell mixing by gentle pipetting. 400㎕ of YEP medium was added to the cuvette and mixture was transferred to a 1.5ml tube and incubated in shaking incubator at 28℃ for 1h before spreading on solidified LB plate with proper antibiotics (50㎍/ml kanamycin, 50㎍/ml gentamicin 25㎍/ml Rifamficin). The plate was incubated at 28℃ for 2 days.

For Arabidopsis transformation, floral dipping method was used. Single colony of transformant *Agrobacterium* was selected and inoculated into 5 ml of LB medium with adequate antibiotics (50㎍/ml kanamycin, 50㎍/ml gentamicin) and grown for 24h at 28℃ shaking incubator. To amplify the bacterium cell, mini-prep cultures were then inoculated again into 300 ml of YEP medium containing same antibiotics and grown in the same condition. Incubated *Agrobacterium* cell was harvested by centrifuge at 4000 rpm for 20min at 20℃ (Eppendorf SOVAL Centrifuge). After discarding supernatant, the cell pellet was resuspended in infiltration media. 300ml of infiltration media contains 0.63 g of MS salt, 15 g of sucrose and 150 ㎕ of Silwet (Vac-In-Stuff, Silwet L-77, LEHLE SEEDS). For successful Arabidopsis transformation, *Pro35S:BIN2* and Col-0 plants were grown on soil until adequate number of inflorescences are generated in a long day condition. Right before transformation, siliques and open flowers were removed. Young buds were then dipped into the infiltration media containing *Agrobacterium* for 10 sec. After floral dipping, plants were laid on a tray and covered with black plastic bag for blocking light for 24 h. Next day, the plants were uncovered, put vertically and grown until their seeds were mature enough to harvest.

5. Protein extraction

For extracting proteins for analysis, seedlings or leaves were

used as a sample. Samples were grinded into a fine powder with liquid nitrogen, and Protein extract buffer was added on the sample. For making 1ml of Protein extraction buffer, the composition was 646㎕ of Diluted Water, 50㎕ of pH7.5 Tris-HCl, 60㎕ of 2.5M NaCl, 200ul of 50% Glycerol, 10ul of 10% SDS, 1ul of 1M DTT, 10ul of 1M MgCl2, 2ul of 0.5M EDTA, 1ul of 0.1% NP40 (Nonidet), 10㎕ of 100x PMSF and 10㎕ of Proteinase Inhibitor (GenDEPOT). 20㎕ of protein extraction buffer were added, and incubated in ice for 10 minute. After the incubation, tubes were centrifuged on 13,000 rpm 4℃ for 15 minute. Supernatant were transferred to new tube. 5X SDS Sample buffer were added, and boiled for 7 minutes.

6. Western blot Analysis

For Detection of Western blot, WesternBright ECL HRP Substrate, 500ml Kit was used as an ECL. FLAG antibody and His antibody used for expression construct. Monoclonal ANTI-FLAG M2 antibody produced in mouse (SIGMA) was used for detection of FLAG antibody, and 6x-His Tag Monoclonal Antibody (4E3D10H2/E3), HRP (ThermoFisher) was used for detection of His antibody.

Table 1. List of primer sequences

Label	Oligo name	Sequences (5' – 3')
NR1	Salk_051605 RP	5'-ACG CGT CGA CCA ACC CTT ACT CCC TTT CTT TC-3'
NR2	Salk_051605 LP	5'-GCC GGC AAG CTT TCG TTA TTA AAT AAA ATG TAG GAG AAA AA-3'
NR3	62970_CDS_ F	5'-GGC CAA GCT TAA TAT CCC TAA TAT CTA ACT ATA TTA AAC C-3'
NR4	62970_CDS_ CACC	5'-GCC GAA TTC AAT TAA CCT CTA TCG TTT CAC CT-3'
NR5	LBb1.3	5'-GCC GAA TTC TGA TTG ATT TTA TGA GTT TTC ACA-3'
NR7	At5g56690q RT_R	5'-GCA GGA TTG ATG TAG ATT TCA G-3'
NR8	At5g56690q RT_F	5'-TAC TAC AGC GAG GAG CAA GA-3'
NR9	At5g62970q RT_R	5'-AAC AAT ATG CTT CCC AGA TG-3'
NR10	At5g62970q RT_F	5'-GTT CAT ACC AGA CAA CAA TGC-3'
NR11	pEarleyGate _202_Nflag_ F	5'-GAC TAC AAA GAC GAT GAC GAC AA-3'
NR12	AT5G62970 _F	5'-CAC CAT GGA CAA GAT CAG TGG GTT TTC TGA T-3'
NR13	AT5G62970 _R	5'-ATA GAA TAC GCG TTT GCA TGT GGG TGA -3'
NR14	SALK_0986 99C_F	5'-TTG GAA CTT AAA CGT GTG AG-3'
NR15	SALK_0986 99C_R	5'-ACA ATA CCA GAA TCA TAC CC-3'
NR16	SALK_0516 05_F	5'-CTT TGT TCC GTT CCT CTC T-3'
NR17	SALK_0516 05_R	5'-ATA GGT TTC GCT GTC AAC C-3'
NR18	SAUR-AC1 RT-F	5'-ATG GCT TTT TTG AGG AGT TTC TTG-3'

NR19	SAUR-AC1 RT-R	5'-ATT GTA TCT GAG ATG TGA CTG TG-3'
NR20	UBQ10(rTime)-F	5'-CCA CCA AAG TTT TAC ATG AAA CGA A-3'
NR21	UBQ10(rTime)-R	5'-TCC AGG ACA AGG AAG GTA TTC C-3'
NR22	CPD rTime F	5'-ATG AAA AGG AGG GAG GAG GA-3'
NR23	CPD rTime R	5'-TGA TCG TGG AGG TTG TTT CA-3'
NR24	CYP85A2 rTime F	5'-AAC GAA TTA CCG CAG TGG AG-3'
NR25	CYP85A2 rTime R	5'-AAG AGC CAT CAT GGA GGT TG-3'
NR26	DWF4RT-F	5'-AGA TGT TCG GTA CAA AGG ATA CGA TAT C-3'
NR27	DWF4RT-R	5'-GTT TAT CAT CTT CTG CTA ATT CCC AAT TG-3'
NR28	PearleyGate 103 His_R	5'-CAC GTG GTG GTG GTG GTG GTG-3'
NR29	Sal_40PRO_ 2.5K_F	5'-ACG CGT CGA CTA GAT GAA AGA TTT TGG GTT GA-3'

Ⅲ. Results

1. Genotypic and phenotypic analysis of T-DNA insert mutant

Previous studies have suggested that BIN2/DWARF12 may regulated by ubiquitin-dependent degradation (Peng et al., 2010). In addition, studies have shown that BIN2 degrades proteasome (Peng et al., 2008). By using Pull-Down Assay, two of the F-box candidate gene has been found. These two genes were At5g62970 and At5g56690 (Figure 3). For the mutant analysis, T-DNA mutants were ordered from the Salk institute. The T-dna mutants Salk_057064 and Salk_098699C of At5g62970 and At5g56690 respectively, were confirmed. The T-DNA insert mutation of the plants were confirmed by genotyping (Figure 4). 5 individual plants of Salk_051605 and 3 individual plants of Salk_098699c were selected as homozygous lines (Figure 5, Figure 6). Compare to the wild type, the root hair grows mature and the root length was about 0.5cm longer than wild type. But mutants didn't show any significant phenotype when they are matured. T-DNA mutant plants were ground and extract RNA. Two RT-PCR primers were prepared to analyze the RNA expression. CDNA normalized with UBQ. In

the case of At5g56690, it was confirmed that the RNA was expressed normally as if it was not mutated, unlike the expectation. At5g62970, it was confirmed that the genes 1, 8 and 10 did not express the gene and the lines 4 and 9 were expressed, and the expression was less than wild type (Figure 7). Thus, the mutation line of At5g56690, Salk_057064, was not used in the subsequent study, and the mutation of At5g62970, the 1st, 8th and 10th mutants, were used. In the case of mutants lacking the F-Box protein, it is expected that BIN2 will not be removed by normal protein production. In order to confirm this, it was cultured in BL-treated medium. In this case, BIN2 was removed by the 26s proteasome along the BL signaling pathway in the wild type, but the mutant was expected to show a different phenotype from that of the wild type since the BIN2 could not be removed. As with the BIN2 overexpression, we expected to show distorted Rosetta leaves, short roots, and dwarf phenotype.

However, there was no specific phenotype other than a slight difference in length at the root (Figure 8). In light of the Semi-RT PCR results, the results show that mutant lines do not express the gene, but BIN2 is degraded through additional pathways or that the candidate gene may not function as an F-box E3 ligase of BIN2.

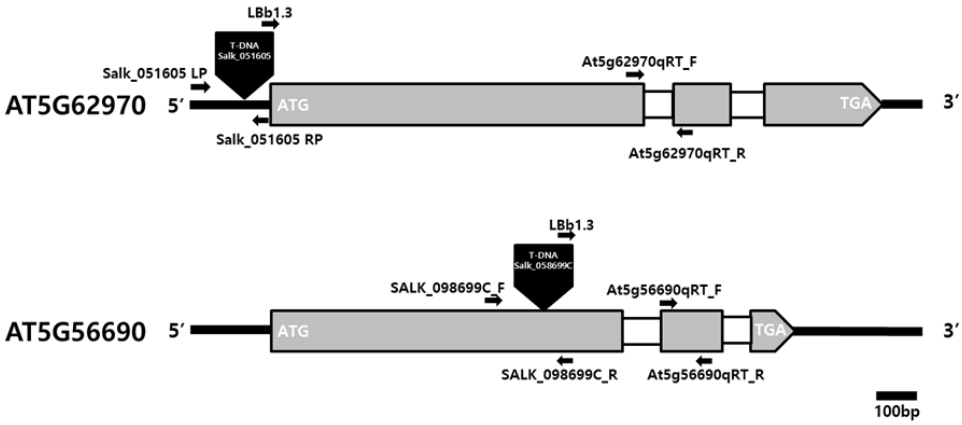


Figure 3. Schematic structure of the At5g62970 and At5g56690 genes.

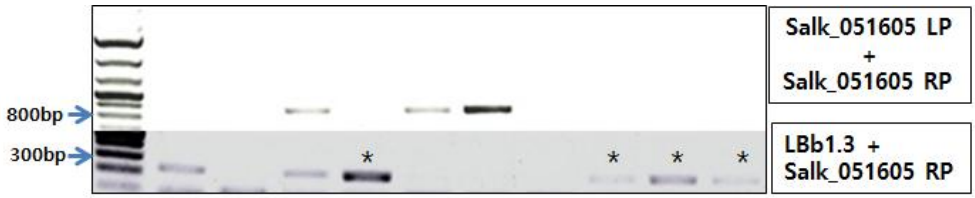


Figure 4. PCR for identification of the Salk_051605 T-DNA insertion

Using Salk_051605 LP and Salk_051605 RP, wild type allele was confrimed (800bp), and by using LBb1.3 and Salk_051605 RP, mutant allele was confirmed (264bp). Asterisks (*) indicates confirmed individual Salk_051605 Plant.

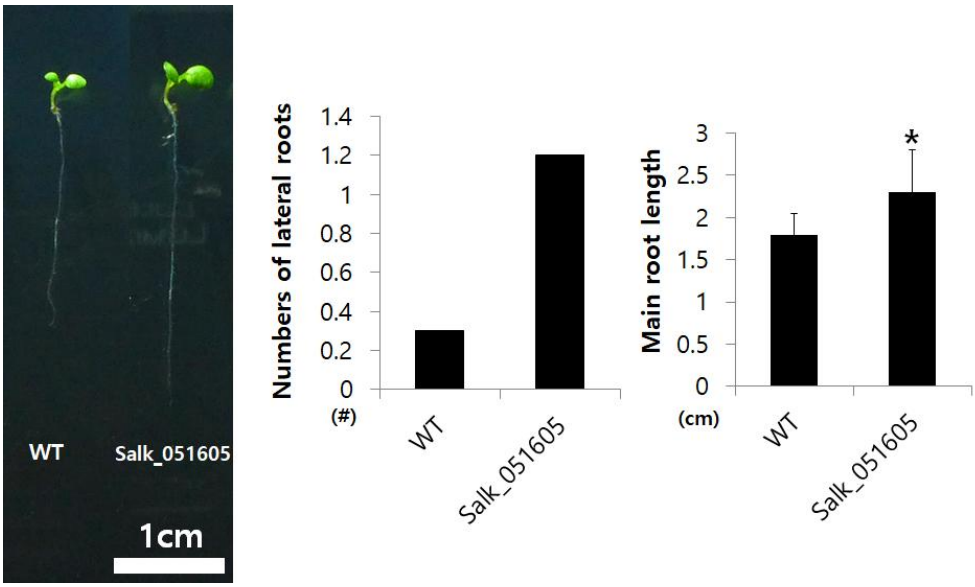


Figure 5. Morphological analysis of At5g62970 T-DNA insert mutant

(A) 6-day-old seedling phenotype of wildtype (Col-0) and At5g62970 T-DNA mutant (Salk_051699). Graph shows main root length of 6-day-old seedling and number of later roots. Asterisks (*) indicate significant differences to the respective Col-0(WT) determined t-test ($p<0.05$)

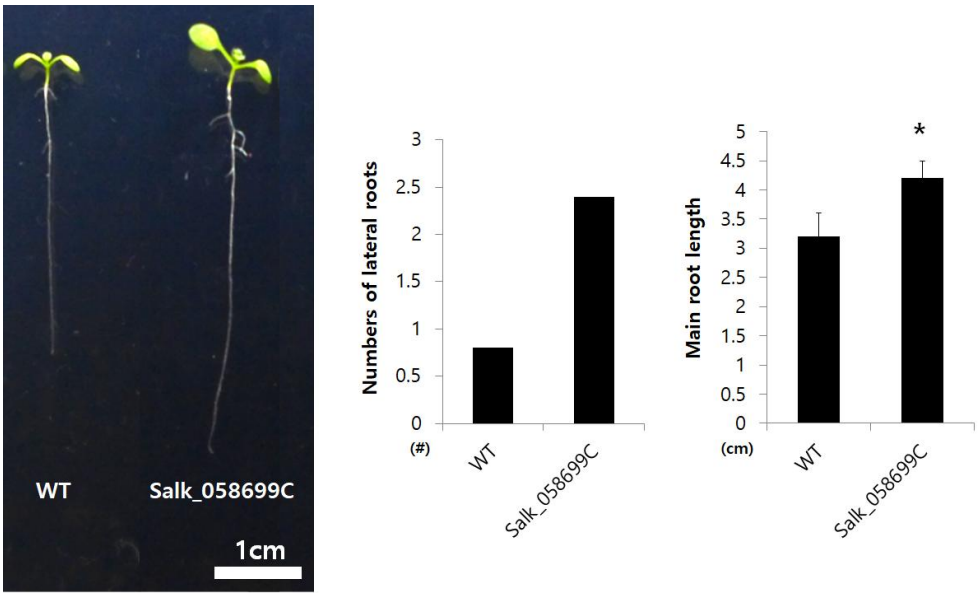


Figure 6. Morphological analysis of At5g56690 T-DNA insert mutant seedlings

6-day-old seedling phenotype of wildtype(Col-0) and At5g56690 T-DNA mutant (Salk_058699C). Graph shows main root length of 6-day-old seedling and number of later roots. Asterisks (*) indicate significant differences to the respective Col-0(WT) determined t-test ($p<0.05$)

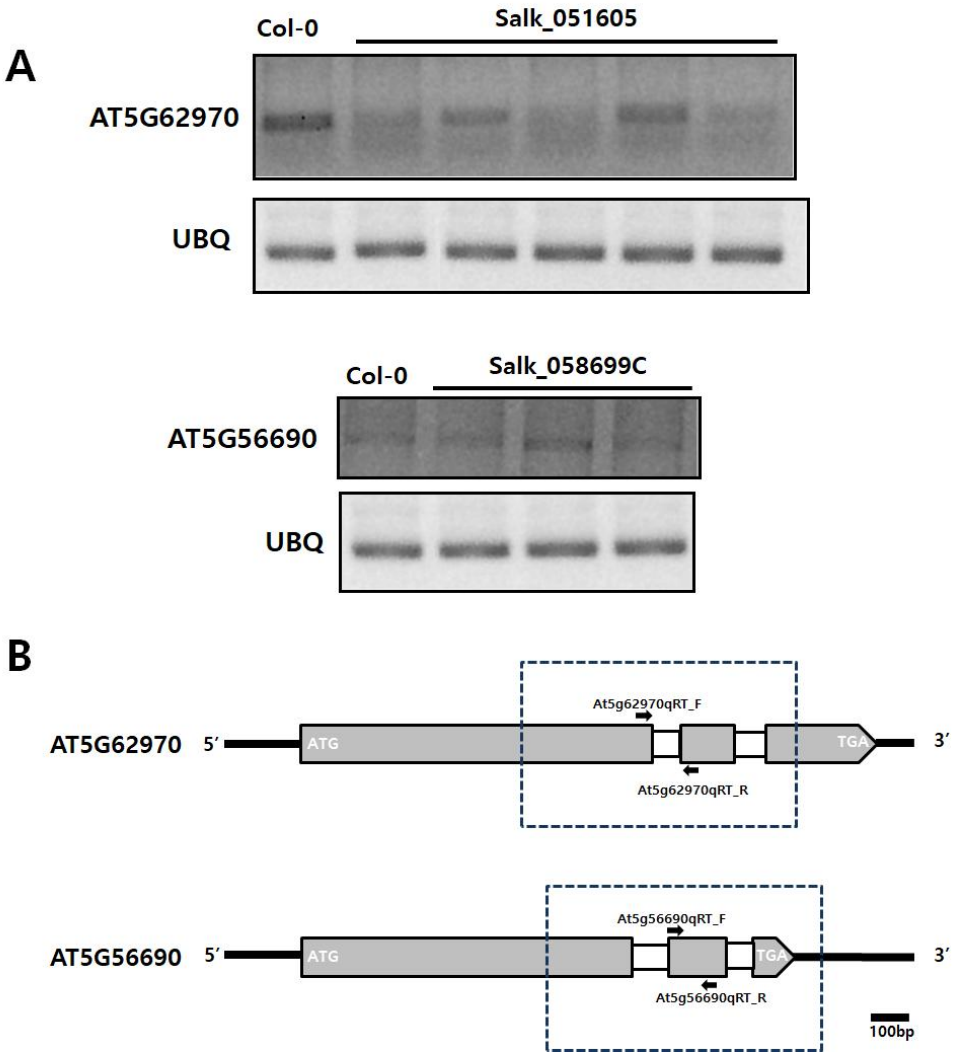


Figure 7. Difference in RNA expression in WT and T-DNA mutant plants

(A) RT-PCR analysis was carried out with total RNA isolated from seedlings. (B) Schematic structure of At5g62970 and At5g56690 gene including location of RT-PCR Primer

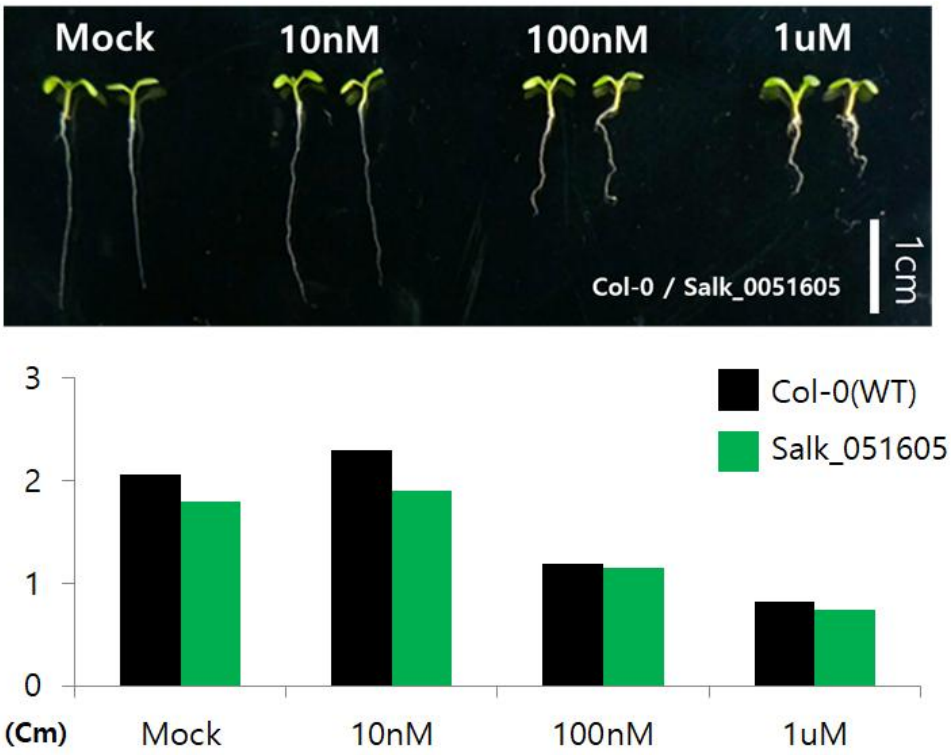


Figure 8. Phenotypes of BL-treated seedlings (Right: WT, Left: mutant)

BL-treated seedling of At5g62970 T-DNA mutant Salk_051605. Graph shows main root length of the seedling.

2. Overexpression construct and protein expression of *Pro35S:BIN2/Pro35S:At5g62970*

In the cloning process, the gene was not amplified through the common Arabidopsis Col-0 RNA. Short-time specific expression of the gene makes hard to get an amplified cDNA. Therefore, Col-0 was grown on a special MS medium supplemented with 10 μM of BL. The 6-days-old seedling RNA was isolated. Both genes were cloned into the pENTR-SD / D-TOPO vector using the Gateway System, and the insertion of the vector was confirmed using restriction enzyme MluI (Figure 9). During this process, At5g56690 had STOP CODON in the middle of the sequence, so though the cloning was completed, the protein was not expressed. Therefore, the interactor candidate gene was narrowed down to At5g62970. The identified template was inserted into pEarleyGate103 (pEG103) and pEarleyGate 202 (pEG202) vectors using LR recombinase. pEarleyGate 103 has a 6xHis tag in the C-Term, and tagged with GFP. In the case of pEarleyGate 202, it is tagged with FLAG in the N-Term. It is assumed that the protein may affected to the function depending on the tagged protein. Subsequently, the vector was inserted into pEG202 and pEG103 vectors using LR Recombinase, and insertion was confirmed using EcoRV and MulI (Figure 10).

Confirmed plasmid was transformed with *Agrobacterium Tumefacience* C.58 Strain and cultured in a medium containing rifampicin, gentamycin, and kanamycin. For transformation, the cells were cultured in YEP medium supplemented with antibiotics to a concentration of 1.3 at OD580. The cultured cells were slowly centrifuged at low temperature to remove the medium. After diluted pallet in the buffer, and transferred to 5-week-old *Pro35S:BIN2* Overexpressed plants with Floral dip method. Seeds were harvested from aged and dried plants, and the collected seeds were selected using Basta. However, since *Pro35S:BIN2* was a transformant prepared using antibiotic Basta, the cloning was successful through protein expression of adult plant (T2) after primary screening with antibiotics. For both pEG103 and pEG202, proteins were identified using Western blot and confirmed using 6xHis antibody and FLAG antibody, respectively (Figure 11). Using this data, we harvested the seeds of the plant with the correct size of the protein.

Pro35S:BIN2 plants used as a background have an extreme dwarf phenotype. But by following the hypothesis, if we insert an overexpression vector to overexpressing the F-box protein, which is thought to be removed by binding to BIN2, we expected that *Pro35S:BIN2* could overcome the dwarf phenotype and assume a wild-type phenotype. Phenotypic analysis was performed, but 4-week-old plants exhibited significantly different phenotypes. There were also plants with a phenotype

similar to that of the Arabidopsis wild type, but the phenotype was not significantly different (Figure 12). In particular, some of the broken rosette leaves, which is the most specific phenotype of BIN2, were fully recovered. However, not every overexpressed plants shows this phenotype. However, most of the plants shows larger phenotype than *Pro35S:BIN2*, so I could speculate that the candidate gene is functioning related to BIN2. Plants transformed with pEG103 also showed similar phenotype to those transformed with pEG202, and nearly half of the plants were similar and recovered. Therefore, it is confirmed that there is no difference in the function of the protein tagged with different marker protein and different terminus.

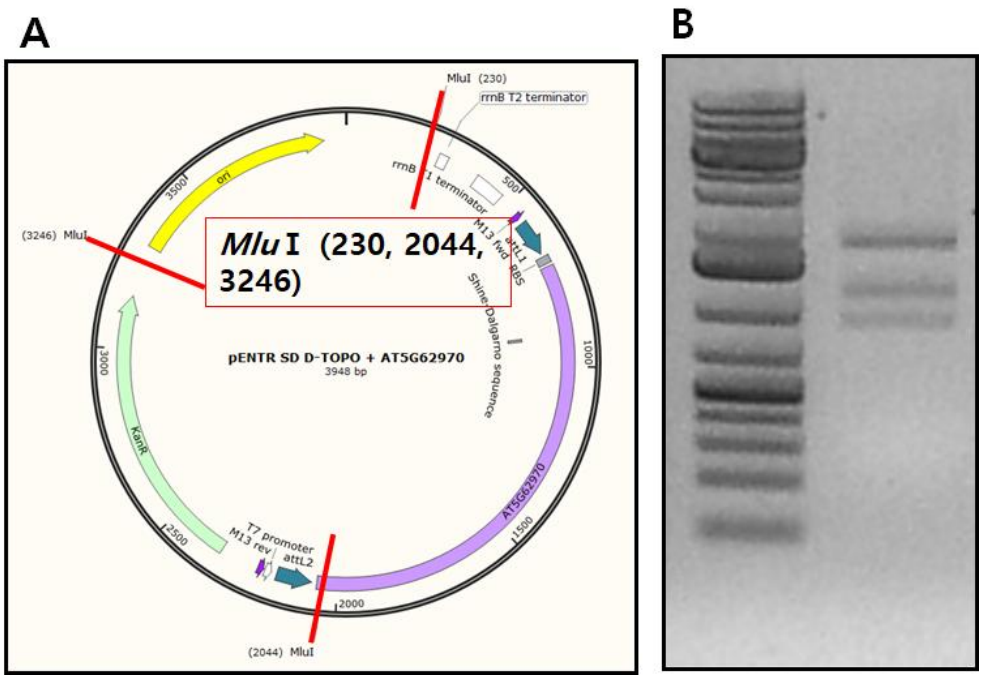


Figure 9. Construct map and DNA fragment analysis

(A) Schematic image of pENTR/SD/D-TOPO Vector ligased with insert (At5g62970)

(B) band image digested with *Mlu*I. Each band located in 1814bp, 1202bp and 932bp.

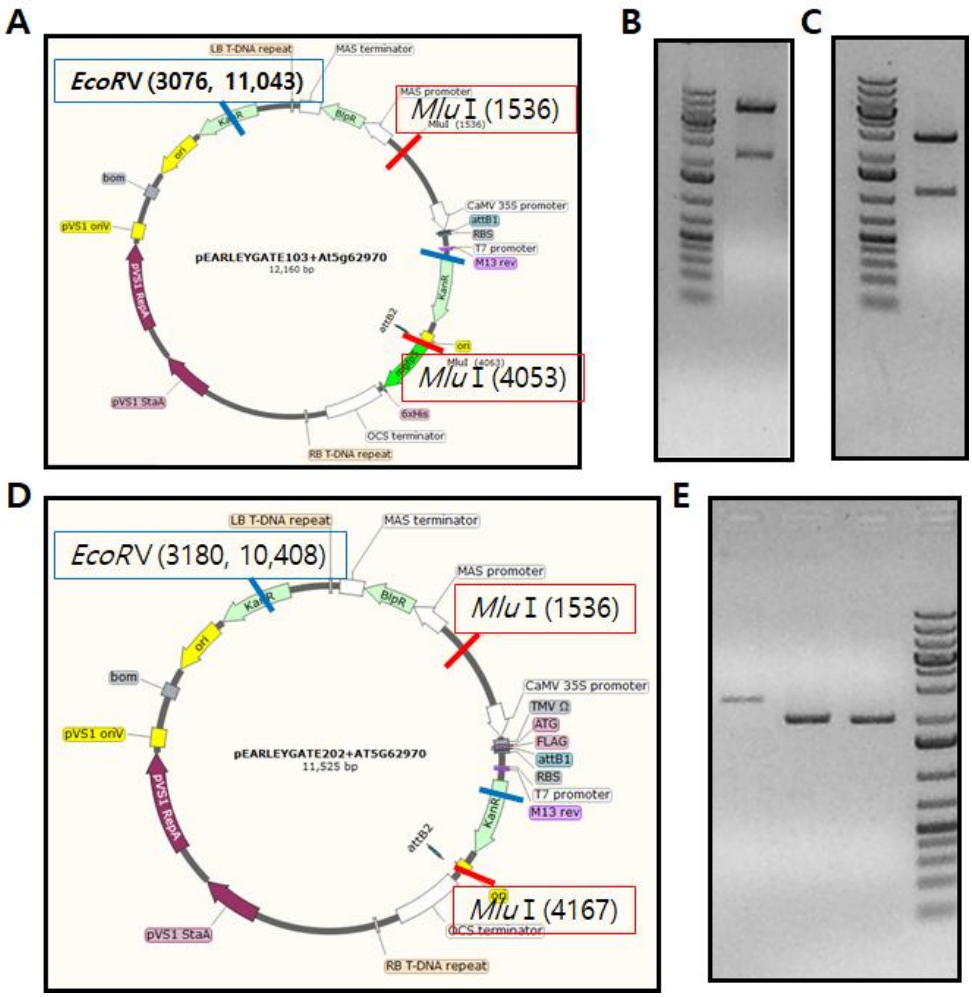


Figure 10. Construct map and DNA fragment analysis.

(A) Schematic image of pEARLEYGATE 103 Vector ligased with insert (At5g62970)

(B) Band image of pEARLEYGATE 103 Vector ligased with At5g62970 digested with *Mlu*I. Each band located in 1814bp,

1202bp and 932bp.

(C) Band image of pEARLEYGATE 202 Vector ligased with At5g62970 digested with *Mlu*I. Each band located in 1814bp, 1202bp and 932bp.

(D) Schematic image of pEARLEY 202 Vector ligased with insert (At5g62970)

(E) Band image of pEARLEYGATE 103 Vector(1st Lane from left) and pEARLEYGATE 202 vector(2nd and 3rd lane from left) ligased with At5g62970 digested with *EcoR*V. Each band located in 1814bp, 1202bp and 932bp.

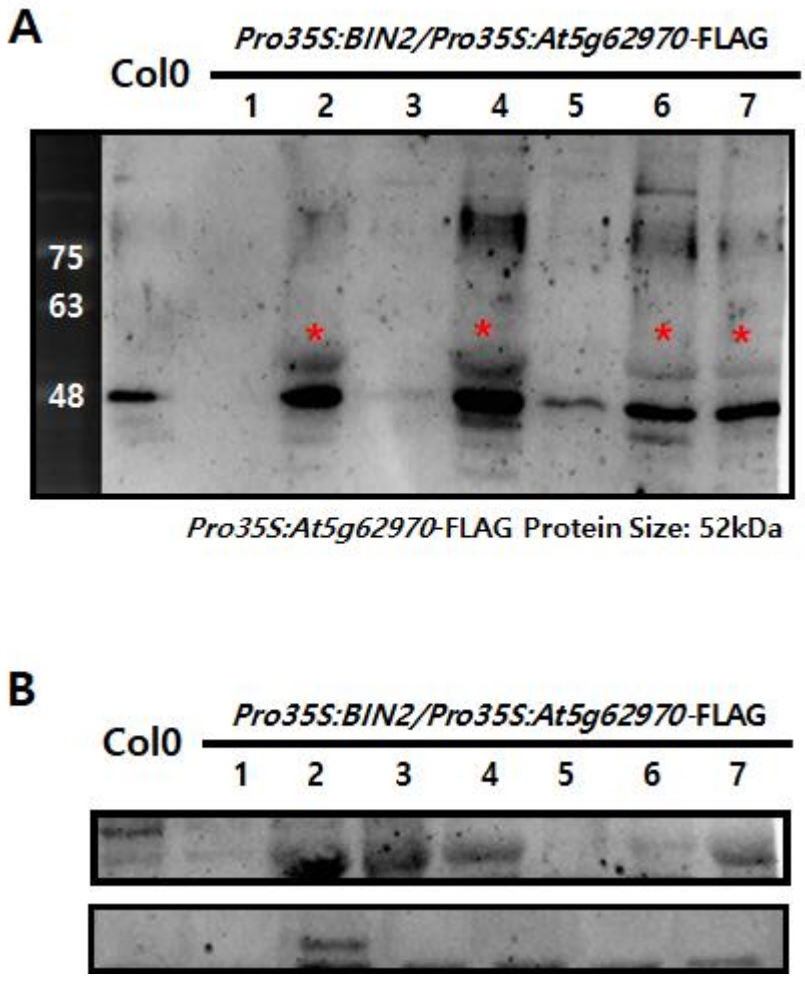


Figure 11. Western blot analysis of *Pro35S:BIN2* background transformants

(A) Western blot analysis using anti-FLAG antibody. Asterisks (*) indicates expressed FLAG protein. Expected protein size was 52kDa. (B) Expressed FLAG protein in various of individual *Pro35S:BIN2/Pro35S:At5g62970*-FLAG transformed plants.

Figure 12. Phenotype of *Pro35S:BIN2* background transformants T2 generation.

Plants were pictured 4 weeks after transfer on the soil. The severe *Pro35S:BIN2* phenotype was partially recovered in some lines, but not equal for all selected T2 generation.

3. At5g62970 overexpression of Col-0 background Arabidopsis

Similarly, two constructs of *Pro35S:BIN2* were also transformed using Col-0. The transformants were transfected into Arabidopsis plants, through the Folral dip method. Transgenic plants were harvested for 24 hours after incubation and awaiting natural aging. Then, the T2 plant was inoculated in the antibiotic medium, and the plants were selected using the Chi-square test. When I checked the proteins of these plants, it was confirmed that FLAG was normally expressed in pEG202, but in case of pEG103, GFP or His tag does not detected. Thus, the phenotypic experiments were performed only with pEG202 vector-transformed plants. The growth rate of the selected plants was faster than that of the existing Col-0, and it was confirmed that the adult size at 5th week was slightly larger than the wild type (Figure 13).

In the case of wild-type transgenic plants, RNA was extracted and analyzed by Semi-RT PCR to confirm whether phenotypes appeared due to the differences in the biosynthetic pathway or the brassinosteroid signaling pathway. Four genes were used as bio markers. The expression levels of CPD, CYP85A2, and DWF4, which affect the biosynthetic pathway, were confirmed, and SAUR-AC1, a biliary-steroid signal transduction gene, was identified (Ren & Gray, 2015) (Figure 14). As a result, there

was no difference in the gene expression in the genes in the biosynthetic pathway, but the expression of SAUR-AC1, which is a signal transduction gene, was higher than that in the wild type. It was concluded that the overexpression of At5g62970 affects the signal transduction of brassinosteroids.

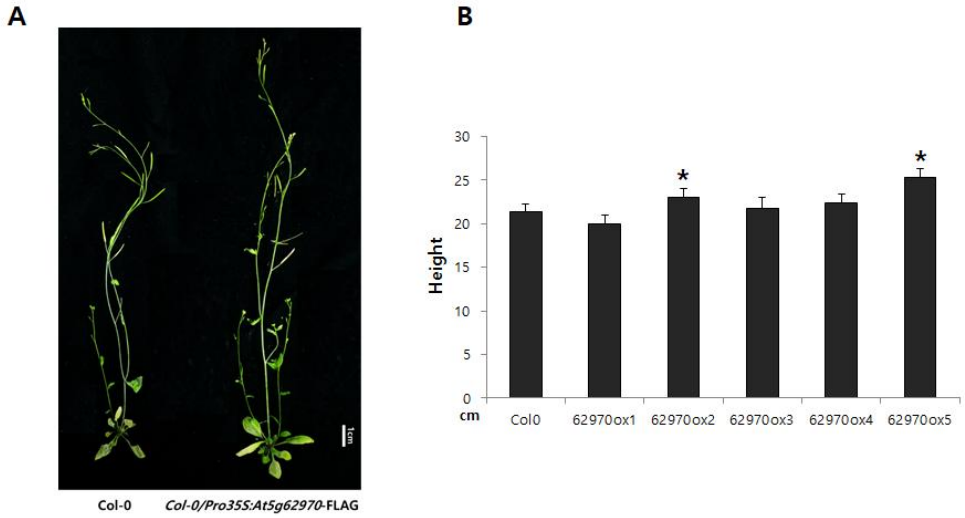


Figure 13. Phenotype of col-0 background transformants and height measurement

(A) Phenotype of col-0 background *Pro35S:At5g62970*-FLAG transgenic plant. Scale Bar= 1cm

(B) Measurement of Plant Height. 62970ox# indicates *Col-0/Pro35S:At5g62970*-FLAG transgenic plant individual lines. Asterisks (*) indicate significant differences to the respective Col-0 determined t-test ($p<0.05$)

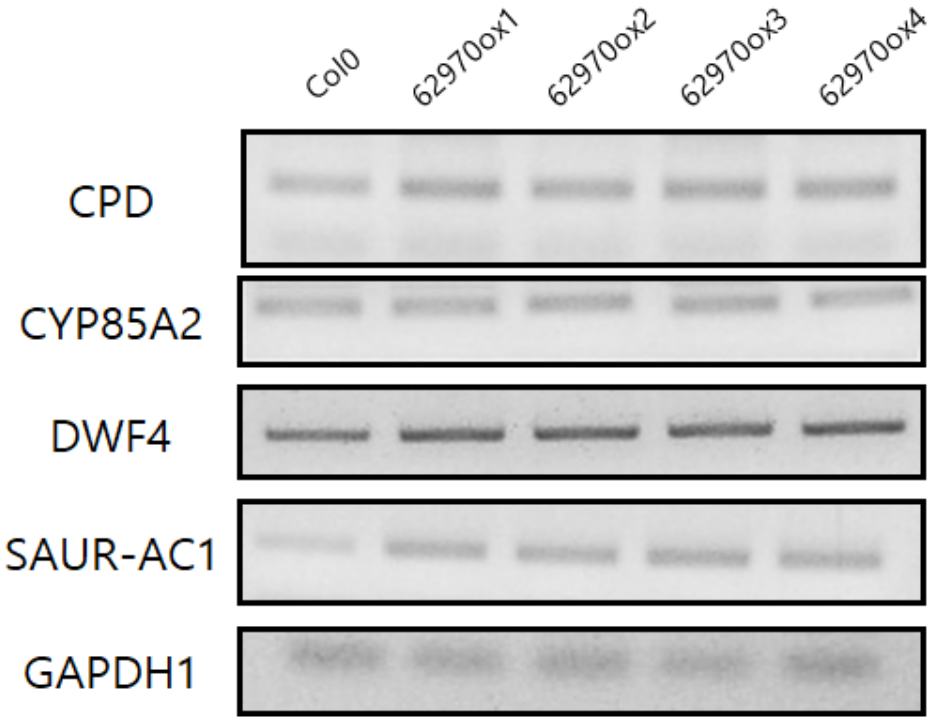


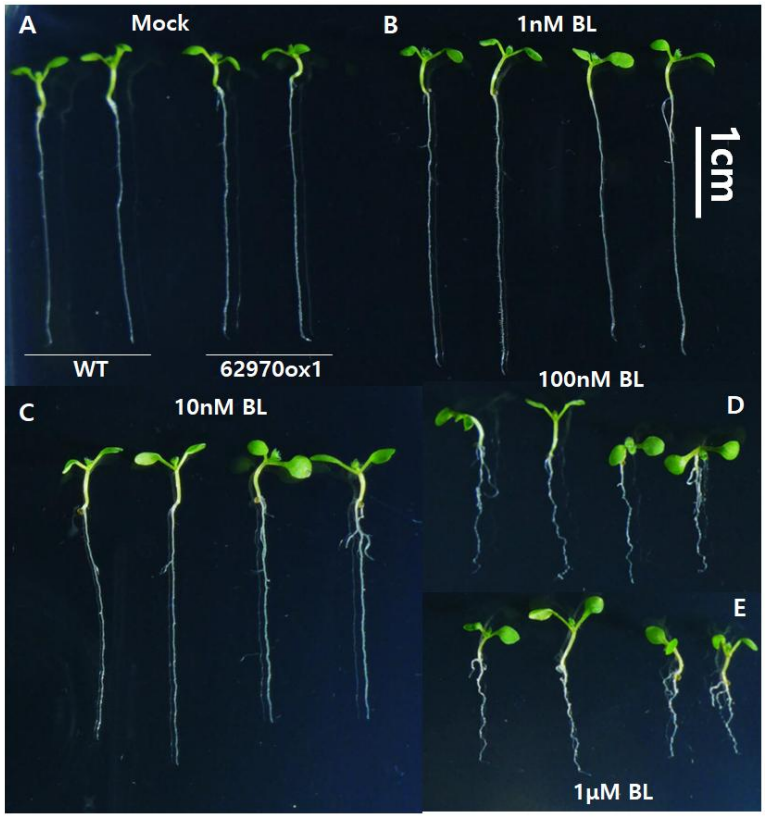
Figure 14. RNA Expression analysis by using RT-PCR

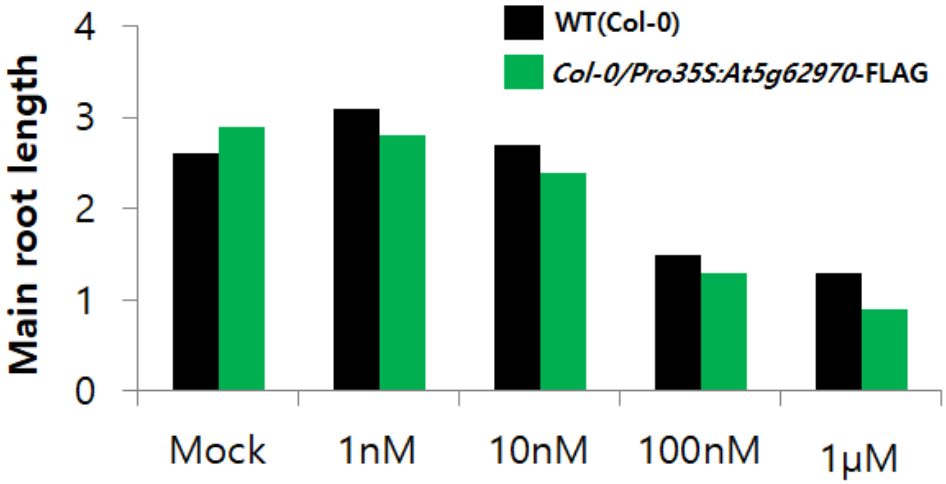
4. Effects of hormone and biosynthesis inhibitor on the phenotypes of seedlings

If At5g62970, the candidate gene, is the correct E3 ligase of BIN2, then both the Col-0/*Pro35S:At5g62970* and *Pro35S:BIN2/Pro35S:At5g62970* transformants will continuously eliminate BIN2 with ubiquitination. This results in continuous activation of brassinosteroid signaling even if the pathway is not active by BR. So the phenotype was expected to be observed as if brassinosteroid is present. Therefore, we tried to confirm this hypothesis by using biosynthesis inhibitor and active form of brassinosteroid. When BL treated to *Col-0/Pro35S:At5g62970* -FLAG and wild-type, the wild-type phenotype was estimated to be the same as *Col-0/Pro35S:At5g62970*-FLAG (Figure 15). However, it was confirmed that the phenotype was not correct with hypothesis, and *Col-0/Pro35S:At5g62970*-FLAG was more sensitive to BL, especially affected by root length and cotyledon length. Also, those phenotypes appeared to be different in each mutant individuals. Because the results on plants cultured for 6 days were difficult to contrast, we used plants cultured for 10 days. As a result, it was observed that the length of the main roots are decreased at lower concentrations.

Pcz is a biosynthesis inhibitor of brassinosteroids. Like Brz, it

is known to have a direct effect on brassinosteroids rather than controlling other growth hormones such as GA. Therefore, in the case of pcz-supplemented medium, the same conditions as BR-free environment is served while the plant is grown. Therefore, the wild-type plant exhibits a phenotype with shortening of roots and hypocotyls with increasing concentration, but in case of the transformant *Col-0/Pro35S:At5g62970*-FLAG, it was assumed that the phenotype would be recovered to the similar to those grown on the general medium. In fact, there was no significant difference in the length of hypocotyls when it grew on dark condition (Figure 16), but the root length was shorter than the wild type (Figure 17). As a result, it was confirmed that the continuous inhibition effect of BIN2 was not particularly high, unlike the prediction.


A
Mock
B
1nM BL
1cm
WT
62970ox1
C
10nM BL
100nM BL
D
E
1μM BL


WT(Col-0)
Col-0/Pro35S:At5g62970-FLAG
Main root length
4
3
2
1
0
Mock
1nM
10nM
100nM
1μM

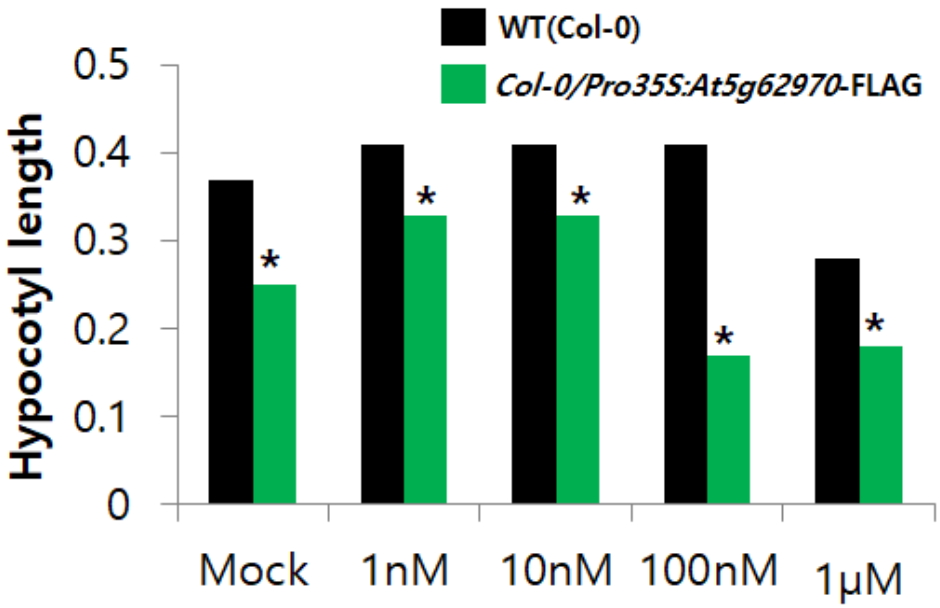


Figure 15. Phenotype of BL treated *Col-0/Pro35S:At5g62970*-FLAG (62970ox1).

(A) 6-day-old seedling grown on Control MS medium. 62970ox1 indicate *Col-0/Pro35S:At5g62970*-FLAG transgenic plant individual lines. In every picutre, Control Wildtype Col-0 is two seedlings on the Left, and 62970ox1 is two seedlings on the Right

(B) 6-day-old seedling grown on MS containing 1nM of BL

(C) 6-day-old seedling grown on MS containing 10nM of BL

(D) 6-day-old seedling grown on MS containing 100nM of BL

(E) 6-day-old seedling grown on MS containing 1 μM of BL

Graph indicates measurement of main root length and measurement of hypocotyl length

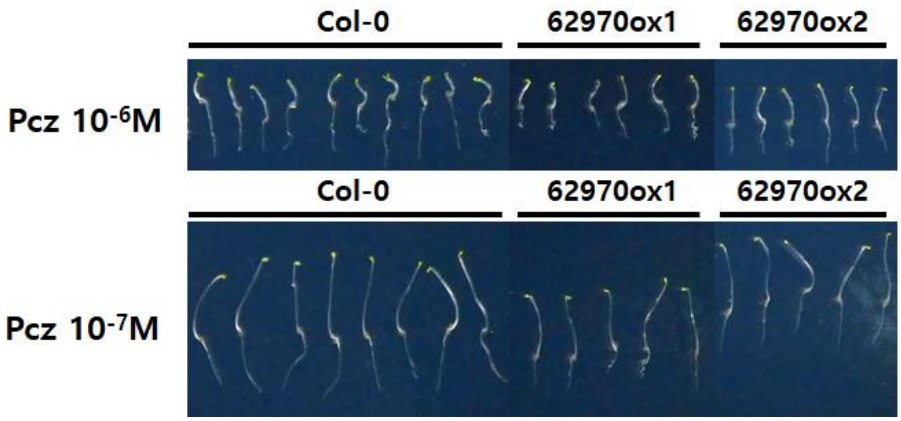


Figure 16. Phenotype of propiconazole treated overexpression seedlings grown on dark condition.

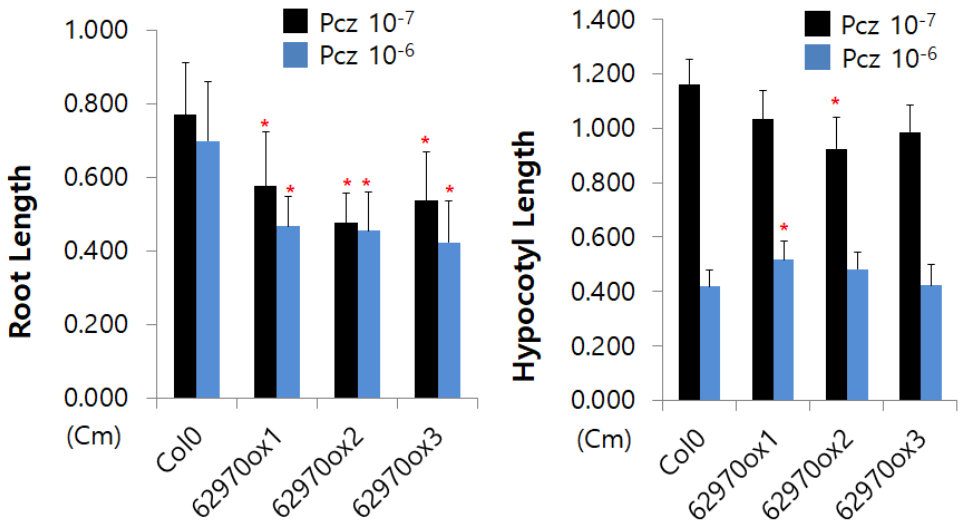


Figure 17. Phenotype analysis of propiconazole treated overexpression seedlings.

Length of hypocotyl started from end of the root, which shows slight hunch of the seedling grown on dark condition. Error bars represent standard deviation. Asterisks (*) indicate significant differences to the respective Col-0 determined t-test ($p<0.05$)

Ⅳ. Discussion

Previous studies have shown that BIN2 is a GSK3 kinase which modulates transcription factors such as BZR1, BZR2, and BES1 by phosphorylation as a negative regulator of BR signaling (Chung & Choe, 2013). In addition, degradation or deficiency of BIN2 is caused by the signal transduction pathway of BIR1/ BAK1. Treatment of MG132, a proteasome inhibitor, inhibits the degradation of BIN2 by BR signaling and also inhibits the function of BIN2 as a kinase (Peng et al., 2008). Research has thus shown that BIN2 is degraded through the proteasome and is ubiquitinated, but the proteins involved remained unclear (Cho et al., 2008).

In this study, candidate genes were selected by previous in vitro pull-down assay and their genotype/ phenotype analyzed to identify the genes involved in proteasome-mediated degradation of BIN2. Two candidate genes were identified: At5g62970 and At5g56690, which were found to have an F-Box like domain. Mutants lacking the function of the two genes were not expected to degrade the BIN2 protein and were thus expected to have phenotypes similar to the MG132-treated phenotype. However, it was confirmed that they did no differ significantly from wild type Arabidopsis, except in relation to root hair growth. Therefore,

RT-PCR was performed to determine whether the gene was genetically mutagenized. It was subsequently confirmed that the mutation Salk_051606 of At5g62970 did not express the gene, but Salk_098699C, a mutant of At5g56690, expressed the same amount of RNA as wild types. Treatment with Blassniolide (BL) for the brassinosteroid reaction in the At5g62970 mutant resulted in no significant differences.

In the case of overexpressed plants, cloning was carried out using a gateway system. At5g56690 was found to have a stop codon during CDS and cloning ceased. Then, cloning was carried out with At5g62970 in the background of BIN2 overexpressing line and wild-type Col-0. When cloning was carried out on BIN2 overexpressing cells, it was difficult to select using antibiotics. Therefore, expression of the labeled protein was confirmed by Western blotting. The selected overexpressing lines showed a phenotype which recovered in a similar manner to wild types, but some lines had a phenotype that did not recover. However, all of the selected plants could be presumed to play a role in BIN2, related to the overexpression of At5g62970 by exhibiting a phenotype that recovered to some degree from overexpressing BIN2. When transformed into Col-0, the transformed plants were confirmed by their BASTA antibiotic resistance. These transformed plants had a slightly larger mature plant size with a faster growth rate. Confirming of the biosynthesis and signaling marker genes of BR through RT-PCR,

the genes affecting biosynthesis did not change substantially, but the genes involved in the signaling pathway changed, confirming the effect of At5g62970 on the signaling pathway.

Based on the above results, transgenic plants were treated with hormones. The transgenic plants were more sensitive to BL and were affected by root length. So I treated with propiconazole, a biosynthesis inhibitor of brassinosteroid. In this experiment, the transformant did not activate signal transduction by continuously removing BIN2, as confirmed by the fact that root length, which was predicted to recover compared with wild types, was instead shortened.

Komander et al. (2009) noted that the process of ubiquitination in an actual cell environment is not simply the process of combining one E3 ligase and one substrate, which is said to be quite dynamic. Many E3 ligases and substrates bind weakly or transiently (Iconomou & Saunders, 2016), and the two outcomes can be deduced from the results described above. It can be inferred that At5g62970 is related to the amount of BIN2 expression in the area where phenotype recovery of Pro35S:BIN2 actually occurs in overexpressing cells. However, we did not observe any significant phenotype changes in lines that do not express At5g62970, thus it was inferred that At5g62970 is not the only E3 ligase that binds to BIN2. It can be assumed that there is another E3 ligase that binds to BIN2 when At5g62970 does not function. Recently, it has been

reported that the gene, KIB1, functions as an E3 ligase of BIN2, suggesting that BIN2 has many E3 ligases (Zhu et al., 2017). This suggests that there are many E3 ligases of BIN2, and At5g62970 is suspected to be a transient or weak-binding E3 ligase in BIN2 degradation, which acts as a minor pathway.

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국문 초록

애기장대 BIN2 분해 후보 유전자 At5g62970 와 At5g56690의 유전학적 분석

유비퀴틴화(ubiquitination)은 세포에서 단백질을 제거하기 위해 사용되는 기작이며, 애기장대에서 브라시노스테로이드(BR)의 합성 및 기능은 수년간의 연구로 상세하게 밝혀져 있으며 BR은 수용체를 따라 최종적으로 BIN2 단백질을 유비퀴틴화하여 분해함으로서 유전자에 직접적 반응을 이끌어 낸다고 알려져 있다. BIN2는 SCF 복합체를 이루는 것으로 알려져 있으며 따라서 F-Box 단백질이 목표 단백질(BIN2)와 결합하여 유비퀴틴화를 진행한다.

F-Box 단백질을 확인하기 위해 선행연구를 통해 확인이 된 두 유전자 후보 At5g62970과 At5g56690를 대상으로 하여 본 수행자는 Gateway System을 사용하여 클로닝을 진행하였고 야생형 Col-0와 *Pro35S:BIN2* 형질전환체에 각각 삽입하고, 표현형을 관찰하였다. 표현형이 야생형과 비슷하게 회복 된 경우도 있었으나 많이 회복되지 않은

표현형도 나타났다. 다수는 BIN2 과발현체보다 회복되는 표현형을 보임으로서 과발현시킨 At5g62970이 BIN2와 관련 된 역할을 하는 것으로 추측해 볼 수 있었다. Col-0를 백그라운드로 형질전환 한 경우에는 성체의 크기가 약간 크고 성장 속도가 빨랐다. 이후 RNA를 통하여 BR의 생합성과 신호전달 마커 유전자들을 확인해 본 결과 생합성에 영향을 주는 유전자들은 크게 변하지 않는 반면 신호전달에 관련 된 유전자는 차이를 보이는 것을 통해 신호전달경로에서 At5g62970이 영향이 있음을 확인할 수 있었다. 형질전환체는 BL에 더 민감하게 반응하여 뿌리의 길이에 영향을 받았지만 생합성 저해제인 Pcz를 처리한 결과, 야생형에 비해 회복될 것이라 예측하던 뿌리길이가 야생형에 비해 짧아진 것을 통해 형질전환체가 BIN2를 지속적으로 제거함으로서 신호전달을 활성화 시키고 있는 것은 아닌 것으로 확인되었다.

과발현체에서 *Pro35S:BIN2* 에 대한 표현형 회복이 나타나는 부분에 있어 At5g62970이 BIN2의 발현량과 관련이 있다고 추측할 수 있다. 하지만 표현형이 나타나지 않은 것을 통해 BIN2에 결합하는 E3 ligase가 한 종류가 아니며, At5g62970이 기능을 하지 않더라도 결합하는 다른 E3 ligase가 있을 수 있음을 유추할 수 있었다. 주요경로로 작용하는 E3 ligase와 일시적이거나 약하게 결합하는 경로가 있을 것이라고 추측될 때 At5g62970은 후자일 가능성이 있다고 예상하는 바이다.

주요어 : BRASSINOSTEROID INSENSITIVE 1 (BIN2), 유비퀴틴화, (GLYCOGEN SYNTHASE KINASE3) GSK3β-Like Kinase, F-Box Protein, 브라시노스테로이드, 애기장대

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