

博士論文

**Physiological and molecular biological studies of
PYR/PYL/RCAR ABA receptors in *Arabidopsis***

(シロイヌナズナ ABA 受容体 PYR/PYL/RCAR の生理学的
および分子生物学的研究)

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Abbreviation

Abbreviation	Full name
2,4-D	2,4-Dichlorophenoxyacetic acid
<i>5^Δdella</i>	pentuple della mutant
AAO3	Arabidopsis aldehyde oxidase 3
ABA	Absciscic acid
ABA2	ABA DEFICIENT 2
ABA3	ABA DEFICIENT 3
ABF	ABRE binding factor
ABI	Absciscic acid-insensitive
ABRE	ABA responsive element
AD	activating domain
AHG	ABA-hypersensitive germination
APS	Ammonium persulfate
AREB	ABA-Response Element Binding Factors
AS2	3'-alkylsulfanilated ABA with two carbons in alkyl
AS6	3'-alkylsulfanilated ABA with six carbons in alkyl
BD	binding domain
BiFC	bimolecular fluorescence complementation
BR	brassinosteroids
BSA	bovine serum albumin
bZIP	basic leucine zipper
BZR1	Brassinazole-Resistant 1
CaMV	Cauliflower mosaic virus
CBB	Coomassie Brilliant Blue
cDNA	Complementary DNA
Col	Columbia ecotype

DAG	day after germination
DL	dim light
DMSO	dimethyl sulfoxide
DREB	Drought Response Element Binding factors
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
<i>ELI</i>	<i>EARLIER FLOWERING 1</i>
<i>ERA1</i>	<i>ENHANCED RESPONSE TO ABA1</i>
EREB	Ethylene Response Element Binding
ETH	ethylene
EYFP	enhanced YFP
GA	Gibberellin
GA ₃	Gibberellin A3
GA ₄	Gibberellin A4
GAI	GA INSENSITIVE
GFP	Green fluorescent protein
GID1	GIBBERELLIN-INSENSITIVE DWARF1
GID2	GIBBERELLIN-INSENSITIVE DWARF2
G-protein	guanine nucleotide-binding protein
GR24	a strigolactone analog
GRAS	GAI, RGA and SCR (SCARECROW) family
GSH	glutathione
GST	Glutathione S-transferase
GTG	GPCR-type G proteins
GUS	β-D-glucuronidase
HAB	Hypersensitive to ABA
HAI	Highly ABA-Induced PP2C
IAA	Indole-3-acetic acid

IPTG	Isopropyl β -D-1-thiogalactopyranoside
JA	jasmonic acid
JAZ	jasmonate ZIM-domain
KAT1	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1
KEG	KEEP ON GOING
LB	Luria-Bertani medium
LEA	Late Embryogenesis Abundant proteins
Ler	Landsberg erecta ecotype
M	molar per liter
MBP	maltose-binding protein
MCS	multiple cloning site
MeJA	Methyl jasmonate
MEP	methylerythritol phosphate pathway
mg	milli gram
ml	milli litre
mM	milli molar per liter
MS	Murashige and Skoog medium
MVA	mevalonic acid
MYC2	a transcription factor in response to ABA and JA
NCED	nine-cis-epoxycarotenoid dioxygenases
OD	optical density
OE	over-expression
OsPYL	AtPYL like in Oryza sativa (rice)
OST	open stomata
OST1	OPEN STOMATA 1
PAC	paclobutrazol
PAGE	polyacrylamide gel electrophoresis
PAMD	a chemical that suppresses PR gene expression induced by SA

PBS	phosphate buffered saline
PIF	PHYTOCHROME INTERACTING FACTOR
PP2C	Protein serine/threonine phosphatases type 2C
PYB	pyrabacin, an agonist of ABA
PYL	PYR-like
PYR	Pyrobactin resistant
RCAR	Regulatory component of ABA receptor
RGA	REPRESSOR OF <i>ga1-3</i>
RGL1	RGA-LIKE1
RGL2	RGA-LIKE2
RGL3	RGA-LIKE3
SA	Salicylic acid
SCF	Skp, Cullin, F-box containing complex
SCL3	SCARCROW LIKE3
SCR	SCARCROW
SE	standard error
SDR1	short-chain dehydrogenase/reductase1
SDS	Sodium dodecylsulfate
SHR	SHORT-ROOT
SL	strigolactone
SLAC1	SLOW-ANION CHANNEL1
SLR1	SLENDER RICE1
SLY1	SLEEPY1
SnRK	Sucrose non-fermenting - related protein kinase
SNZ	SNEEZY
SOC	Super Optimal broth with Catabolic repressor
SPY	SPYNDLY, O-linked N-acetylglucosamine transferases
START	StAR-related lipid transfer

TBS	Tris-buffered saline
TEMED	N,N,N,N'-tetramethylethylene diamine
Tween-20	polysorbate-20
w/v	weight per volume
WB	western blotting
WT	Wild-type
Y2H	Yeast two hybrid
Y3H	Yeast three hybrid
YFP	Yellow fluorescent protein
ZEP	Zeaxanthin epoxidase
μg	micro gram
μM	micro molar

CHAPTER 1 Introduction

Plants, as sessile organisms, are unable to search for favorable circumstance or avoid stresses by moving like animals. Consequently plants have to utilize or endure everything around them. To regulate their growth and development in response to variable circumstances, plants have evolved very flexible mechanisms, which are induced by many kinds of small molecules called plant hormones (Wolters and Jurgens 2009). Until now, at least nine kinds of plant hormones have been reported (Figure 1-1), including indole-3-acetic acid (IAA), cytokinin (CK), abscisic acid (ABA), gibberellins (GA), ethylene, salicylic acid (SA), jasmonic acid (JA), brassinosteroids (BR) and strigolactones (SL) (Santner *et al.* 2009). To study the functions of plant hormones and uncover their mechanisms of action will facilitate the understanding of the plant growth and responses to biotic and abiotic stresses and improve the technologies in agricultural production and plant protection.

1.1 Absciscic acid (ABA)

ABA was discovered in 1960s by the studies of the compound (denoted abscisin II) that accelerated leaf abscission from cotton fruit (Ohkuma *et al.* 1963) and the compound (denoted dormin) that induced bud dormancy in sycamore (Cornforth *et al.* 1965). Later comparative structural analysis of these two compounds confirmed that they were the same compound, a 15-carbon sesquiterpenoid, and nominated it as abscisic acid (ABA) (Addicott *et al.* 1968). There are three types of ABA including (S)-*cis*-ABA (natural type), (R)-*cis*-ABA (unnatural type) and (S)-2-*trans*-ABA (Figure 1-2). Nowadays ABA is known as one of well-studied plant hormones.

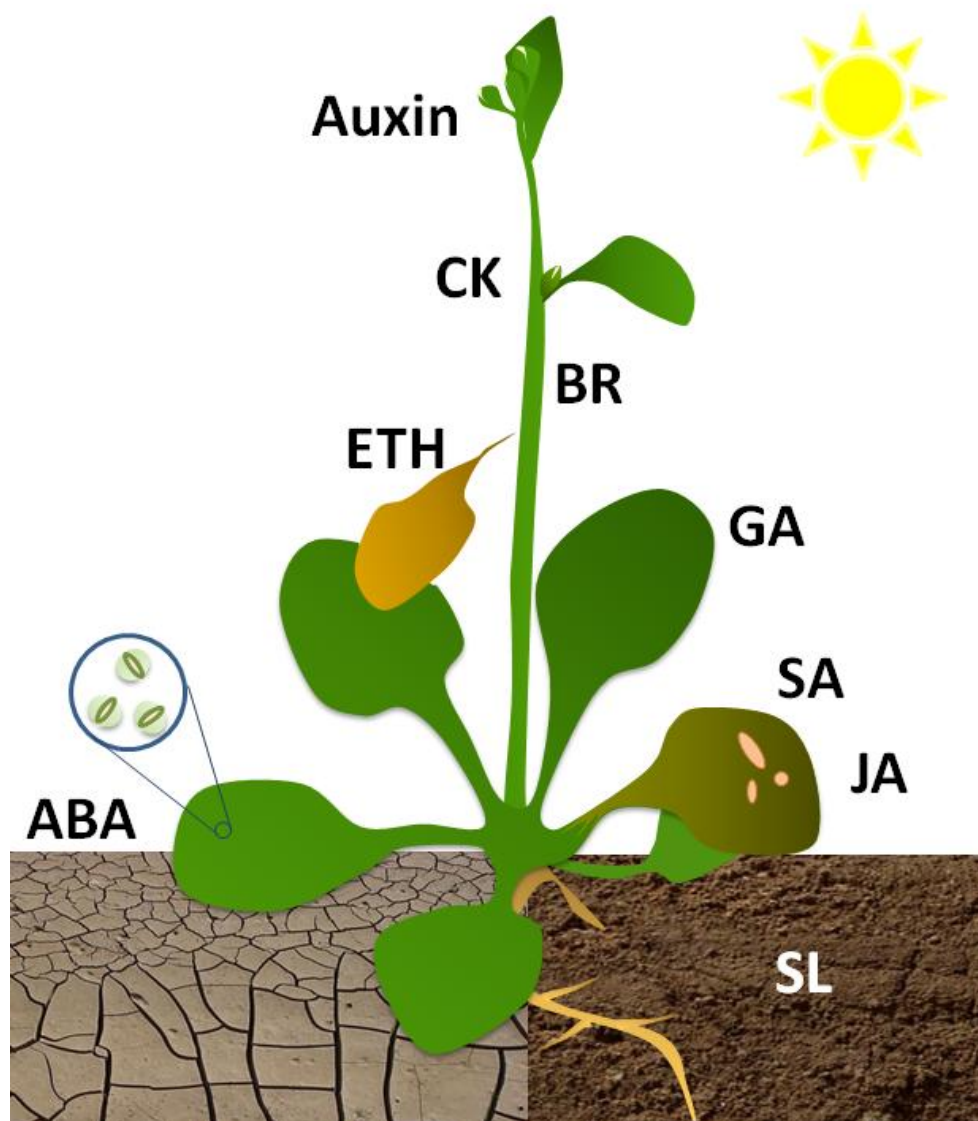


Figure1-1 Plant hormones that regulate plant growth and development
 Auxin promotes the plant growth and apical dominance. Cytokinin (CK) stimulates cell division and releases apical dominance. Brassinosteroid (BR) is essential for plant vegetative and reproductive growth and development, including cell elongation and proliferation. Ethylene (ETH) stimulates leaf and fruit abscission. Gibberellin (GA) stimulates stem elongation by promoting cell division and elongation. Absciscic acid (ABA) increases the tolerance of plant to stresses such as drought and salt by inducing stomatal closure and stress related protein expression. Salicylic acid (SA) and Jasmonic acid (JA) are responsible for plant responses to pathogens. Strigolactone (SL) regulates branching and root growth. Besides these basic functions mentioned above, increasing roles and crosstalk among these hormones are reported.

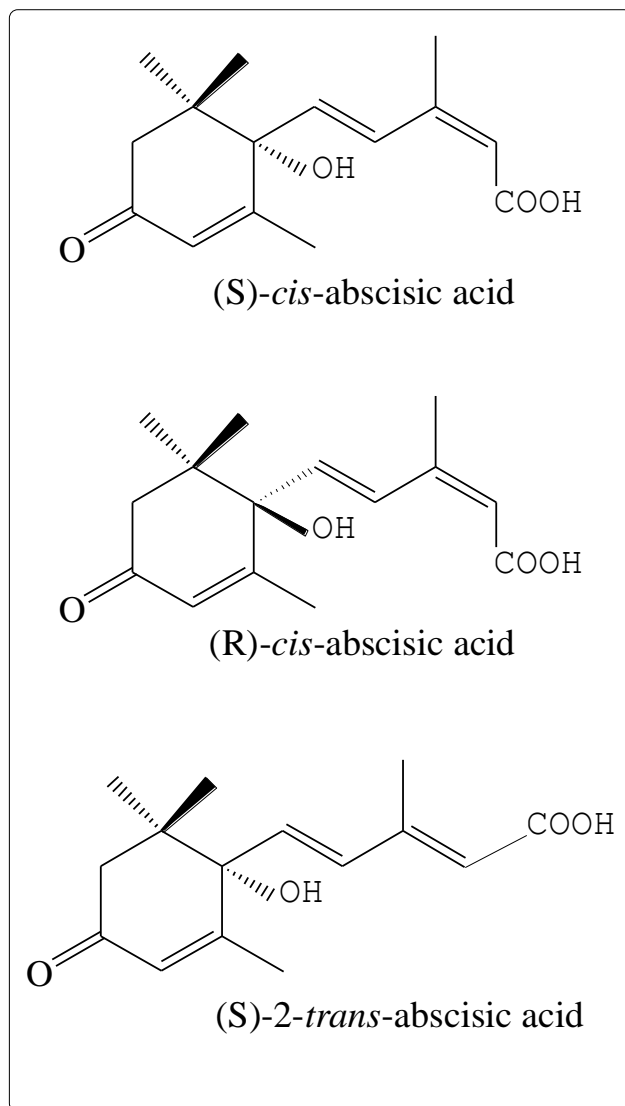


Figure1-2 Enantiomer and isomer of ABA

1.2 Physiological roles of ABA in plant

Large number of studies showed ABA plays many physiological roles in plant growth and development such as regulating seed dormancy and germination, heterophylly (leaves of different shapes on the same plant), root growth (including primary root and lateral root), senescence (De Smet *et al.* 2006) and floral transition (Domagalska *et al.* 2010).

In fact ABA is apt to be considered as a stress responsive plant hormone because its endogenous level and functions are related to the abiotic stresses such as osmotic stress (induced by drought and high salinity) and cold stress. In plants suffering from these stresses, endogenous ABA level will be elevated and ABA responsive genes will be induced, including the genes encoding the late embryogenesis abundant (LEA) protein, some regulatory proteins, transporters and enzymes (Fujita *et al.* 2011). The increasing ABA level is also able to induce stomatal closure thereby alleviating water loss during the drought stress (Israelsson *et al.* 2006).

Recently, an increasing number of studies has indicated that ABA also regulates plant defenses to diseases. ABA acts as a negative regulator of disease defenses in plants by antagonizing against SA-, JA- and ETH-mediated signaling (Mauch-Mani and Mauch 2005). In addition, ABA plays positive roles in defenses by activating stomatal closure (Lee and Luan 2012).

Elucidation of the action mechanisms of ABA in plants will facilitate the regulation of plant growth and stress responses that can increase the agricultural productivity. For instance, ABA is related to the regulation of stomatal movement, which is important for both drought resistance and absorption of CO₂ individually. Regulation of ABA signaling will facilitate to maintain balance flexibly between stress responses and utilization of the CO₂.

1.3 ABA metabolism

ABA can be synthesized in all vascular plants and also in mosses and all algal

classes. In addition, some fungi can synthesize ABA (Oritani and Kiyota 2003). To date, ABA catabolism has been well studied including its biosynthesis and metabolism.

1.3.1 ABA biosynthesis

As shown in Figure 1-3, ABA is synthesized by cleavage of carotenoids, which mainly derivate from the methylerythritol phosphate (MEP) pathway in plastid (Rodríguez-Concepción and Boronat 2002, Milborrow and Lee 1998), although there are some other pathways including mevalonic acid (MVA) that synthesize carotenoid in cytosol during specific developmental stages, such as in etiolated seedlings (Rodríguez-Concepción *et al.* 2004). There are many significant genes that encode the enzymes for ABA biosynthesis from carotenoids. First, in chloroplast, zeaxanthin epoxidase (ZEP), which is encoded by *ABA1* in *Arabidopsis* converts zeaxanthin to violaxanthin (Marin *et al.* 1996). Then nine-cis-epoxy-carotenoid dioxygenases (NCEDs), cleave the *cis*-isomers of violaxanthin and neoxanthin to xanthoxin (Schwartz *et al.* 2003). Last the *cis*-xanthoxin is converted to active ABA by the sequential reactions catalyzed by SDR1, which is encoded by *ABA2*, and AAO3 in cytoplasm. During this process, the sulfation of AAO3 molybdenum cofactor by ABA3 is necessary (Seo *et al.* 2000, González-Guzmán *et al.* 2002, Hedden and Thomas 2008).

1.3.2 ABA catabolism

Except for biosynthesis, ABA homeostasis in the plant is also affected by its catabolism (Figure 1-4). The first step in main catabolic pathway is the hydroxylation of 8' position of ABA by a cytochrome P450 mono-oxygenase, which is encoded by *CYP707A* family genes in *Arabidopsis* (Krochko *et al.* 1998, Kushiro *et al.* 2004, Saito *et al.* 2004). Then the unstable 8'-OH ABA is cyclized to form phaseic acid (PA) and reduced to dihydrophaseic acid (DPA) and *epi*-DPA successively. There are also a few hydroxylations at 7' or 9' position of ABA, which produce of 7'-OH ABA and

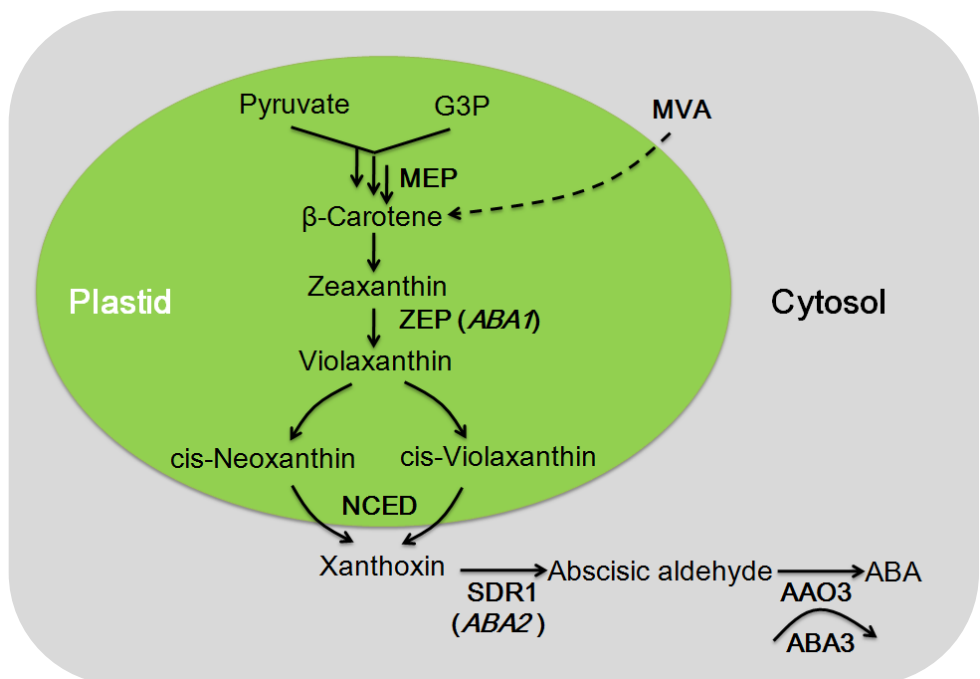


Figure 1-3 Schematic of abscisic acid biosynthetic pathways

The main steps catalyzed by ZEP, NCED, SDR1, AAO3 and ABA3 are indicated. All of these catalytic steps excluding the last two, occur in the plastids.

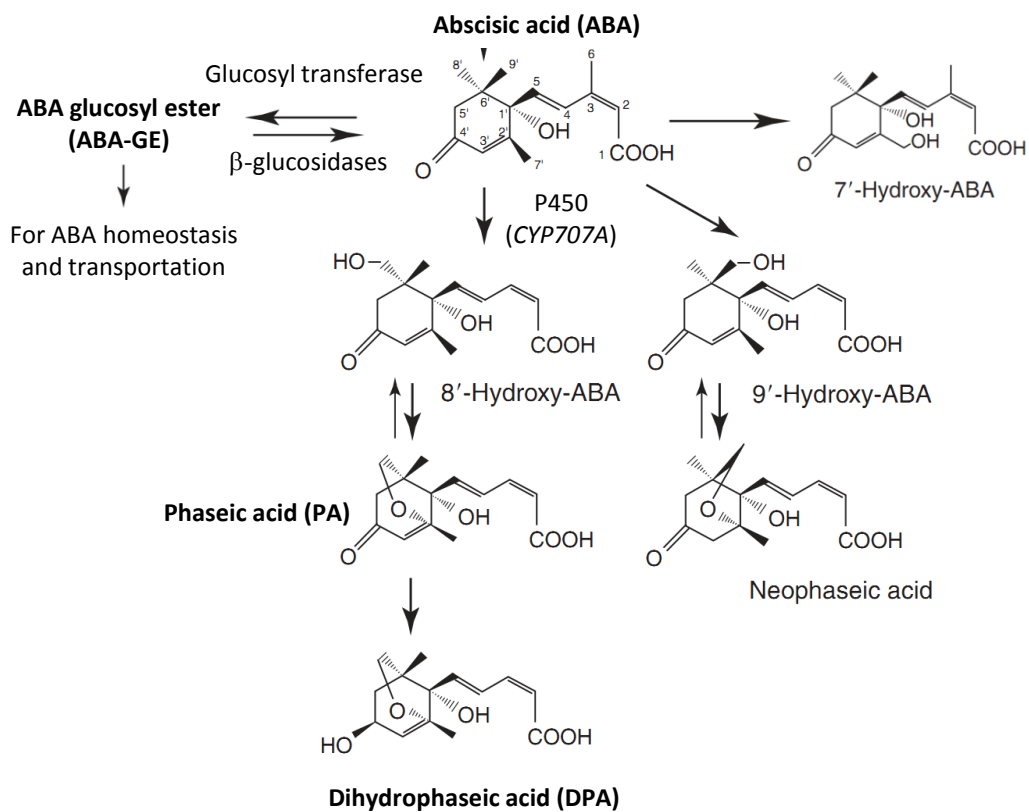


Figure 1-4 ABA catabolism and conjugation

ABA activity is reduced by hydroxylation. DPA loses all ABA activity. Glucosylation is the main conjugation type of ABA and this process can be reversed by degradation. This reversible process may be important for ABA homeostasis and transportation. (Extracted and modified from Annual Plant Reviews, Volume 24: Plant Hormone Signaling, Wiley. Com)

9'-OH ABA. Excluding the DPA, all catabolites mentioned above are still active although their activities are weaker than ABA. In addition, although there is still in debate, glycosylation of ABA, which produces ABA glucosyl ester (ABA-GE) by glucosyl transferase (Lim *et al.* 2005, Xu *et al.* 2002), and its hydrolysis by β -glucosidases are supposed to affect the ABA homeostasis and transportation (Hartung *et al.* 2002, Dietz *et al.* 2000, Lee *et al.* 2006, Sauter *et al.* 2002, Gilbert *et al.* 2013).

1.4 Significant elements in ABA signaling

Four decades have passed since the discovery of ABA. During this period great efforts have been made to investigate the ABA signaling pathway. The isolation of ABA-insensitive mutants based on the inhibition of seed germination by ABA treatment has identified *ABA-insensitive* (ABI) loci *ABI1* to *ABI5* and other elements. Characterization of genes reside in these loci have revealed that they are very important elements in ABA responses (Koornneef *et al.* 1984, Finkelstein 1994b, Nambara *et al.* 1995, Ooms *et al.* 1993) as described below.

Type 2C protein phosphatase (PP2C)

There are more than 80 members in PP2C family in *Arabidopsis* including 9 members in clade A. *ABI1* and *ABI2* are two homologous genes that encode clade A of Type 2C protein phosphatase (PP2C). *abi1-1* and *abi2-1* showed resistance to ABA in various physiological phenomena and developmental stages such as inducing dormancy, inhibition of seed germination and seedling growth, stomatal movement and drought responses (Finkelstein and Somerville 1990, Koornneef *et al.* 1984, Schnall and Quatrano 1992, Finkelstein 1994a, Leung *et al.* 1997). However, in contrast to ABA resistant mutant *abi1-1*, novel alleles of *ABI1* gene as revertants of *abi1-1* mutant showed more sensitivity to ABA than wild type indicating that PP2Cs act as a negative regulator in ABA signaling (Gosti *et al.* 1999). HAB1 (Homology to

ABI1 1) and HAB2 (Homology to ABI1 2) were identified based on sequence similarity to ABI1 and were characterized to be two negative regulators in ABA signaling like as ABI1 (Rodriguez *et al.* 1998, Saez *et al.* 2004). AHG1 (ABA Hypersensitive Germination 1) and PP2CA/AHG3, which are also classified as PP2C and negative regulators in ABA signaling, are predominantly expressed in seed and mainly function in seeds (Nishimura *et al.* 2007, Yoshida *et al.* 2006b). The physiological roles of the rest three members, HAI1 (Highly ABA-Induced PP2C), HAI2 and HAI3, only showed ABA hypersensitivity in double or triple mutants. Recently, they have been confirmed to affect low water potential phenotypes independent of ABA signaling (Bhaskara *et al.* 2012).

Transcription factors

In addition to PP2Cs, the screening of ABA-insensitive mutants also identified some transcription factors such as ABI3, ABI4 and ABI5, which are all positive factors in ABA signaling. *ABI3* encodes a B3 domain transcription factor. In addition to as a transducer of ABA, ABI3 is also considered as one of the major regulators of the transition between embryo maturation and early seedling development as an essential embryogenesis factor because loss of function of ABI3 makes seed remain green, fail to establish desiccation tolerance, and germinate at a developmental stage (Nambara *et al.* 1995). *ABI4*, encoding a APETALA2 domain transcription factor (Finkelstein *et al.* 1998), belongs to a larger transcription factor family which includes Dehydration-Responsive element-binding protein (DREBs) and Ethylene Response Element Binding Factor (ERF/EREBP) (Reeves *et al.* 2011). Although it was identified by the ABA insensitive seed germination screening, recent increasing studies indicate that ABI4 can also mediate many aspects of signaling transduction including sugar signaling, lipid break down, plastid-to-nucleus and regulate plant growth in rosette growth, cell wall metabolism and the lateral root development (Wind *et al.* 2013). *ABI5* encodes a bZIP (basic leucine zipper) domain

transcription factor that binds to ABA responsive element (ABRE) and regulates downstream genes (Finkelstein and Lynch 2000, Finkelstein *et al.* 2005). ABI5 plays important roles in regulating seed germination, postgermination seedling growth and floral transition (Finkelstein and Lynch 2000, Lopez-Molina *et al.* 2001, Lopez-Molina *et al.* 2002, Wang *et al.* 2013). There are also some other transcription factors such as ABA-Responsive Element Binding Factors (AREBs/ABFs), which are ABI5 homologous clade of bZIPs (Choi *et al.* 2000, Uno *et al.* 2000).

ENHANCED RESPONSE TO ABA1 (ERA1), which encodes the protein farnesyltransferase β -subunit regulates development of plants (Cutler *et al.* 1996, Yalovsky *et al.* 2000). ERA1 plays roles at the downstream of ABI1 and ABI2 but functions at the upstream of ABI3 and ABI5 (Brady *et al.* 2003).

SnRK2

Sucrose non-fermentation response kinases (SnRKs) are homologs of AMPK/SNF1 in animal and yeast. SnRKs can be divided into three families, including SnRK1, SnRK2 and SnRK3. SnRK2 and SnRK3 are special in plants. SnRK2s can be further divided into three groups. Group I and group II of SnRK2s are not or weakly activated by ABA. Group III of SnRK2 including SnRK2.2/SnRK2D, SnRK2.3/SnRK2I and SnRK2.6/SnRK2E are strongly activated by ABA and regulate many aspects of plant growth and responses. SnRK2.6/SnRK2E/OST1, as one of most studied members of the SnRK2 group phosphorylates SLAC1 (Slow-anion channel1) and KAT1 (an inward K⁺ channel), which are two significant channels for stomatal movements in response to ABA (Kulik *et al.* 2011). The functions and redundant roles among SnRK2.2/2.3/2.6 in ABA responses were well uncovered by the success of the Arabidopsis triple mutant *srk2d/i/e* (*snrk2.2/snrk2.3/snrk2.6*) (Nakashima *et al.* 2009, Fujita *et al.* 2009, Fujii and Zhu 2009). SnRK2.2/2.3/2.6 can phosphorylate bZIP transcription factors such as ABI5 and some other AREBs that play important roles in ABA responses (Nakashima *et al.*

2009). In addition, SnRK2 show the ability to interact with PP2Cs that inactivate SnRK2 by dephosphorylation (Umezawa *et al.* 2009, Yoshida *et al.* 2006a).

Based on the above results the ABA signaling has been uncovered to some extent. That is, PP2Cs act at the upper stream and play negative roles by dephosphorylating the SnRK2s (group III), which are positive regulators in ABA signaling by activating their downstream factors such as bZIP transcription factors. ABA responses and ion channels that mediate stomatal movements are induced by the activation of these transcription factors.

RCAR/PYR/PYL Receptors

Although a great deal of work had been done to characterize the significant factors in ABA signaling pathway by using the forward genetics, which characterized PP2Cs and significant transcription factors, and reverse genetics, which uncovered the significance and redundancy of group III of SnRK2 as mentioned before. Most factors characterized by these methods are categorized as downstream factors of ABA signaling pathway. How ABA is sensed and how ABA signal is activated were largely to be investigated.

Many studies have indicated that ABA perceptions occur in both intracellular and extracellular domains (Cutler *et al.* 2010). Recently ABA receptors were identified by the combination of chemical genetics and yeast screen. The breakthrough has been made by the finding of PYR/PYL/RCAR, a group of soluble ABA receptors, by two independent groups, although there are other candidates of ABA receptors such as GPCR-type G proteins (GTGs) (Pandey *et al.* 2009). In 2009, Park *et al.* screened a mutant that is insensitive to pyrabactin, a synthetic growth inhibitor which functions as an agonist of ABA, and named it as PYRABACTIN RESISTANT1 (PYR1). PYR1 and its homologs PYR-like (PYLs) can bind to PP2Cs and inhibit dephosphorylation of SnRK2. PYR/PYLs function redundantly in regulating ABA

responses (Park *et al.* 2009). Meanwhile, by yeast two hybrid screening, Ma *et al.* identified regulatory component of ABA receptor 1 (RCAR1, corresponds to PYL9) which can interact with ABI1 and ABI2 and inhibit their enzymatic functions in an ABA dependent manner (Ma *et al.* 2009). In the same year, Umezawa *et al.* investigated the relationship between PP2Cs and SnRK2s by reconstituting the PYL, PP2C and SnRK2 signaling complex *in vitro* (Umezawa *et al.* 2009). As a result the major ABA signaling pathway induced by RCAR/PYR/PYL (hereafter will be designated as PYL) had been established. The schematic of this signaling pathway was briefly summarized in Figure 1-5. In the presence of ABA, the complex of ABA and PYL binds to PP2C and inhibits its activity for the dephosphorylation of SnRK2. After being released from the inhibitory effect of PP2C, SnRK2s phosphorylate themselves (or are phosphorylated by other factors) and their targets such as transcription factors and ion channel proteins, ABA responses are exerted.

Structural biological studies on PYL receptors

Immediately following by the discovery of PYL receptor family, the structure of PYL proteins and the mechanism of their interactions with ABA or PP2Cs were well studied by many groups (Nishimura *et al.* 2009, Yin *et al.* 2009, Sun *et al.* 2012, Zhang *et al.* 2012, Miyazono *et al.* 2009, Melcher *et al.* 2009, Dupeux *et al.* 2011). PYLs, which are homologous to the Bet v 1-fold and START domain proteins, contain a ligand-binding pocket (Klingler *et al.* 2010). By binding to the internal pocket of PYL, ABA induces the structural changes of two conserved loops, gate loop and latch loop, and leads to a formation of interface on PYL that facilitates the interaction of PYL with PP2C (Figure 1-6). It is proposed that the catalytic site of PP2C is sealed by the interaction with ABA-PYL complex and then PP2C loses its inhibitory effect on SnRK2 function (Miyakawa *et al.* 2013). In addition, tryptophan residue on the hairpin of PP2C is very important for the interaction between PYL and

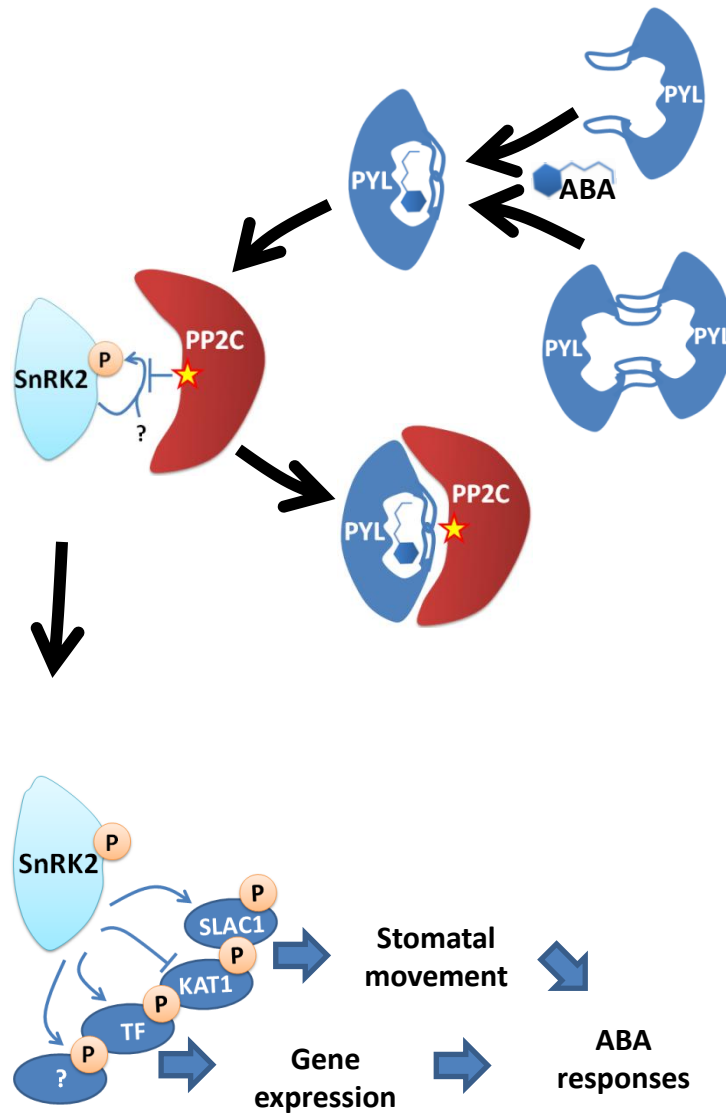


Figure 1-5 The ABA signaling pathway induced by PYL.

The binding of ABA into PYL pocket induces PYL conformational changes and promotes its interaction with PP2C, which inhibits the activity of SnRK2 by dephosphorylation. After released from the inhibition by PP2C, SnRK2 phosphorylates itself and activates downstream targets, such as SLAC1, KAT1, TFs and other unknown factors. Then the ABA responses including stomatal movement and gene expression are induced.

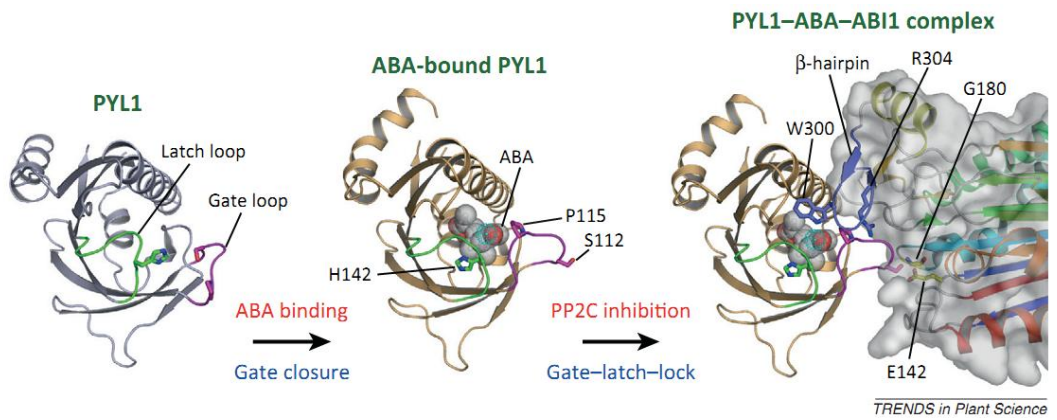


Figure 1-6 Structural mechanism of interaction between PYL and PP2C
 An example (PYL1) for explanation that how ABA induces structural changes of PYL and induces the interaction with PP2C (Figure is extracted from Miyakawa, *et al.*, 2013).

PP2C but not important for the catalytic activity of PP2C (Miyazono *et al.* 2009). This discovery gave a clear answer to the long lasting mystery that why *abi1-1* and *abi2-1* mutants showed constitutive insensitivity to ABA despite they are negative regulators in ABA signaling.

There are 14 PYLs in Arabidopsis. Except for PYL7, PYL11 and PYL12, which have not been confirmed yet and PYL13 that showed no binding activity of ABA, the rest of PYL members can be divided into three groups based on oligomeric state in the absence of ABA, including homodimeric group (PYR1, PYL1 and PYL2), monomeric group (PYL4-PYL6, PYL8-PYL10) and transdimeric group (PYL3) (Zhang *et al.* 2012). Both of homodimeric PYLs and transdimeric PYL inhibit the activity of PP2C in an ABA dependent manner by undergoing dissociation of dimeric state caused by ABA binding and successive formation of PYL-ABA-PP2C heterotrimer successively. The monomeric PYLs show inhibitory effects on PP2C in an ABA independent manner (Hao *et al.* 2011). A recent study showed that PYL13 can interact with and inhibit PP2CA (AHG3, ABA-Hypersensitive Germination3) in an ABA independent manner. Moreover, the interaction between PYL13 and PYL10 and their antagonism in the ABA-independent inhibition of PP2Cs were confirmed (Li *et al.* 2013).

Physiological roles of PYL receptors

The functions of PYL in physiological level have also been studied. PYR1, PYL1, PYL2 and PYL4 showed some redundant functions in ABA responses and regulating seed germination (Park *et al.* 2009). Further studies using sextuple mutant indicated that PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8 play redundant roles in regulating seed germination and establishment, root elongation, stomatal movement and vegetative and productive growth (Gonzalez-Guzman *et al.* 2012). Except for redundant roles, PYL8 also plays a nonredundant role in regulation of root in response to ABA (Antoni *et al.* 2013). Over-expression of PYL5 and PYL8

individually increased the sensitivities of *Arabidopsis* to ABA in seed germination and root elongation (Santiago *et al.* 2009, Saavedra *et al.* 2010) . In addition to *Arabidopsis*, the existence and functions of PYL are also reported in rice, tobacco, citrus, *Artemisia annua* (Romero *et al.* 2013, Zhang *et al.* 2013, Lackman *et al.* 2011, Kim *et al.* 2011).

1.5 Objectives

There are 14 members in the *PYL* gene family in *Arabidopsis*. They show various spatio-temporal expression levels (Figure 1-7) and redundant physiological roles with each other as mentioned before. PP2C genes are also expressed in different patterns in cell. For example, *ABI1* and *ABI2* are expressed in both the cytoplasm and nucleus, however, *AHG1* and *AHG3* are mainly expressed in the nucleus. SnRK2s may not be the only targets of PP2Cs and there may be more targets of SnRK2s in addition to the transcription factors identified recently. Although the functions of PYL were greatly characterized by biochemical and structural biological approaches, there is a great deal of work to be done to uncover the mechanisms of PYLs in regulating the ABA signaling pathway and the functions of PYLs in plant growth and development. In addition, it is worth mentioning the co-operative crosstalk between plant hormones. PYL, as receptor of ABA identified recently, may play much more roles than we know at present.

The main work of this research is to characterize the physiological and molecular biological roles of PYLs in *Arabidopsis* and investigate the potential roles of PYL in plant hormone crosstalk.

(D) *Arabidopsis thaliana* Tiling Array Express (At-TAX) expression data of the PYR/PYL/RCAR gene family.

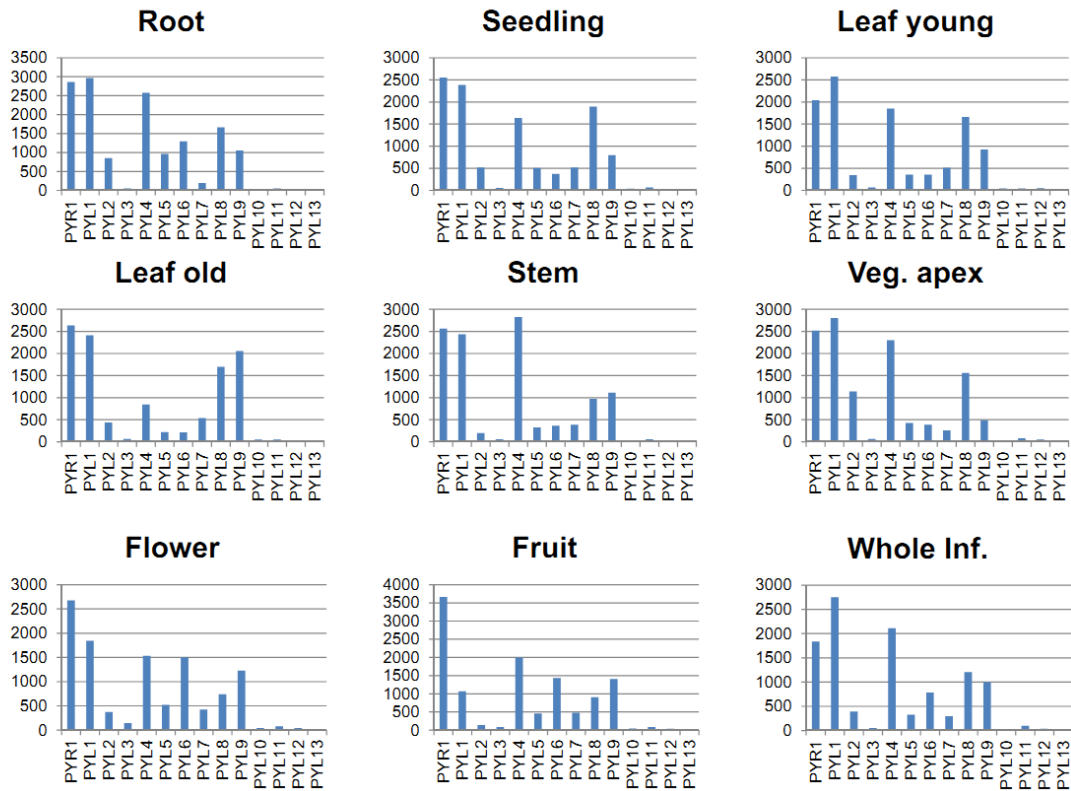


Figure 1-7 Spatio-temporal expression level of each PYL in Arabidopsis
 PYLs show various spatio-temporal expression levels(extracted from Gonzalez, et al. (2012))

CHAPTER 2 Observation of the phenotypes of PYL over-expression lines in response to different conditions

2.1 Introduction

There are 14 members in the PYL family in *Arabidopsis*. Each PYL shows distinct abilities in binding to ABA and PP2Cs. The expression levels of each *PYL* are not in the same manner. As shown in Figure 1-7, *PYL1* and *PYL1* are almost constantly expressed in all parts of *Arabidopsis*. *PYL6* is expressed preferentially in root, flower and fruit. Some *PYLs* such as *PYL10*, *PYL11*, *PYL12* and *PYL13* show low expression level in all over the tissues. Consequently it is still unclear that what the molecular and physiological functions of PYLs are in plant. To investigate the functions of each PYL, we tried to construct the *PYL* over-expression lines of *Arabidopsis* using pBIG2113SF vector, which is driven by 35S promoter (Ichikawa *et al.* 2006). Except for *PYL1*, *PYL9* and *PYL10*, all the rest of the over-expression lines of *PYLs* were established and homo-lines were screened (Figure 2-1). The physiological responses of these over-expression lines to ABA and other treatments have been investigated.

2.2 Results

2.2.1 Germination rate

First the germination rate of *PYL* over-expression (*PYL*-OE) lines in response to ABA treatment was investigated. The germination rate was counted at 36h after transferring to 22 °C. The result showed that over-expression lines of *PYL1*, *PYL2*, *PYL4* and *PYL8* were much more sensitive to ABA. *PYL4*-OE and *PYL8*-OE lines showed lower germination rate than the Vec line (stands for control containing empty pBIG vector without fragment insertion) even without ABA treatment (Figure 2-2). These results consist with the results from Park, et al. to some extent that *PYL1*

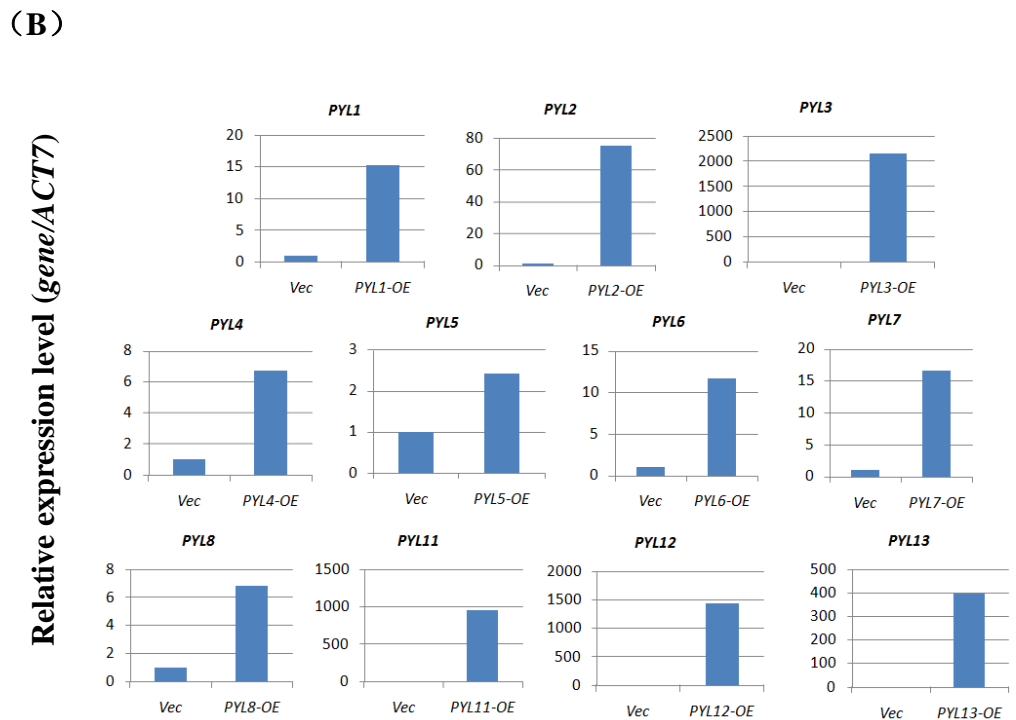
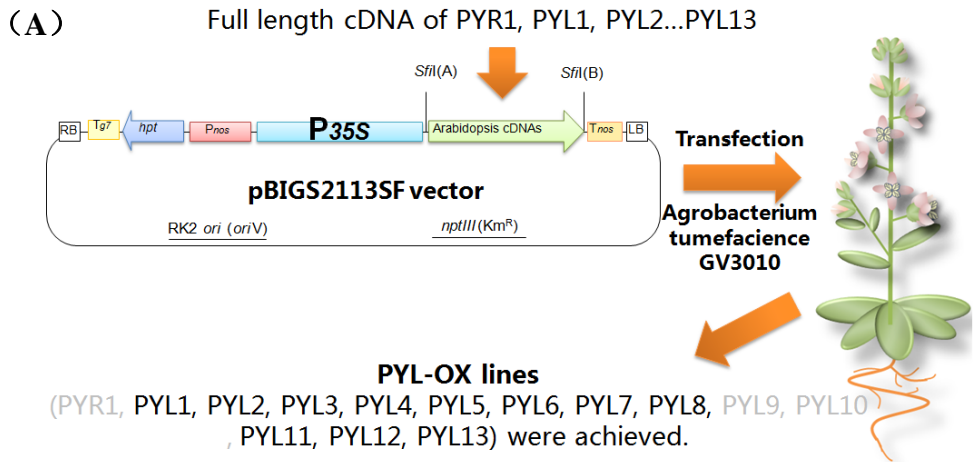


Figure 2-1 Strategy and results of construction of *PYL*-OE lines

- (A) Strategy for each *PYL* over-expression (*PYL*-OE) lines construction
- (B) Expression level of each *PYL* relative to actin7 (*ACT7*) in each *PYL*-OE line gene. Vec stands for the control line containing empty pBIG verctor without fragment insertion.

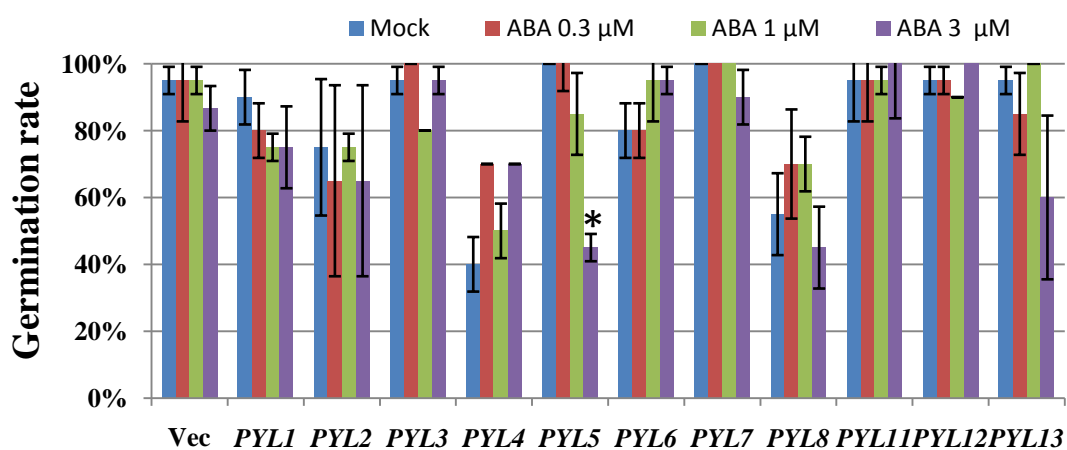


Figure 2-2 Seed germination rate of *PYL*-OE lines

The results are from the average of three replicates. Values represent the means SE of more than ten seeds. *, significant difference ($P < 0.05$)

PYL2 and PYL4 play redundant roles in inhibiting seed germination (Park *et al.* 2009). However, on the contrary, over-expression of *PYL3*, *PYL6*, *PYL7*, *PYL11* and *PYL12* showed no increase in or even decrease in the sensitivity to ABA, which indicated that these PYLs might be not in charge of regulating the seed germination.

2.2.2 Seedling establishment

In addition to seed germination, ABA also shows inhibitory effect on seedling establishment. Some *PYL*-OE lines showed increasing sensitivity to ABA during seedling establishment such as *PYL1*, *PYL2*, *PYL5* and *PYL7* (Figure 2-3) that partially consisted with the results of seed germination.

2.2.3 Root growth

ABA is important for the regulation of primary root growth and lateral root formation. I examined the responses of *PYL*-OE lines to ABA and found that the primary root elongation of *PYL2*-, *PYL5*- and *PYL7*-OE lines were more suppressed by ABA compared to that of control line (Figure 2-4A). However, the results of lateral root responses were unstable because of the high sensitivity of lateral root to environment.

It is reported that high salinity, which induces ABA level, can also inhibit the primary root elongation and the ABA signaling is necessary for this inhibition (Achard *et al.* 2006). To study the roles of PYL receptor in salinity responses, we tested the root elongation of *PYL* over-expression lines under treatment of 100 mM NaCl or both of 100 mM NaCl and 30 mM abamineSG, an ABA biosynthesis inhibitor (Kitahata *et al.* 2006). The results (Figure 2-4B) showed that some *PYL* over-expression lines such as *PYL5*-, *PYL7*-, *PYL11*- and *PYL13*-OE were much sensitive to NaCl than the Vec line. However, *PYL1*, *PYL2* and *PYL3* lines showed no obvious changes after the treatment of NaCl. Furthermore, the inhibition caused by NaCl can be reduced by abamineSG in some *PYL*-OE lines, including

(A)

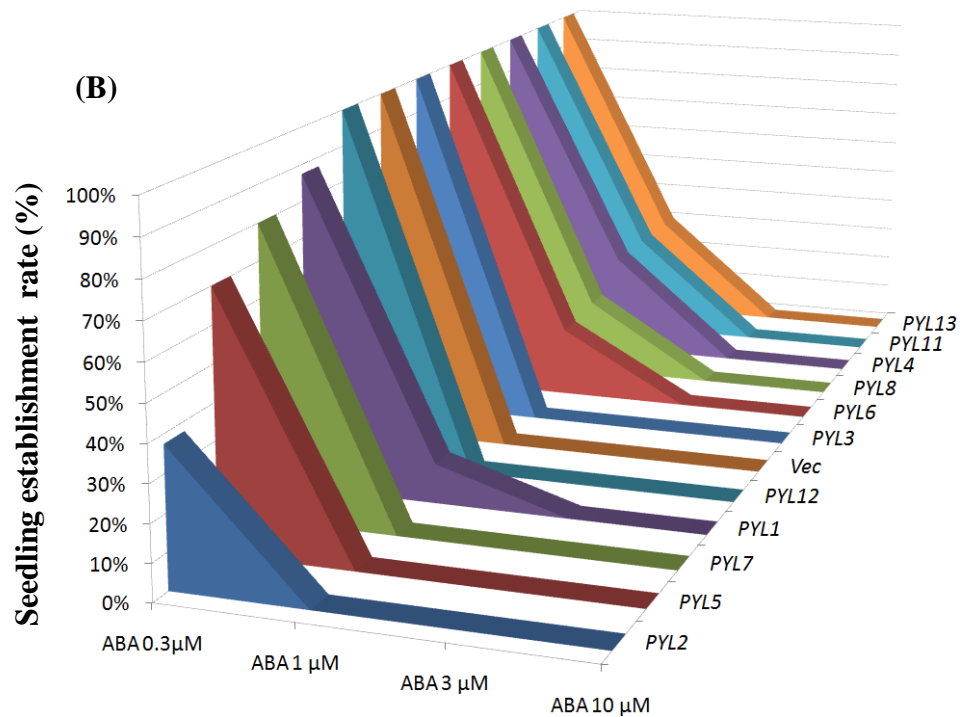
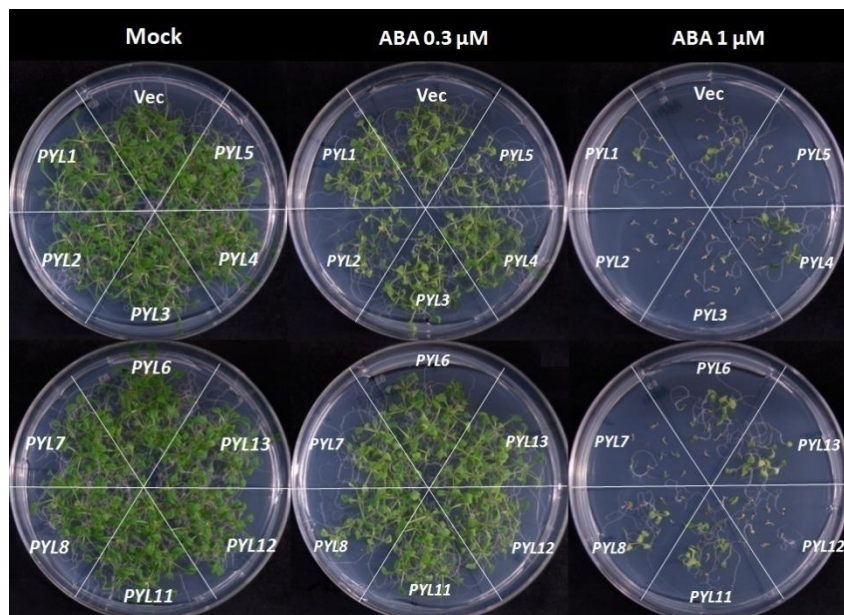


Figure 2-3 Seedling establishment rate of *PYL*-OE lines.

Seedling establishment was judged by the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves on 14 DAG (day after germination). The (A) phenotype and (B) establishment rate were observed and estimated.

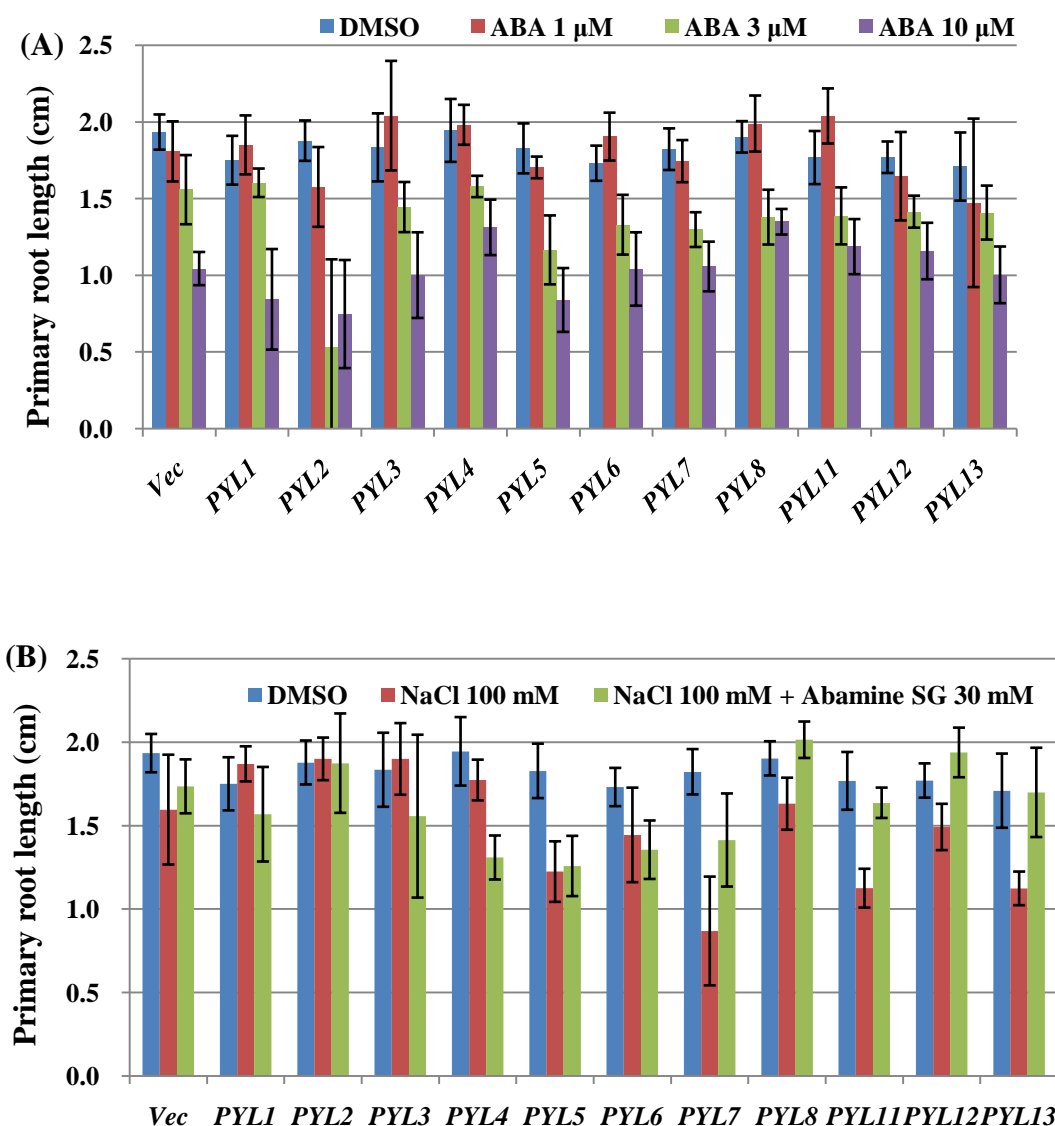


Figure 2-4 Primary root growth of *PYL*-OE lines under various treatments. 3-day-old seedlings were transferred to half MS medium containing compounds as indicated and further cultured for 4 days. The responses of each line to (A) gradient ABA concentration and (B) salt stress (NaCl 100 mM) were detected. To investigate the relationship between salt and ABA in regulating the root growth, an inhibitor of ABA biosynthesis, abamineSG, was cotreated with NaCl. Values represent the means SE of ten seedlings.

PYL7-, *PYL8*-, *PYL11*-, *PYL12*- and *PYL13*-OE lines, which indicated that the inhibitory effects might be dependent of ABA level. On the contrary, abamineSG treatment showed no recovery effects on root elongation inhibited by NaCl in *PYL5* and *PYL6*-OE lines. This result gave a hint that *PYL5* and *PYL6* could play roles in responses of *Arabidopsis* to NaCl independent of or less dependent of increase in ABA level induced by NaCl.

2.2.4 Hypocotyl growth

Although the inhibitory effect of ABA on hypocotyl elongation is known, the mechanism is still to be uncovered. We postulated that *PYL*, as ABA receptor, could be involved in this inhibition. As sucrose also affects the hypocotyl elongation (Zhang *et al.* 2010), we chose the half MS medium without sucrose as the culture medium for this experiment to avoid the effects of sucrose. There was no obvious difference in hypocotyl length between *PYL*-OE lines and the Vec line under constant light and dark treatment (Figure 2-5). However, I found that *PYL2*-OE, *PYL6*-OE and *PYL11*-OE individually inhibited hypocotyl elongation (Figure 2-6B) under special procedure (hereafter this procedure is called JK1 for convenience), which gave a 48h light pretreatment after cold treatment at 4 °C for 2 days and is followed by dark treatment for two to four days (Figure 2-6A). *PYL6*-OE lines were chosen for further study. The results showed that higher expression level of *PYL6* resulted in the stronger inhibitory effect on hypocotyl elongation (Figure 2-7), which meant *PYL6* play negative roles in regulating hypocotyl growth.

To confirm the importance of light pretreatment, the effects of 3 h, 48 h and 60 h light pretreatment were compared each other (Figure 2-8). After all I found that only 48h light pretreatment induced inhibitory effect on hypocotyl elongation in *PYL6*-OE lines. The seeds treated for 3 h by light were transferred into dark before germination. However, the seeds treated for 48 h or 60 h by light had already germinated and started photomorphogenesis. In 48 h light treatment, the cotyledons were light green,

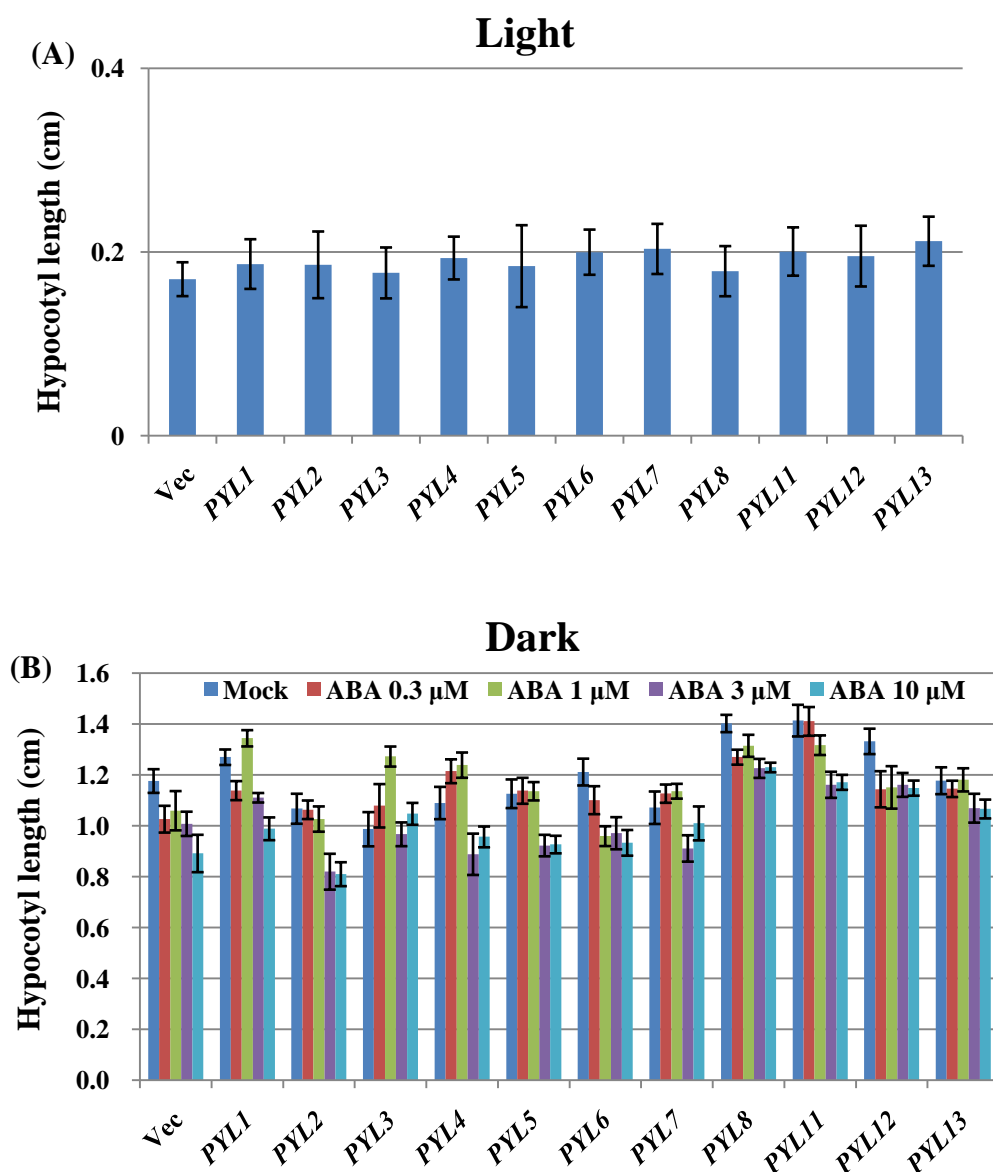


Figure 2-5 Hypocotyl length of *PYL*-OE lines under different conditions.

The seeds were germinated on half MS medium (-sucrose). After cold treatment for 3 days and 6h light pulse treatment, (A) the seeds were grown under constant white light treatment for 3 days before measurement; (B) the seeds were grown under dark for 36h and then transferred to the half MS medium (-sucrose) containing different concentration of ABA and followed by 3 days cultivation under dark before measurement. Values represent the means SE of ten seedlings.

(A) JK1 treatment

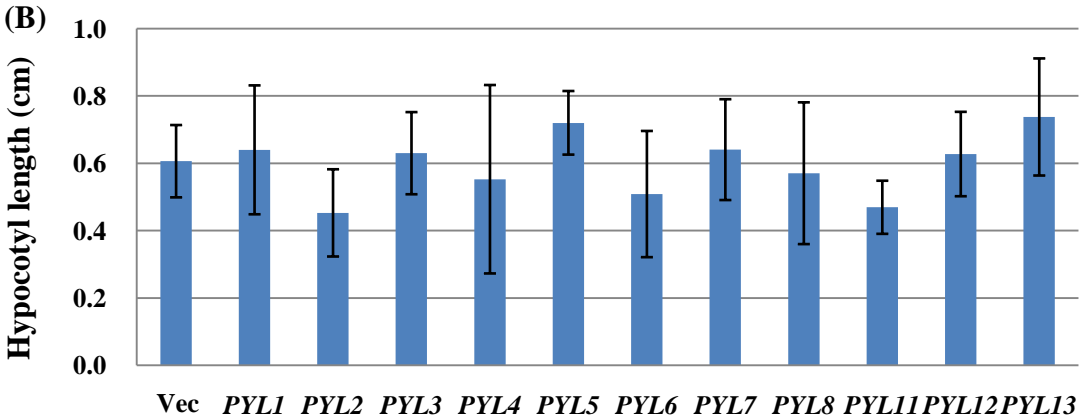


Figure 2-6 Hypocotyl length in Vec line and each *PYL*-OE line under JK1 treatment

(A) Schematic representation for JK1 treatment procedure: the seeds were germinated on half MS medium (-sucrose). After cold treatment for 3 days and 48h light treatment, the seedlings were grown under dark treatment for 3 days before measurement. (B) The hypocotyl length of each line. Values represent the means SE of ten seedlings.

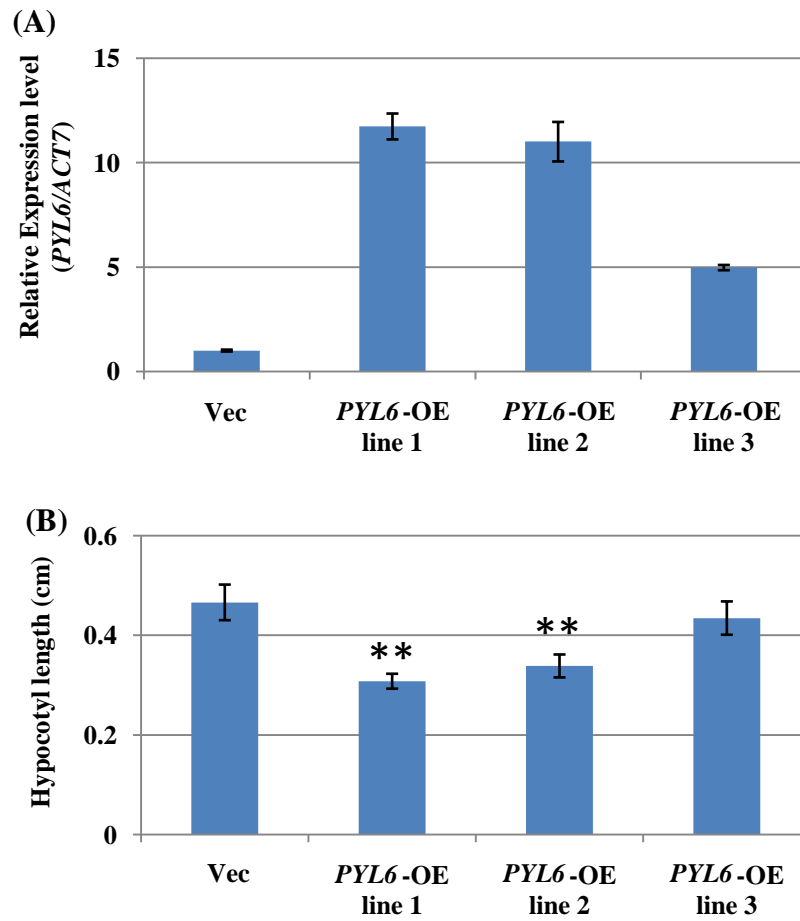


Figure 2-7 Hypocotyl length in Vec line and *PYL6*-OE lines with different expression level

(A) *PYL6* expression levels in different lines relative to ACT7. (B) The hypocotyl length of each line by JK1 treatment as mentioned in figure 2-6. Values represent the means SE of ten seedlings.

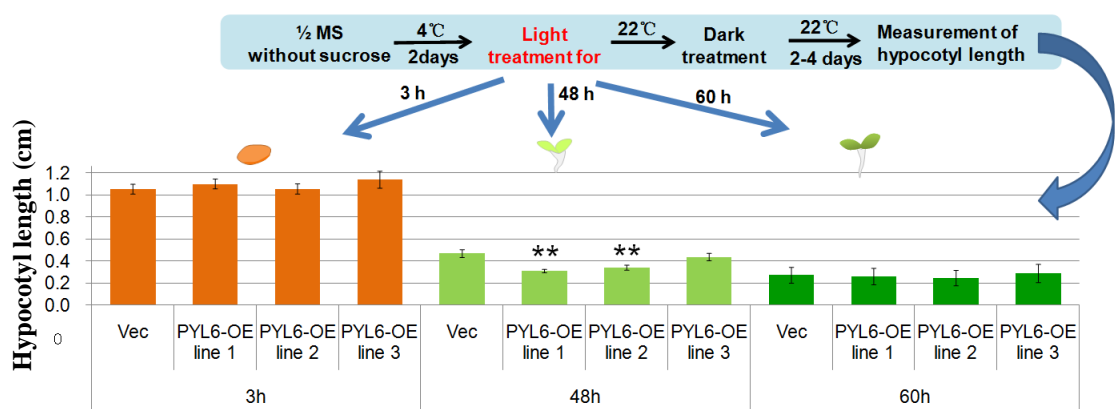


Figure 2-8 Hypocotyl length dependent on light treatment after cold treatment

The seeds of *PYL6*-OE lines and control (Vec) lines were treated by light for 3h, 48h and 60h individually and followed by dark treatment for additional 3 days and measurement. Values represent the means SE of ten seedlings. **, significant difference ($P < 0.01$) from Vec line (Control).

however, the cotyledons turned into green in the 60 h treatment, which stands for the different photomorphogenesis status. In addition, the same result of inhibition on hypocotyl elongation in *PYL6* over-expression lines was also observed in a constant dim light treatment experiment (Figure 2-9). A GUS line in which the expression of *GUS* genes promoted by the promoter of *PYL6* showed that the *PYL6* expression level is high in cotyledon and hypocotyl (Antoni *et al.* 2013). So here I would suggest that *PYL6* should play roles in regulating hypocotyl growth during a specific photomorphogenesis procedure.

2.2.5 Function of *PYL6* in regulating hypocotyl elongation

2.2.5.1 Relationship between *PYL6* and ABA signaling factors

ABA showed strong inhibition activity on hypocotyl elongation (Figure 2-5). However, there is no information about which ABA receptor functions in the inhibitory effect of ABA on hypocotyls growth. The results in the previous section suggest that *PYL6*, as an ABA receptor, should negatively regulate hypocotyl elongation through ABA signaling pathway. To uncover this hypothesis, the hypocotyl growth of a variety of mutants in ABA biosynthesis and signaling pathway such as *aba2-2* and *aba3-1*, two mutants in ABA biosynthesis, *abi1-1* and *abi2-1*, PP2C mutants, *abi4-5* and *abi5-7*, mutants of transcription factors at downstream of ABA signaling were investigated under different light treatment conditions. The results showed that under the JK1 treatment, the hypocotyls of the *aba2-2*, *aba3-1* and *abi5-7* were longer than wild type (Col) (Figure 2-10). While *abi1-1* and *abi2-1* showed no obvious difference compared to wild type in hypocotyl length. The results from dim light treatment revealed that mutation of *ABI5* (*abi5-7*) dramatically increased the hypocotyl elongation compared to wild type and the other mutants (Figure 2-11). Furthermore, the hypocotyl responses of these mutants to ABA and PAC, a GA biosynthesis inhibitor, under JK1 treatment were studied (Figure 2-12). Both *abi4-5* and *abi5-7* showed longer hypocotyl than wild type and other mutants

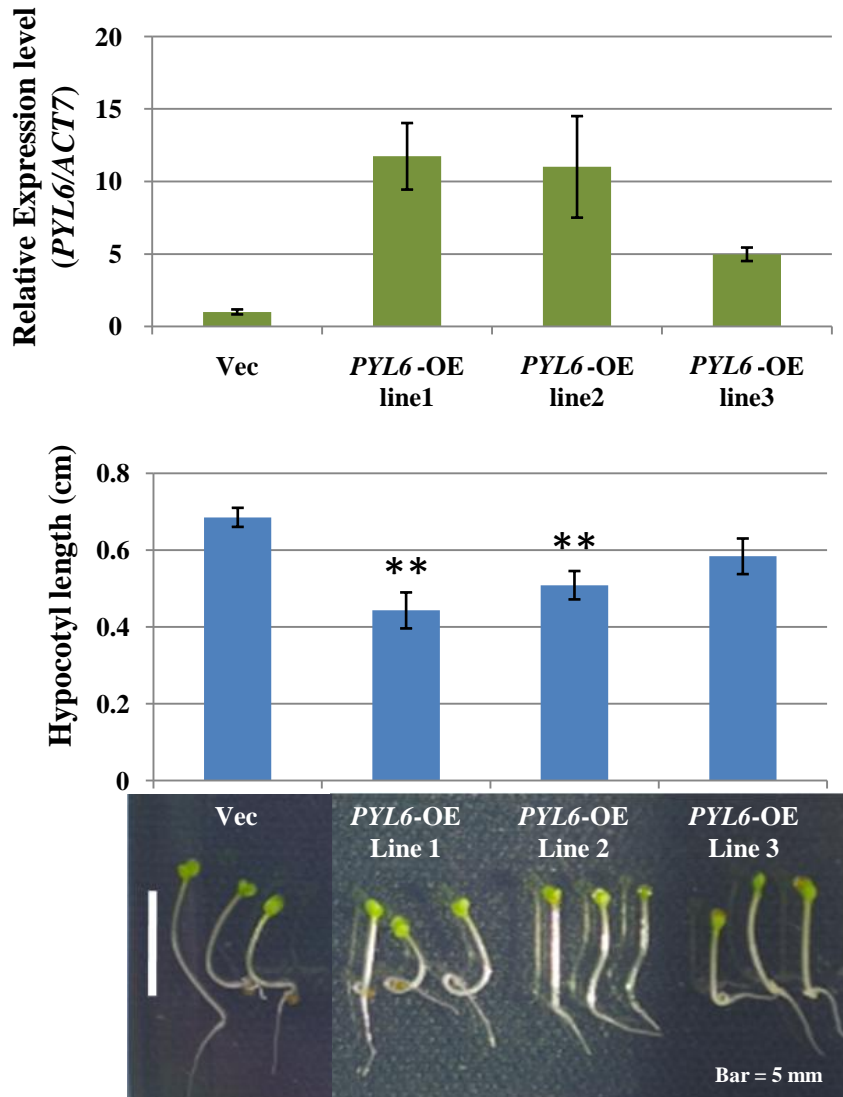


Figure 2-9 Hypocotyl length grown under dim light treatment

The seeds were germinated on half MS medium (-sucrose). After cold treatment for 3 days and 6h light treatment, the seedlings were grown under dim light treatment for 3 days before measurement. Values represent the means SE of ten seedlings.

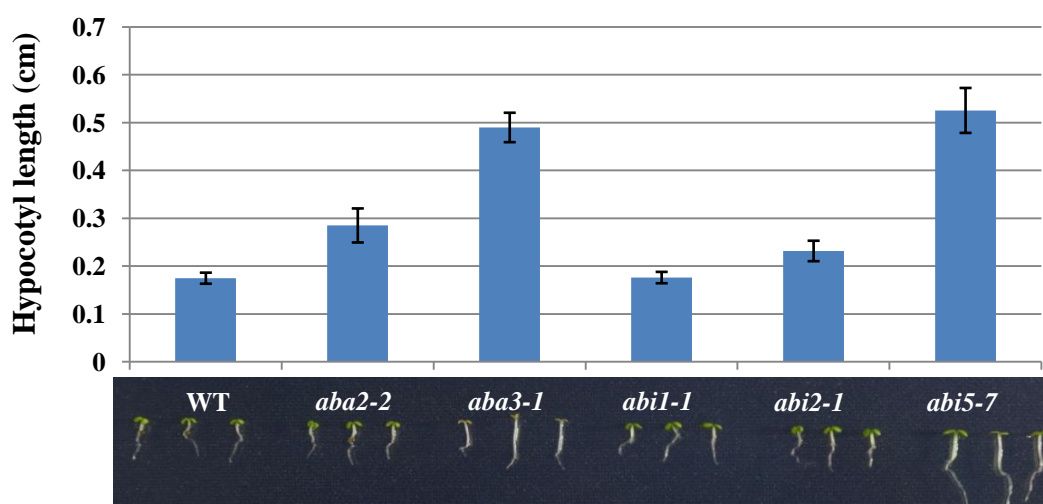


Figure 2-10 Hypocotyl length grown under JK1 treatment

The seeds were germinated on half MS medium (-sucrose) followed by JK1 treatment as mentioned in figure 2-6. Values represent the means SE of ten seedlings.

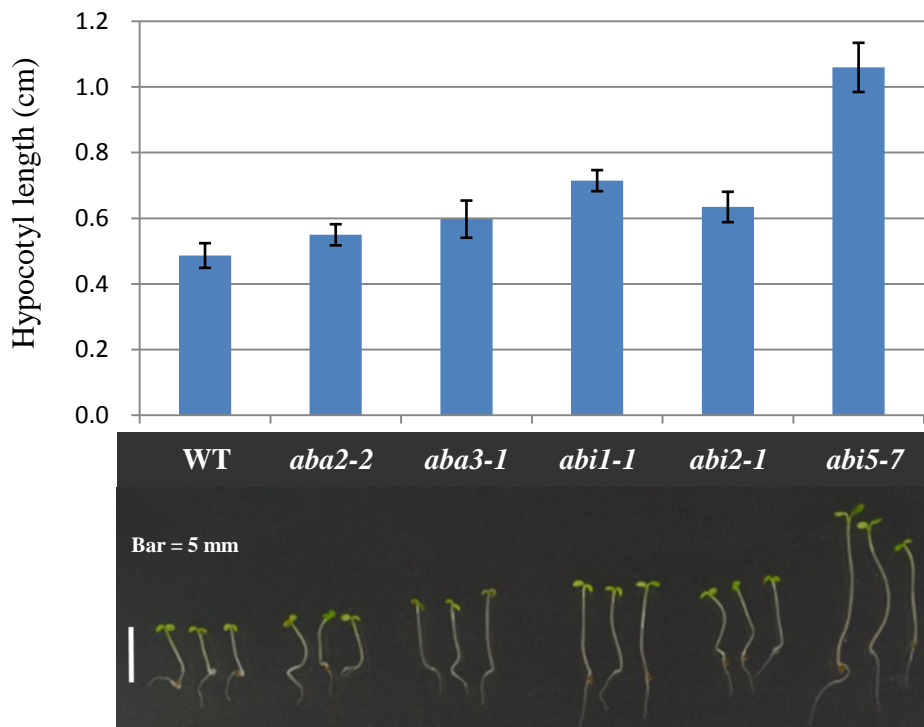


Figure 2-11 Hypocotyl length grown under dim light treatment
The seeds were germinated on half MS medium (-sucrose). After cold treatment for 3 days and 6h light pulse treatment, the seeds were grown under constant dim light treatment for 3 days before measurement. Values represent the means SE of ten seedlings.

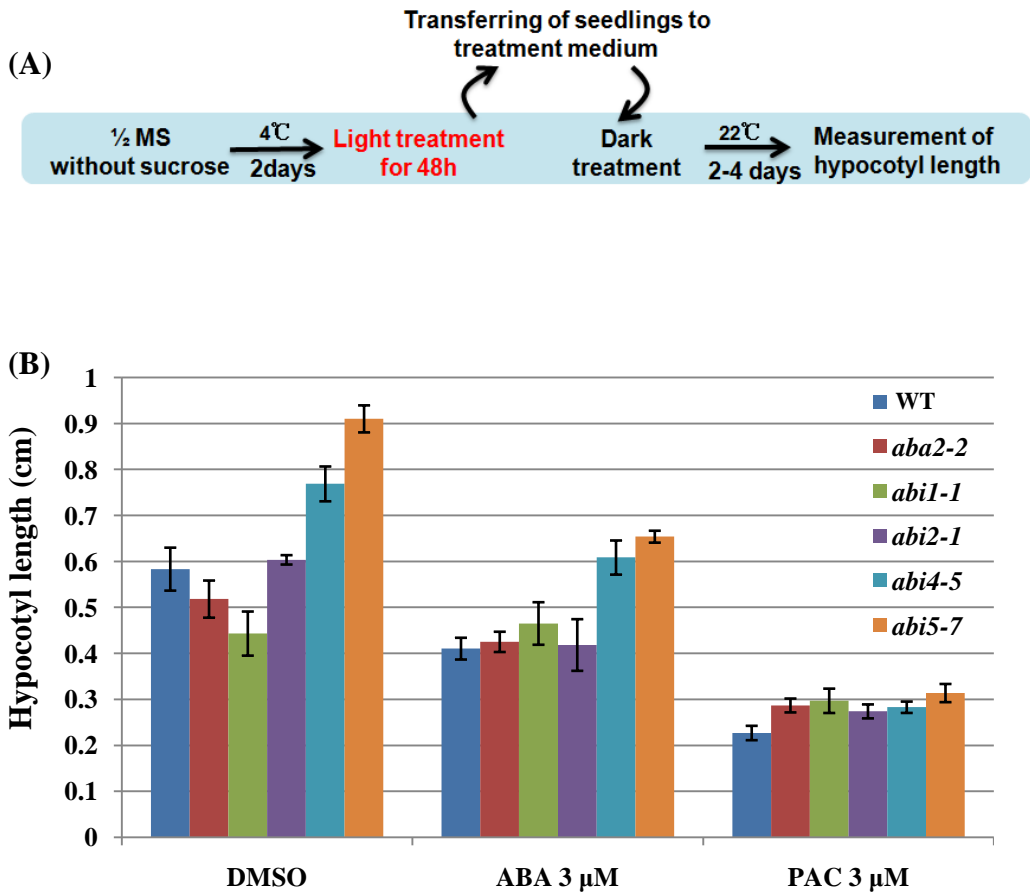


Figure 2-12 Hypocotyl length grown under JK1 treatment

(A) Schematic for procedure: the seeds were germinated on half MS medium (-sucrose) followed by JK1 treatment the same as mentioned in figure 2-6 with the additional step of transferring the seedlings to new half MS medium (-sucrose) with or without ABA or GA after 48h light treatment. (B) The hypocotyl length of each mutant. Values represent the means SE of ten seedlings.

even in the presence of 3 μ M ABA, although hypocotyl elongation of the mutants showing here was more or less all inhibited by PAC treatment. Above all results indicate that GA is necessary for hypocotyl elongation, but it is worthy to note that the hypocotyls of all of the mutants tested here were longer than that of wild type. The difference is tiny but significant, which gave a hint that there may be a regulatory function in ABA signaling independent of GA functions in regulating hypocotyl growth.

The above results indicate that ABI5 but not PP2C (ABI1 and ABI2) acts as important negative regulators in inhibiting hypocotyl growth under both JK1 and dim light treatment conditions. In addition, it is reported that the ABA-dependent ABI5 accumulation occurs within a limited developmental time window between 48 h and 60 h post-stratification (Lopez-Molina *et al.* 2001) which is consistent with the time window of PYL6 dependent inhibition of the hypocotyl elongation. Then I checked the *ABI5* expression level and found that its transcription level was increased in *PYL6*-OE line compared to the Vec line (Figure 2-13). This result could explain the hypocotyl phenotype of *PYL6* over expression line.

2.2.5.2 Relationship between PYL6 and GA signaling

Hypocotyl elongation is regulated by DELLA (Feng *et al.* 2008, Stavang *et al.* 2009). To investigate the roles of GA signaling in hypocotyl responses to JK1 and dim light treatments, I used *5Adella*, a pentuple della mutant with loss of all five functional DELLA proteins and therefore in which GA signaling is constitutively expressed to show resistance to PAC. *5Adella* showed longer hypocotyl compared to its wild type *Ler* (Lansberg) under both JK1 and dim light treatments (Figure 2-14). Former results also indicated that PAC strongly inhibited the hypocotyl growth even in all ABA mutants (Figure 2-12). It is well known that ABA and GA always play antagonistic roles in regulating the plant growth and development. So I further investigated whether the inhibition of PYL6 on hypocotyl is related to GA signaling

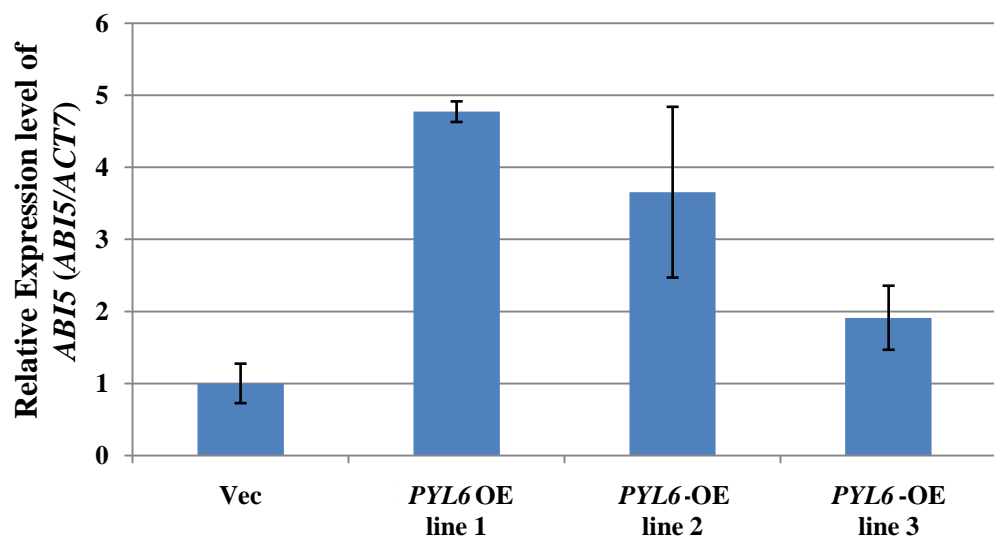


Figure 2-13 *ABI5* expression level in *PYL6*-OE lines

The real-time PCR was performed by using cDNA from 15-day-old seedlings without any treatment. Transcription level of each *ABI5* is relative to *ACT7*.

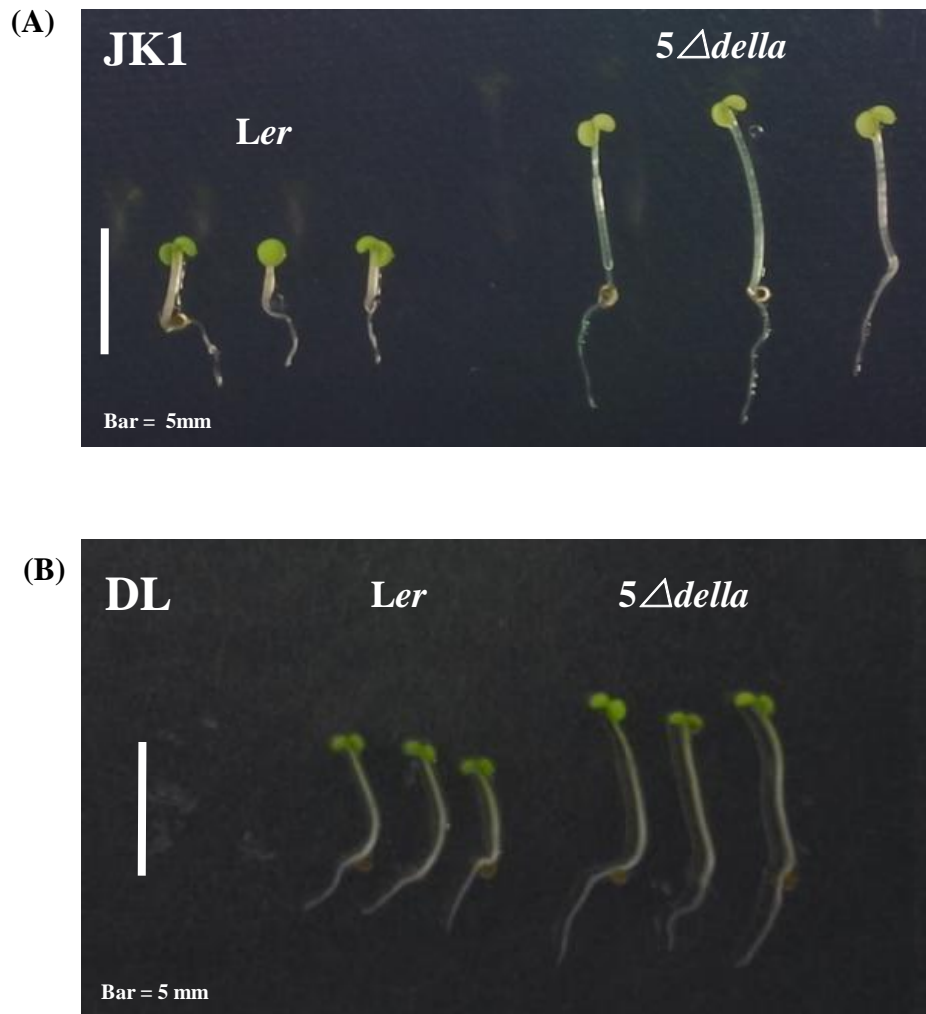


Figure 2-14 Hypocotyl responses of *5Δdella* and wild type (*Ler*) under different treatments

(A) The responses of *5Δdella* and *Ler* under JK1 treatment

(B) The responses of *5Δdella* and *Ler* under dim light (DL) treatment.

pathway. The results showed that GA treatment rescued the inhibitory effect of over-expression of *PYL6* on hypocotyl elongation (Figure 2-15). Furthermore, hypocotyl elongation inhibited by 3 μ M ABA was recovered by 10 μ M GA₃ in the Vec line but not in *PYL6*-OE. These results indicated that *PYL6* should function as a modulator between ABA and GA signaling in regulating hypocotyl during a specific photomorphogenesis procedure (JK1).

2.3 Discussion

The above data support the recent published results that PYLs play redundant and distinct roles in regulating seed germination, root elongation and seedling establishment. In addition, I initially found that *PYL6* functions as a negative regulator in hypocotyl elongation during photomorphogenesis. Further studies revealed that this regulation could be caused by the effects of *PYL6* on the increase of the *ABI5* expression level. However, the mechanism of these effects of *PYL6* is still unknown. Moreover, the activity of *ABI5* is not only related to its expression level, but also related to other factors. For example, the phosphorylation of *ABI5* by SnRK2 is necessary for its activity (Lopez-Molina *et al.* 2001, Piskurewicz *et al.* 2008) and *ABI5* could be degraded by KEG (KEEP ON GOING) (Stone *et al.* 2006, Liu and Stone 2013). According to the communicational data from Dr. Yazaki, the direct interaction between *PYL6* and *ABI5* was confirmed by *in vitro* pull down assay (unpublished data). Therefore I think it is worthy to study further the relationship between *PYL6* and *ABI5* in both transcriptional and post-transcriptional levels.

In addition, it is also important to validate the regulation of hypocotyl growth by *PYL6* in relation to the crosstalk with GA signaling pathway. In fact *RGL2*, a DELLA protein that plays negative roles in GA signaling, was reported to stimulate ABA biosynthesis and *ABI5* accumulation (Piskurewicz *et al.* 2008, Piskurewicz *et al.* 2009). The roles of *PYL6* in GA signaling pathway and the relationship between

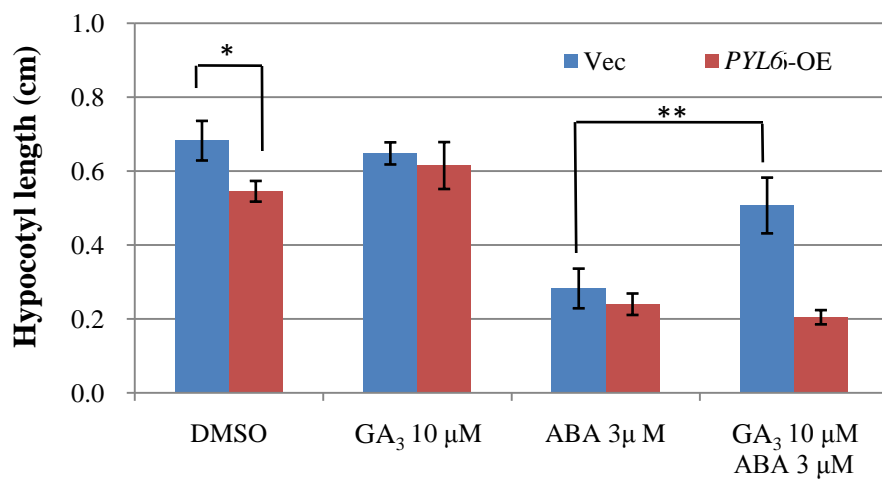


Figure 2-15 Hypocotyl length grown under JK1 treatment

The same procedure was performed as mentioned in figure 2-12 with the difference of treatment in medium. Values represent the means SE of ten seedlings. **, significant difference ($P < 0.01$). *, significant difference ($P < 0.05$), from Vec line (Control).

PYL6 and DELLA protein in regulation of ABI5 expression level are still to be investigated (Figure 2-16).

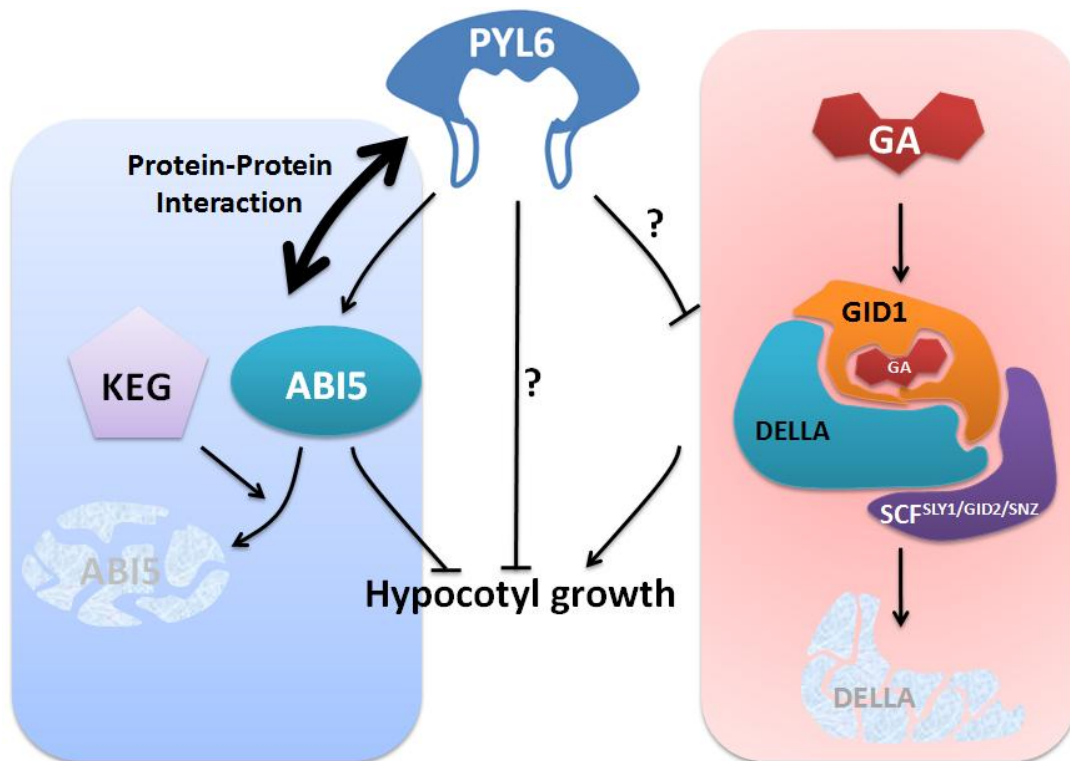


Figure 2-16 A model for regulation mechanisms of hypocotyl growth by PYL6, ABI5 and DELLA.

PYL6 inhibits hypocotyl growth through promoting ABI5 expression level or interaction with ABI5, while the function of PYL6 in regulating hypocotyl growth by GA pathway is still a mystery. There is crosstalk between ABA signaling (blue background) and GA signaling (red background) in regulating hypocotyl growth.

2.4 Materials and methods

2.4.1 Plant materials

The *PYL*-OE lines were constructed by transformation of pBIG2113SF vector with insertion of each *PYL* into *Arabidopsis thaliana* ecotype Columbia (*Col-1*). Empty pBIG2113SF vector was also transformed into *Col-1* as control and named as the Vec line (All prepared by Dr. Seo, Seo 2012). *aba2-2*, *aba3-1*, *abi1-1*, *abi2-1*, *abi4-5* and *abi5-7* in *Col-1* background are kindly provided by Dr. Hirayama T. (Okayama University), pentuple *della* mutant (*5Adella*) in the Landsberg background (*Ler*) is kindly provided by Prof. Blázquez MA (Instituto de Biología Molecular y Celular de Plantas, Spain).

2.4.2 Culture medium

Half-strength Murashige and Skoog (half MS) culture medium: MS Plant Salt Mixture (Wako, Japan), 1% sucrose (excluding sucrose in hypocotyl experiment), 0.8% agar, 1/1000x vitamin mixture (500 mg/L nicotinic acid, 500 mg/L pyridoxine•HCl, 200 mg/L thiamine•HCl, 2000 mg/L glycine, folic 500 mg/L acid, 50 mg/L biotin) and 0.1 mg/L *myo*-Inositol (Wako, Japan). Solution was adjusted to pH5.7 and autoclaved at 121 °C for 15 min.

2.4.3 Standard culture condition

The seeds were surface-sterilized by 70% ethanol for 20 min, rinsed by 99% ethanol, dried on sterilized filter paper and then placed onto half MS medium. After cold treatment at 4 °C under dark for 2-3 days, seeds were grown under constant fluorescent light ($18.2\text{--}46.5\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) at $22\pm1\ ^\circ\text{C}$. This method has been used in all experiments if there is no special description.

2.4.4 Seed germination

The germination rate was observed by 12 h per interval from 24 h to 120 h after

cold treatment at 4 °C. Germination was defined as the protrusion of radicle.

2.4.5 Seedling establishment

The seeds were germinated on half MS medium with or without compounds treatments under constant light condition. Seedling establishment was judged by the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves on 14 DAG (days after germination).

2.4.6 Root growth

The seeds were germinated on half MS medium and precultured for 3 days under long day light condition (16 h light and 8 h dark). 3-day-old seedlings were then transferred onto half MS medium containing compounds indicated individually and further cultured for 4 days under the same condition as before. At the time of transfer, the position of root tip was marked as the original point to evaluate root elongation on treatment medium. The pictures of roots were taken by digital camera (Pentax K30) and the root length was measured using ImageJ (NIH).

2.4.7 Hypocotyl growth

The seeds were sterilized and placed on half MS medium without sucrose. After cold treatment for 2 days, the seeds were treated by light indicated in different experiments (for dark treatment, there is no additional light treatment; for JK1 treatment, plus 42 h which results in 48 h light treatment in sum), then transferred back to dark condition followed by additional culture for 3 days. For constant light treatment and dim light treatment, the seeds were grown under constant light conditions with $18.2\text{--}46.5\ \mu\text{mol}/\text{m}^{-2}\ \text{s}^{-1}$ for light treatment and $1.2\ \mu\text{mol}/\text{m}^{-2}\ \text{s}^{-1}$ for dim light treatment. The pictures of hypocotyls were taken by digital camera and measured by ImageJ.

2.4.8 Real-time PCR

2.4.8.1 Preparation of cDNA templates

(1) RNA extraction

RNA from *Arabidopsis* was extracted by Total RNA Extraction Kit Mini (Plant) (RBC Bioscience). 50–100 mg frozen sample was homogenized into powder and mixed with 500 µl RB buffer and 5 µl mercaptoethanol by vortex followed by 5 min incubation at room temperature. The sample was centrifuged with 13,000 rpm for 2 min. The supernatant was transferred to a new tube and mixed with 1/2 volume of ethanol by vortex. The mixture was transferred into RB column and followed by centrifugation 13,000 rpm for 2 min. The column was washed by W1 buffer and Wash buffer successively by centrifugation and dispose of flow through every time. After washed by Wash buffer, additional centrifugation was performed at 13,000 rpm for 3 min to dry the ethanol. Then RNase-free water was added into column and followed by incubation for 3min. The total RNA sample was collected by centrifugation at 13,000 rpm for 3 min.

(2) Reverse transcription

PrimeScript[®] RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa) was used for reverse transcription. RNA extraction buffer containing 700 ng total RNA was mixed with 2 µl 5×gDNA Eraser buffer, 1 µl gDNA Eraser and distilled water that made the total volume up to 10 µl. The reaction was performed at 42 °C for 2 min. The reverse transcription was performed by mixing 10 µl reaction buffer from last step and 10 µl Premix (5×PrimeScript buffer 2 (for Real Time) 4 µl, PrimeScript RT Enzyme Mix I 1 µl, RT Primer Mix 1 µL, distilled water 4 µl). PCR reaction was performed using a process with 37 °C, 15 min followed by 85 °C, 5 sec for 1 cycle by TaKaRa PCR Thermal Cycler Dice Gradient[®].

2.4.8.2 Real-time PCR analysis

The reaction mixture contained 12.5 μ l SYBR *Premix Ex TaqII* (Tli RNaseH plus) (2 \times), 0.4 μ l 5'primer (10 μ M), 0.4 μ l 3'primer (10 μ M), 2.5 μ l cDNA template and 9.5 μ l distilled water. Each sample was added into 96 wells PCR plate of ABI type (WATSON) and was performed by Thermal Cycler Dice RealTime System TP800 (TaKaRa). The reaction protocol was shown in the table below and followed dissociation. The relative expression level was calculated by Δ Ct method.

1 cycle	95 $^{\circ}$ C	30 sec
40 cycles	95 $^{\circ}$ C	5 sec
	60 $^{\circ}$ C	30 sec

CHAPTER 3 Characterization and investigation of the PYLs and DELLAs interactions

3.1 Introduction

GA is a plant hormone that promotes the growth of plants. GA is essential for normal *Arabidopsis* life cycle at many different stages such as seed germination, growth of hypocotyls, stem internodes, leaves, stamens, and petals (King *et al.* 2001).

To date, the studies of GA signaling pathway has experienced great development by the finding of significant elements including DELLAs (GAI, RGA, RGL1, RGL2 and RGL3 in *Arabidopsis* and SLR1 in rice) (Peng *et al.* 1997, Silverstone *et al.* 1997, Wen and Chang 2002, Lee *et al.* 2002, Ikeda *et al.* 2001), F-box proteins (SLY1 and SNZ in *Arabidopsis* and GID2 in rice) (McGinnis *et al.* 2003, Strader *et al.* 2004, Gomi *et al.* 2004) and GID1 (GIBBERELLIN INSENSITIVE DWARF1, GA receptor) (Ueguchi-Tanaka *et al.* 2005, Nakajima *et al.* 2006). DELLA, as negative regulator in GA signaling pathway, inhibits seed germination, flower transition, photomorphogenesis and so on (Tyler *et al.* 2004, Achard *et al.* 2007, Hou *et al.* 2008). Recent reports indicate that DELLA also mediates the plant responses to abiotic stress and biotic stress (Navarro *et al.* 2008, Achard *et al.* 2006). An F-box protein represented by SLY1/GID2 plays positive roles in GA signaling by binding to DELLA and catalyzing the poly-ubiquitination of DELLA, and then facilitating the degradation of DELLA by 26S proteasome (Dill *et al.* 2004, Fu *et al.* 2004, Sasaki *et al.* 2003). The finding and the structural studies of GID1, a receptor with GA binding pocket, established the model of GA signaling pathway as shown in Figure 3-1. The binding of GA to GID1 induces the conformational changes of GID1 and its interaction with DELLA, which then subsequently promotes the conformational changes of DELLA and facilitates the recognition and polyubiquitination of DELLA

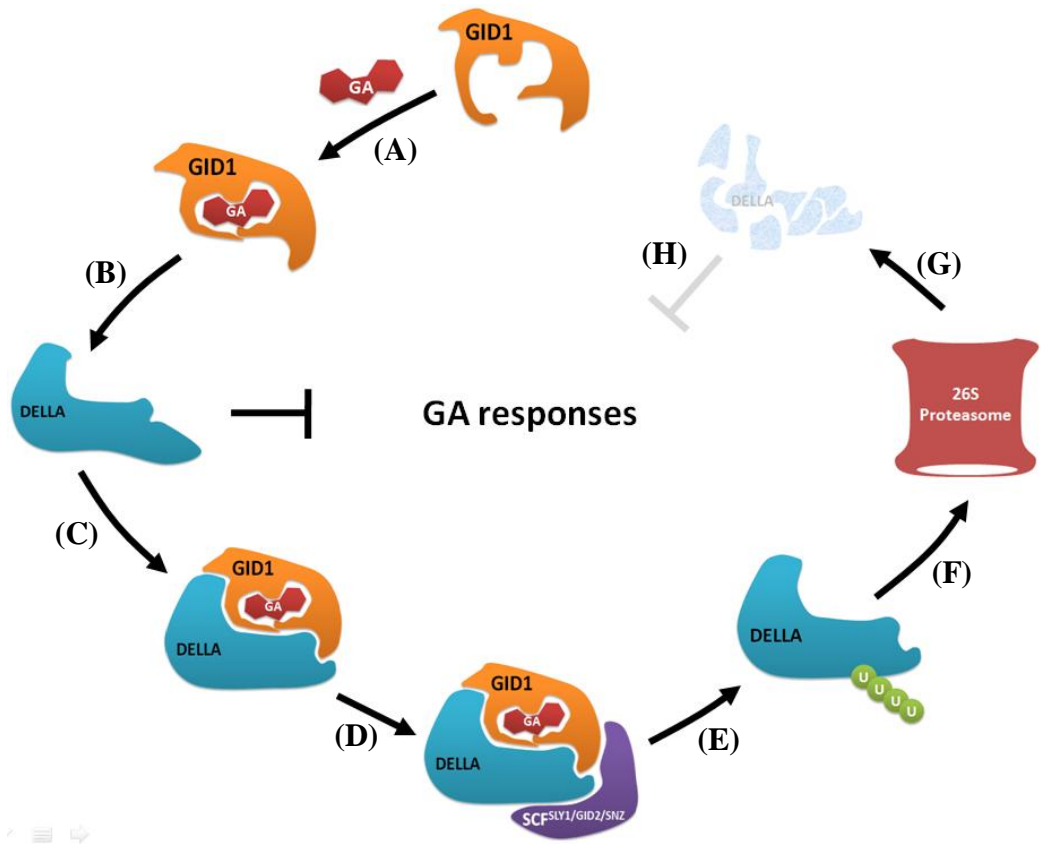


Figure 3-1 The model of GA signaling pathway

DELLA as a negative regulator suppresses the GA responses in plant. GA binds to the GID1 and induces lid closure of GID1 (A) then facilitates its interaction with DELLA (B, C) which leads to a surface on the GA-GID1-DELLA complex for recognition by SCF^{SLY1/GID2/SNZ} (D). Following ubiquitination by SCF^{SLY1/GID2/SNZ} (E), DELLA is degraded by 26S proteasome (F, G) and loses its inhibitory effects on plant (H) which induces the GA responses.

by SCF^{SLY1/GID2/SNZ}. Then DELLA is degraded by 26S proteasome and the GA responses being suppressed by DELLA start. In addition to the main elements mentioned above, the activity of DELLA is also regulated by some other factors such as SPY (SPYNDLY, O-linked N-acetylglucosamine transferases), which activates DELLA by GlcNAc-modification (Shimada *et al.* 2006), and EL1(EARLIER FLOWERING 1), which activates DELLA by phosphorylation (Dai and Xue 2010). DELLA proteins localize only in nucleus, which is consistent with their roles in regulating gene expression (Zentella *et al.* 2007, Hou *et al.* 2008, Gallego-Bartolomé *et al.* 2011). However, no canonical DNA-binding domain has been found yet in DELLA proteins. Increasing studies indicated that DELLAs play roles as significant regulators by binding to transcription factors and other regulators such as PIFs, which are important for photomorphogenesis (Feng *et al.* 2008, de Lucas *et al.* 2008), JAZ, which functions as a repressor in jasmonate (JA) signaling (Hou *et al.* 2010), SCL3, a positive regulator in GA signaling (Zhang *et al.* 2011, Heo *et al.* 2011) and BZR1, a transcription factor activated by BR signaling (Bai *et al.* 2012, Li *et al.* 2012, Gallego-Bartolomé *et al.* 2012).

GA and ABA play antagonistic roles in many aspects of plant growth and development such as germination, root elongation, floral transition and so on (Weiss and Ori 2007, Razem *et al.* 2006). Most of these functions of GA are negatively regulated by DELLA proteins (Harberd *et al.* 2009). Moreover, increasing studies indicate that DELLA is related to the plant responses to salt and osmotic stresses which are mainly regulated by ABA signaling. Quadruple DELLA mutant showed increasing sensitivity to salt compared to wild type (Achard *et al.* 2006). Salt induction of *ABI5* expression may require the initial induction of *RGL2* (Yuan *et al.* 2011).

Above all, DELLA protein interacts with many factors in other plant hormone signalings and mediates the plant growth and stress responses that shared with ABA signaling. In addition, former results in this thesis showed that PYL could function as

a modulator between ABA and GA signaling. Based on these results, I hypothesized that the crosstalk between ABA and GA may be induced by the interaction between PYL and DELLA to some extent (Figure 3-2).

3.2 Results

3.2.1 Finding of interactions between PYLs and DELLAs by yeast-two-hybrid assay

To verify the hypothesis, the yeast-two-hybrid (Y2H) assay was performed to prove the interactions between AtPYLs and AtDELLAs. Due to the transcription activation activity of DELLA in yeast, the full length cDNA of five *AtDELLAs* (*GAI*, *RGA*, *RGL1*, *RGL2* and *RGL3*) were constructed into pGADT7 plasmid separately. The full length cDNA of 12 *AtPYLs* (all the *PYL* members excluding *PYL8* and *PYL10*) were constructed into pGBKT7 individually. All 70 combinations between PYLs and DELLAs were transformed into AH109 and examined on SD-His, Ade medium with or without 50 μ M of ABA. The results (Table 3-1) showed that PYL5 interacted with GAI and RGL1. PYL6 showed clear and broader interactions with GAI and RGA than PYL5. PYL11 interacted with all five DELLAs. These interactions were all strictly dependent on ABA. PYL9 interacted with RGL1 independent of ABA. However, in contrast to the PYLs mentioned above, PYL3 interacted with GAI in the absence of ABA and this interaction was inhibited by the presence of ABA, which indicated that the mechanism of this interaction might differ from the interactions mediated by PYL5, PYL6, PYL11 and PYL9.

Further studies showed that both the inhibitory effect of ABA on the interaction between PYL3 and GAI and the promotion effect of ABA on the interaction between PYL5 and GAI were dependent on ABA concentration (Figure 3-3A). Pyrabactin (PYB) is a synthetic sulfonamide and inhibits germination and hypocotyl growth and induces stomatal closure in a similar pattern to ABA (Zhao *et al.* 2007, Park *et al.* 2009, Puli and Raghavendra 2012). PYB mimics ABA in promoting the interaction

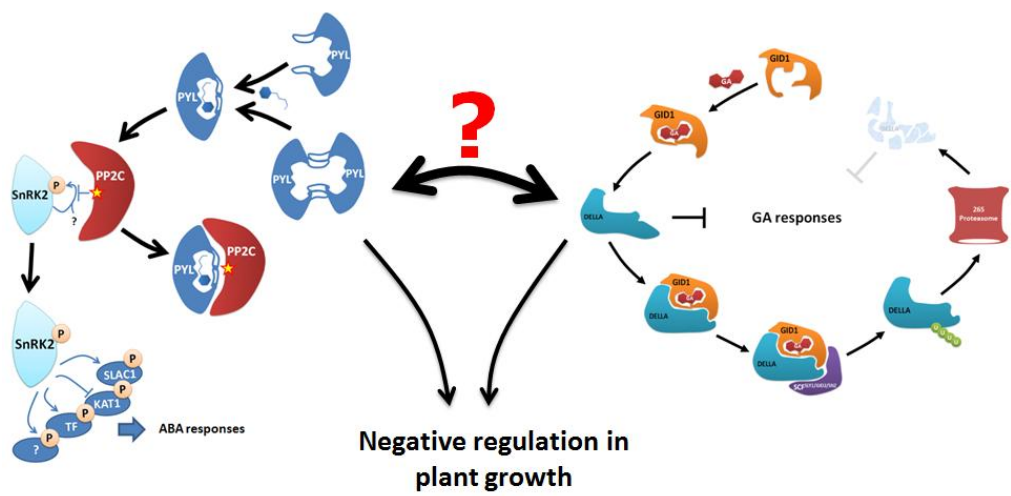


Figure 3-2 A hypothesis for interaction between ABA and GA signaling

BD	AD	DMSO	ABA 50 μ M	BD	AD	DMSO	ABA 50 μ M	BD	AD	DMSO	ABA 50 μ M
Vec	Vec	-	-	PYL4	Vec	-	-	PYL11	Vec	-	-
	GAI	-	-		GAI	-	-		GAI	+	+++
	RGA	-	-		RGA	-	-		RGA	-	+++
	RGL1	-	-		RGL1	-	-		RGL1	-	+++
	RGL2	-	-		RGL2	-	-		RGL2	-	+++
	RGL3	-	-		RGL3	-	-		RGL3	-	+++
PYR1	ABI1	-	-	PYL5	ABI1	-	+++	PYL12	ABI1	+++	+++
	Vec	-	-		Vec	-	-		Vec	-	-
	GAI	-	-		GAI	-	+++		GAI	-	+
	RGA	-	-		RGA	-	+		RGA	-	-
	RGL1	-	-		RGL1	-	+++		RGL1	+	+
	RGL2	-	-		RGL2	-	+		RGL2	-	-
PYL1	RGL3	-	-	PYL6	RGL3	-	-	PYL13	RGL3	-	-
	ABI1	-	+++		ABI1	+++	+++		ABI1	+++	+++
	Vec	-	-		Vec	-	-		Vec	-	-
	GAI	-	-		GAI	-	+++		GAI	-	-
	RGA	-	-		RGA	-	+++		RGA	-	-
	RGL1	+	+		RGL1	-	++		RGL1	-	-
PYL2	RGL2	-	-	PYL7	RGL2	-	+	PYL13	RGL2	-	-
	RGL3	-	-		RGL3	-	+		RGL3	-	-
	ABI1	+++	+++		ABI1	+++	+++		ABI1	+++	+++
	Vec	-	-		Vec	-	-		Vec	-	-
	GAI	-	+		GAI	-	-		GAI	-	-
	RGA	-	-		RGA	-	-		RGA	-	-
PYL3	RGL1	-	-	PYL9	RGL1	+	+	PYL13	RGL1	-	-
	RGL2	-	-		RGL2	-	-		RGL2	-	-
	RGL3	-	-		RGL3	-	-		RGL3	-	-
	ABI1	-	+++		ABI1	-	+++		ABI1	+++	+++
	Vec	-	-		Vec	-	-		Vec	-	-
	GAI	+++	-		GAI	-	-		GAI	-	-
PYL4	RGA	-	-	PYL10	RGA	-	-	PYL11	RGA	-	-
	RGL1	-	-		RGL1	++	++		RGL1	-	-
	RGL2	-	-		RGL2	-	-		RGL2	-	-
	RGL3	-	-		RGL3	-	-		RGL3	-	-
	ABI1	-	+++		ABI1	+++	+++		ABI1	+++	+++
	Vec	-	-		Vec	-	-		Vec	-	-

Table 3-1 Y2H screening of interactions between PYLs and DELLAs

The interactions between 12 AtPYLs (all PYLs in *Arabidopsis* excluding PYL8 and PYL10) and 5 AtDELLAs (GAI, RGA, RGL1, RGL2 and RGL3) were investigated. The interactions between PYLs and ABI1 played as control. -, no interaction. +, interaction. Vec stands for empty vector of pGADT7 or pGBKT7.

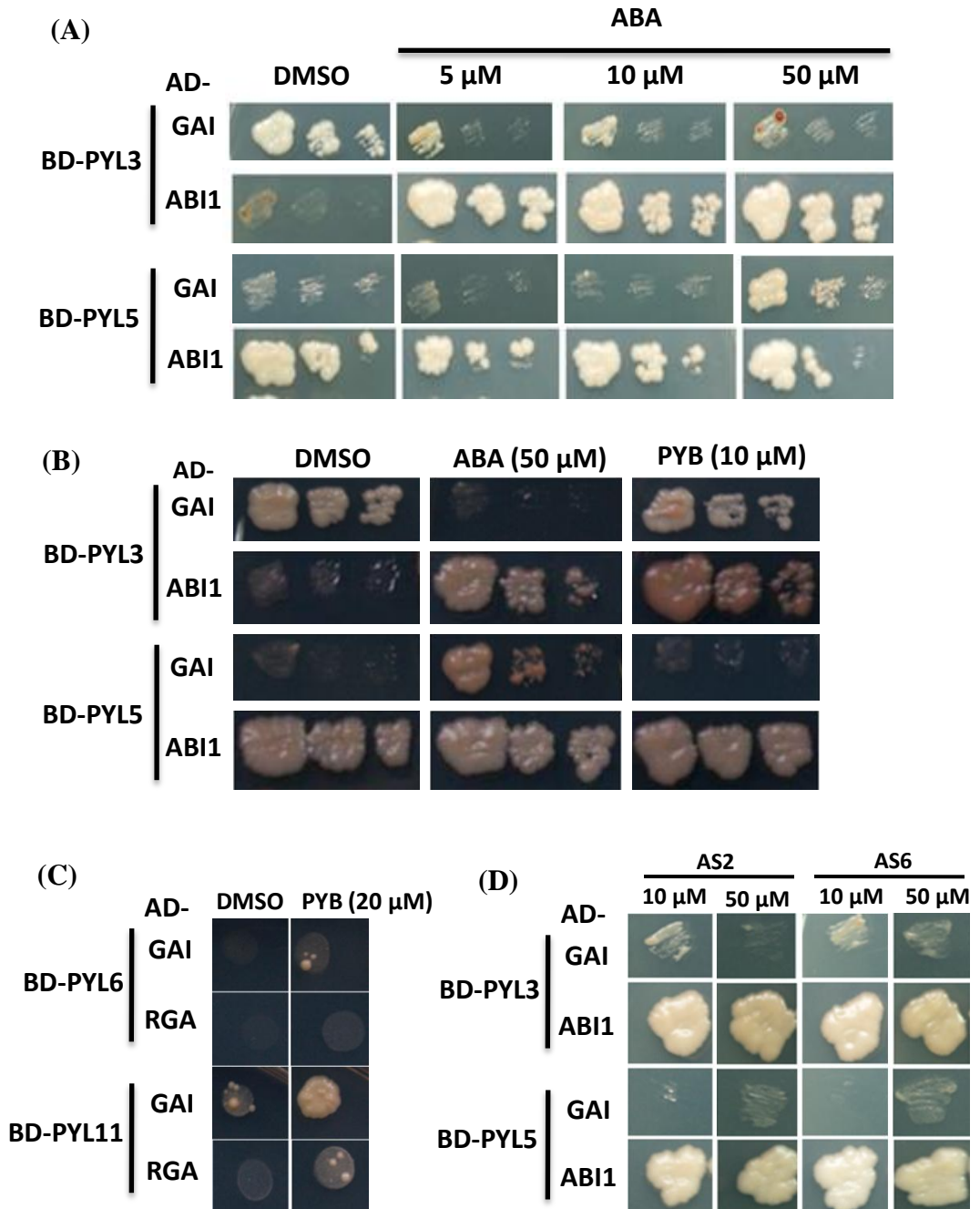


Figure 3-3 The ABA or agonist dependency of interactions between PYL and DELLA

The dependency of interactions between PYL3/PYL5 and GAI/ABI1 on ABA and its agonist and antagonist were determined in yeast two-hybrid system. (A) The effects of ABA concentration on interactions. (B, C) The effects of PYB on interactions (Figure C is extracted from the Figure 3-22). (D) The effects of AS2 and AS6 on interactions.

between PYLs (PYR1 and PYL1) and PP2Cs (HAB1, ABI1 and ABI2) (Park *et al.* 2009, Hao *et al.* 2010). However, PYB did not mimic the function of ABA in the interactions of PYL3 and PYL5 with GAI (Figure 3-3B). In addition no interaction was observed between PYL6 and GAI/RGA in the presence of PYB. However, PYB showed weak promotion effect on the interaction between PYL11 and GAI/RGA (Figure 3-3C). 3'-alkylsulfanilated ABA (AS_n, where n stands for the carbon number of an alkyl chain) is designed to develop an antagonist of PYLs. AS_n mimics ABA activity when $n < 4$ while it functions as an antagonist when $n > 4$ (Takuya *et al.* 2011, Sakae *et al.* 2012, Jun *et al.* 2013). The effect of AS2 (agonist) and AS6 (antagonist) in the interaction between PYL and DELLA were investigated. The AS2-dependent interactions between PYL3/5 and ABI1 as positive control were also detected. As expected, AS2 mimicked the activity of ABA and promotes the interactions between PYL3/PYL5 and ABI1 (Figure 3-3D). Surprisingly, AS6, being reported as an antagonist of ABA, also promoted these interactions in Y2H assay. For the interaction of PYL and DELLA, both AS2 and AS6 mimicked the ABA activity to inhibit the interaction between PYL3 and GAI. Moreover, the inhibitory effect of AS2 and AS6 on this interaction was even stronger than ABA by judging from the comparison of the yeasts growth on the medium containing these two compounds with that on the medium containing ABA. While none of these two compounds promoted the interaction between PYL5 and GAI. These complicated results indicate that these interactions are very sensitive to the conformational changes of PYL because several reports have reported that ABA and its agonist or antagonist induce the tiny changes in the structures of pocket or latch of PYL, and which should affect the PYL-PP2C binding (Yuan *et al.* 2010, Hao *et al.* 2010).

3.2.2 Confirmation of the interaction between PYL and DELLA in rice

In addition to *Arabidopsis* whether this interaction exists in rice was also investigated by Y2H assay. Although SLR1 is the only DELLA in rice and therefore

has been widely studied, there are still few reports of PYL in rice. To search for the potential PYL candidates from rice, AtPYL1 were submitted to the database of rice and accessed the sequence similarity to PYLs in blast. Eleven candidates with high homology with AtPYL were selected and aligned by MEGA5 (Tamura *et al.* 2011) (Figure 3-4). Among these rice *PYL* homologues, six candidates, named as *OsPYL1-6*, were constructed into pGBKT7 and served as BD in Y2H assay. SLR1 served as AD. The results from Y2H showed that two OsPYLs, OsPYL4 and OsPYL5 interact with SLR1 (Figure 3-5). The interaction of OsPYL4 with SLR1 was independent of ABA while OsPYL5 was dependent on ABA. These results showed that the interaction between PYL and DELLA not only exists in *Arabidopsis* (dicot), but also exists in rice (monocot).

3.2.3 Confirmation of the interaction between PYL and DELLA *in vivo* by BiFC

The bimolecular fluorescence complementation (BiFC) of split enhanced YFP (EYFP) was used to confirm the interactions between PYLs and DELLAs *in vivo*. PYL3 and PYL5, which interact with GAI and show contrary result of interaction in response to ABA, were constructed into vectors and transiently transformed into protoplasts of *Arabidopsis*. The result showed that the interactions between PYLs and DELLAs were observed in the nucleus (Figure 3-6), which was consistent with the localization of DELLA (Silverstone *et al.* 2001). However, both nEYFP-PYL3 and cEYFP-PYL5 interacted with GAI-nEYFP each other constantly independent of exogenous ABA treatment (Table 3-2). This result was not consistent with the results in the Y2H experiment showing that the interaction of PYL3 with GAI was inhibited by ABA while the interaction of PYL5 with GAI was dependent on ABA. The interaction of PYL5 with GAI, which was independent of exogenous ABA, could be explained by the existence of ABA in protoplast used in this experiment, while the ABA independent interaction of PYL3 with GAI is still a mystery. These results

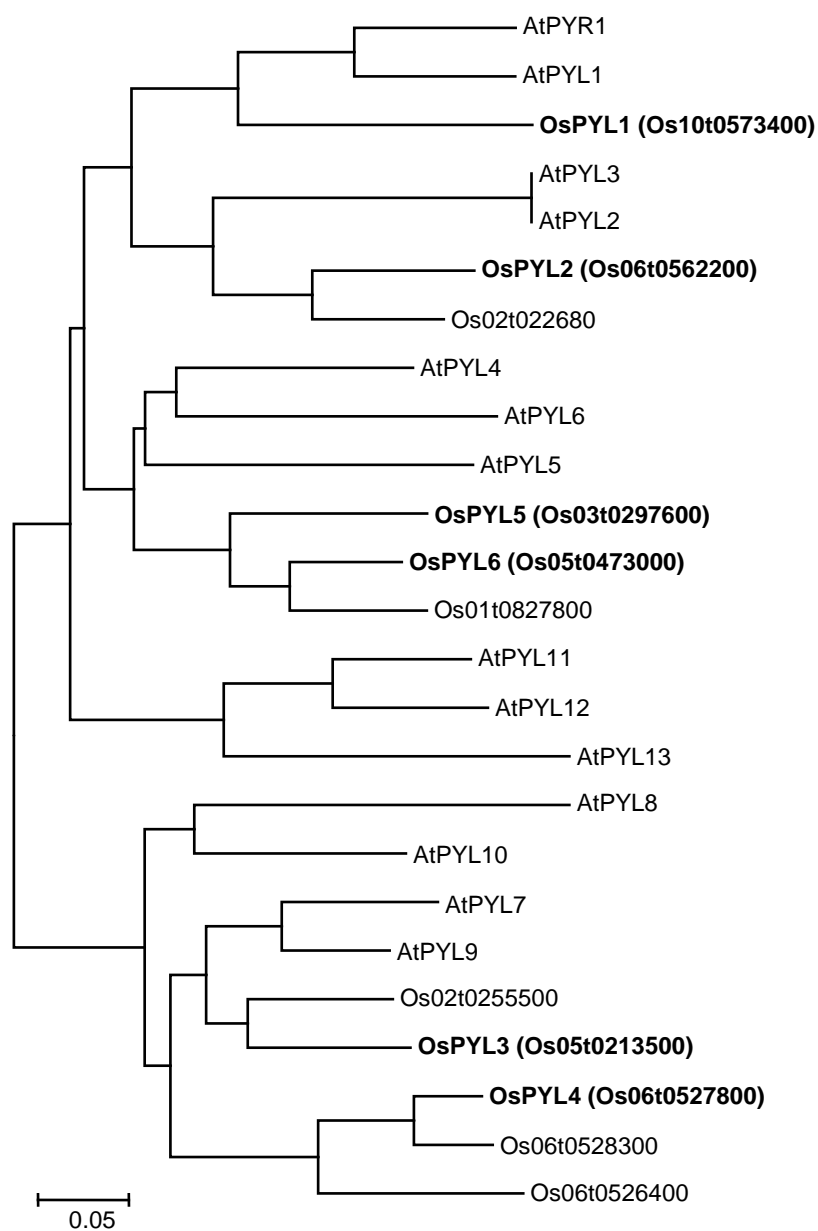
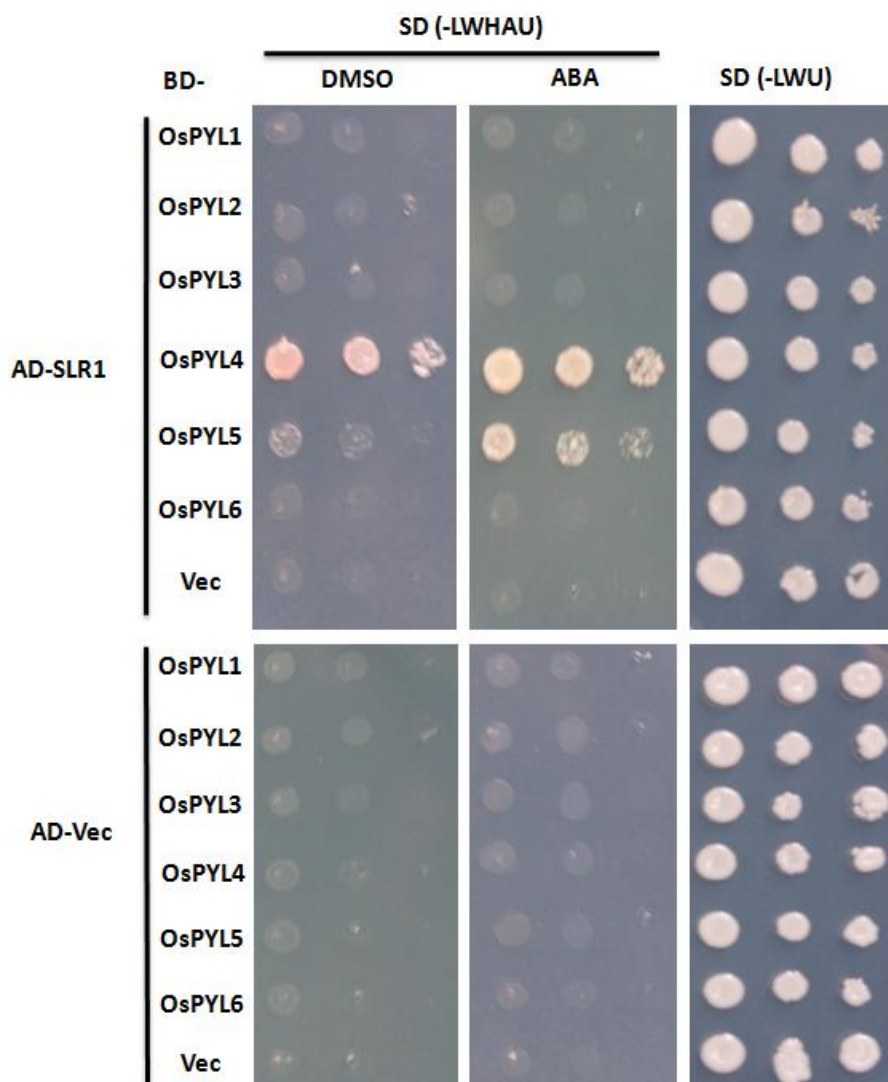


Figure 3-4 Phylogenetic tree of AtPYLs and rice orthologue OsPYLs

Phylogenetic tree was analyzed by MEGA 5 and tested by neighbour-joining method. Six of these OsPYLs were selected and named as OsPYL1-6 indicated in bold. These six OsPYLs were studied in the further experiment.



3-5 Screening of interactions between OsPYLs and SLR1 in rice by Y2H assay

OsPYLs served as BD and SLR1 served as AD were transformed into yeast AH109. The screening was performed on SD (-LWHAU) with or without ABA. AD-Vec and BD-Vec served as negative control. The yeasts were diluted by three grades (1, 1/10, 1/100 from left to right) and loaded on SD medium. OsPYL4 showed interaction with SLR1 independent of ABA. OsPYL5 showed interaction with SLR1 dependent on ABA. While both BD-Vec and AD-Vec showed no interaction with any AD or BD partners.

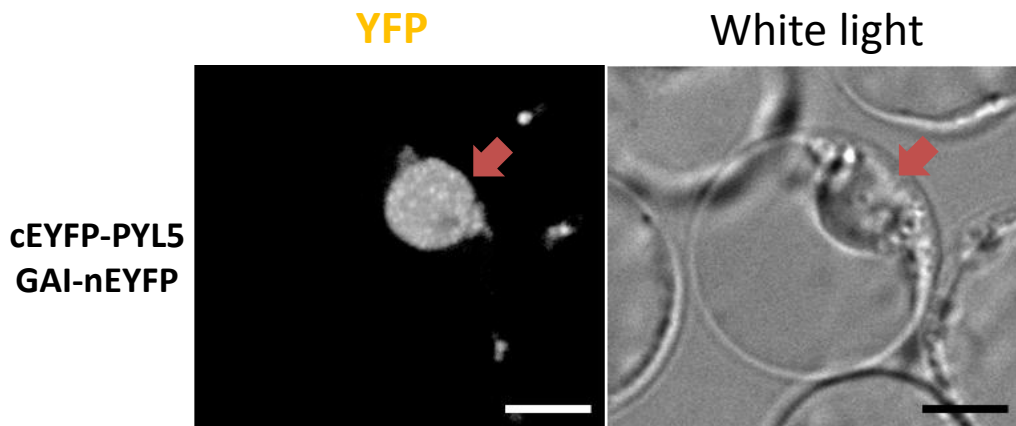


Figure 3-6 Observation of interaction between PYL and DELLA in plant protoplast by BiFC

All combinations of PYL5 and GAI were transiently transformed into Arabidopsis suspension culture and observed by an LSM710 confocal microscope. The interaction between PYL5 and GAI resided in nucleus.

Table 3-2

ABA (50 μM)	nEYFP-PYL3		ABA (50 μM)	cEYFP-PYL5	
	-	+		-	+
GAI-cEYFP	+	+	GAI-nEYFP	+	+

Table 3-2 Interactions between PYL3/PYL5 and GAI from BiFC assay

Arabidopsis suspension culture was transiently transformed with all combinations of PYL3/PYL5 and GAI with complementary fusions and cultured in the presence or absence of ABA

validated the interaction in plant *in vivo* even though the mechanism of interaction in plant may differ from the one in yeast.

3.2.4 Confirmation of the interaction between PYL and DELLA *in vitro* by pull down assay

Next I further investigated the interaction between PYL and DELLA *in vitro* by pull down assay. The *PYL3*, *PYL5* and *PYL6* were constructed into pGEX-6P-1 vector, which produces GST tagged protein, and expressed in *E. coli* (Rosetta DE3, Novagen) (Figure 3-7). To check whether the PYLs function normally, Trx-His tagged ABI1 was expressed by pET-32a vector in *E. coli* (Rosetta DE3) (Figure 3-8) and the pull down between GST-PYLs and Trx-His-ABI1 was tested. The result showed that GST-PYL3 interacted with Trx-His-ABI1 in an ABA dependent manner, GST-PYL5 and GST-PYL6 interacted with Trx-His-ABI1 in an ABA independent manner (Figure 3-9). These results were consistent with the former results and indicated that all GST-PYLs expressed *in vivo* functioned normally.

The DELLAs (GAI, RGA and RGL1) fused with Trx-His-tag showed poor solubility no matter how many trials for searching for better expression conditions (Figure 3-10). In consideration of the instability of DELLA proteins, the pull down assays of Trx-His-GAI with GST-PYL3 or GST-PYL5 were done without protein purification and enrichment. The result showed that both of GST-PYL3 and GST-PYL5 pulled down the Trx-His-GAI and the interaction was independent of ABA (Figure 3-11). However, the interaction between GST-PYL3 and Trx-His-GAI was strengthened by ABA, which was opposite to the results from Y2H.

To confirm these results, I further tried to search for a better vector that can increase the solubility of DELLA. Finally, pMAL-c2X, which fuses a maltose binding protein (MBP) to the target protein, gave a perfect solubility to DELLA proteins (Figure 3-12). GID1s fused with Trx-His tag were expressed to be a positive

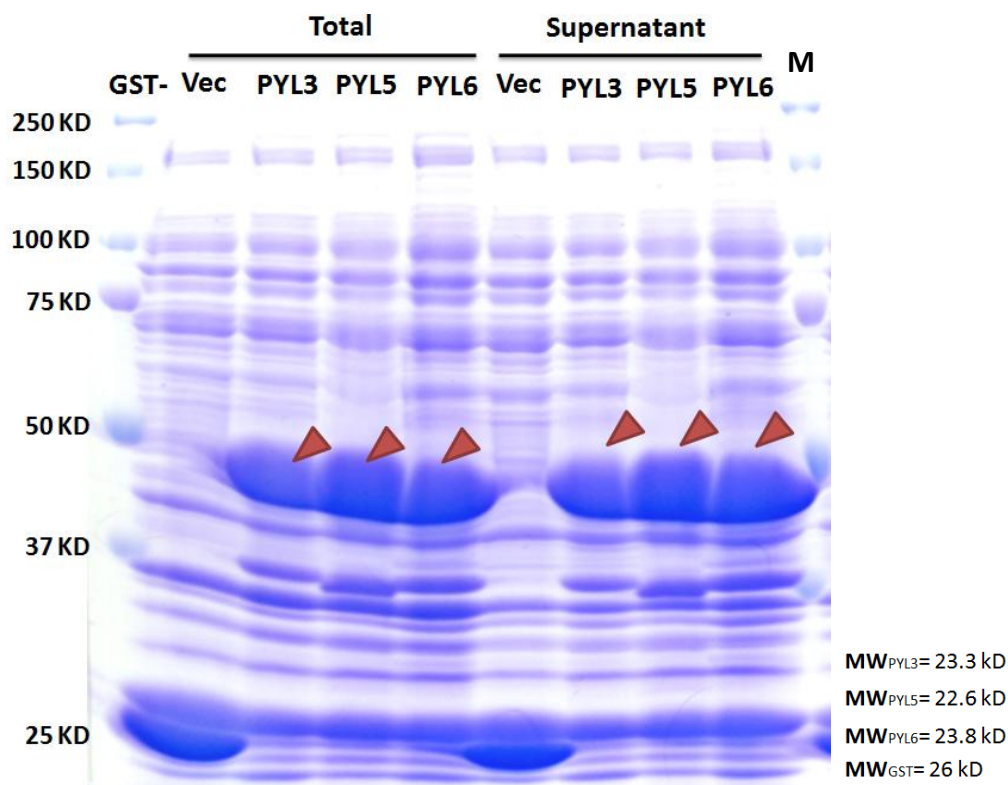


Figure 3-7 Expression of PYL3, PYL5 and PYL6 proteins fused with GST tag in vitro.

PYL3, PYL5 and PYL6 were fused with GST-tag by pGEX-6P-1 and expressed by Rosetta DE3. Expressed proteins were detected by SDS-PAGE and CBB staining. vec stands GST tag expressed by empty vector. M stands for marker. All these proteins showed good solubility (arrow heads).

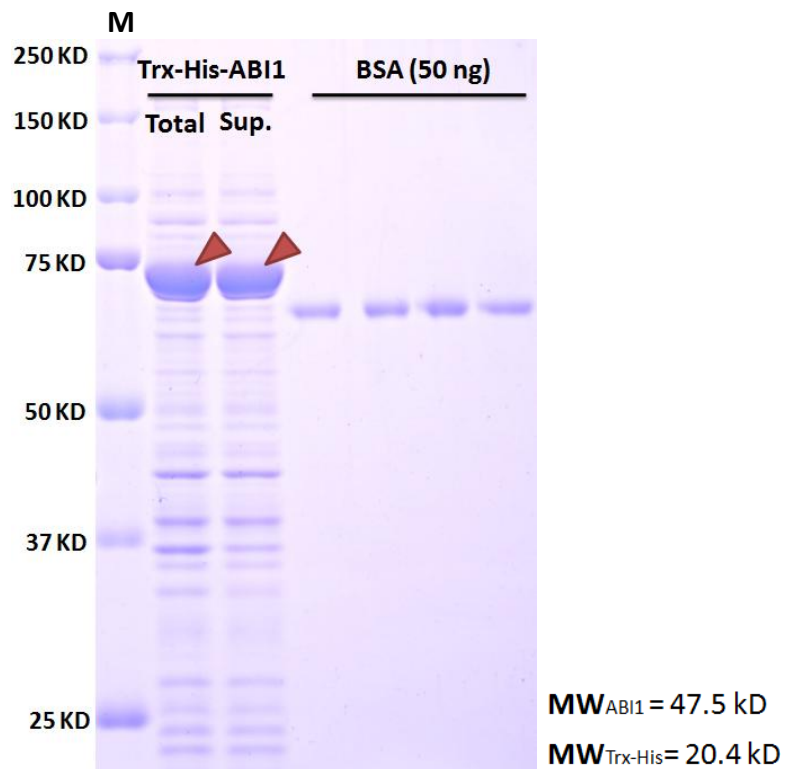


Figure 3-8 Expression of ABI1 proteins fused with Trx-His-tag *in vitro*
 ABI1 was fused with Trx-His-tag by pET-32a and expressed by Rosetta DE3. SDS-PAGE and detection by CBB staining indicated good solubility of ABI fusion protein. Sup. stands for supernatant of expressed protein solution after centrifugation. M stands for marker.

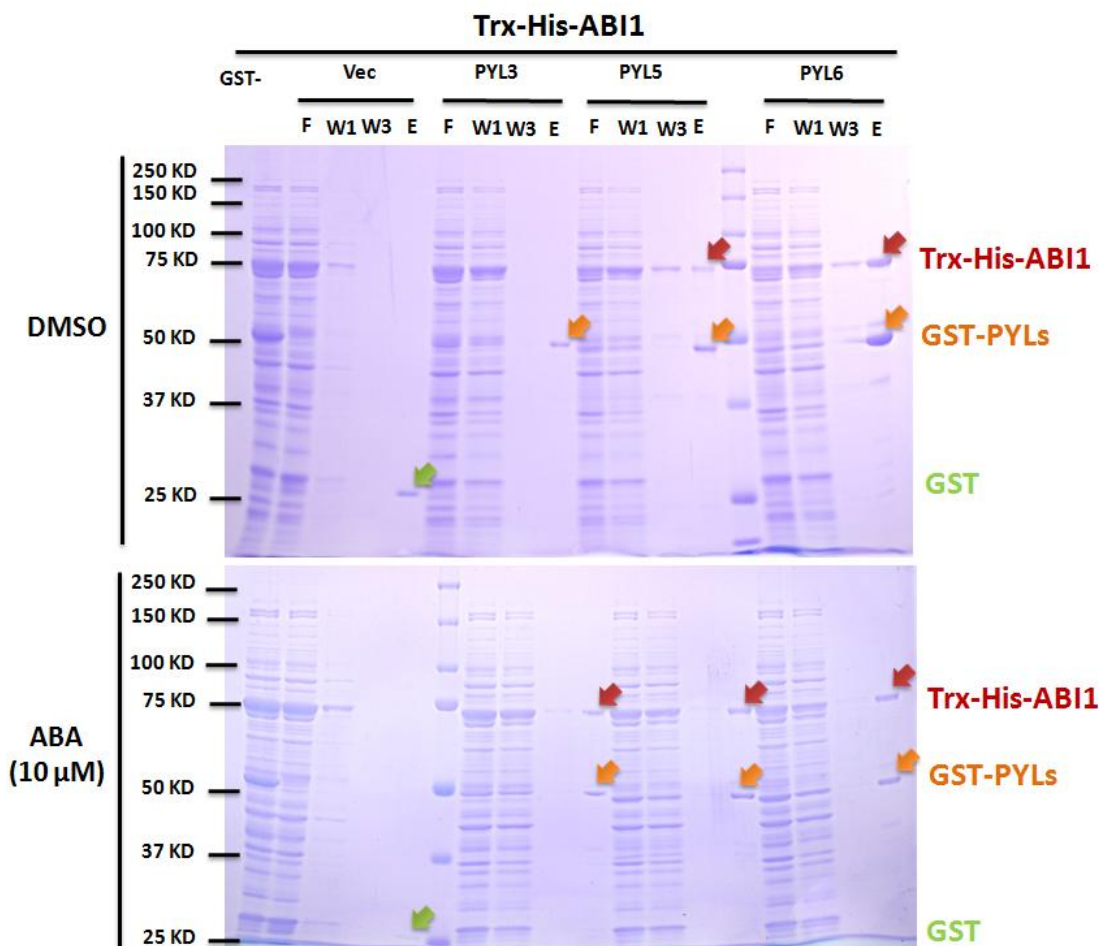


Figure 3-9 The pull down assay by Glutathione sepharose 4B beads

The crude proteins of GST-PYLs/vec and Trx-His-ABI1 were mixed separately and followed by incubation with or without ABA (10 μ M) at room temperature for 90 min. The reaction buffer was loaded into the column prepared by glutathione sepharose 4B beads, which immobilized GST-tag and then performed the standard procedure of gravity column method. After collection of the flow through (F), the column was washed by 10 bed volumes of 1XPBS buffer for three times and collected (W1, W2 and W3). Then the column was incubated with elution buffer for 15 min. Finally the elution (E) together with flow through and wash were collected and tested by SDS-PAGE. The gel was stained by CBB. The upper gel showed that both GST-PYL5 and GST-PYL6 pulled down the ABI1 in the absence of ABA while GST-vec and GST-PYL3 did not. The gel below revealed that all GST-PYLs showing here pulled down the Trx-His-ABI1 in the presence of ABA excluding the GST-vec.

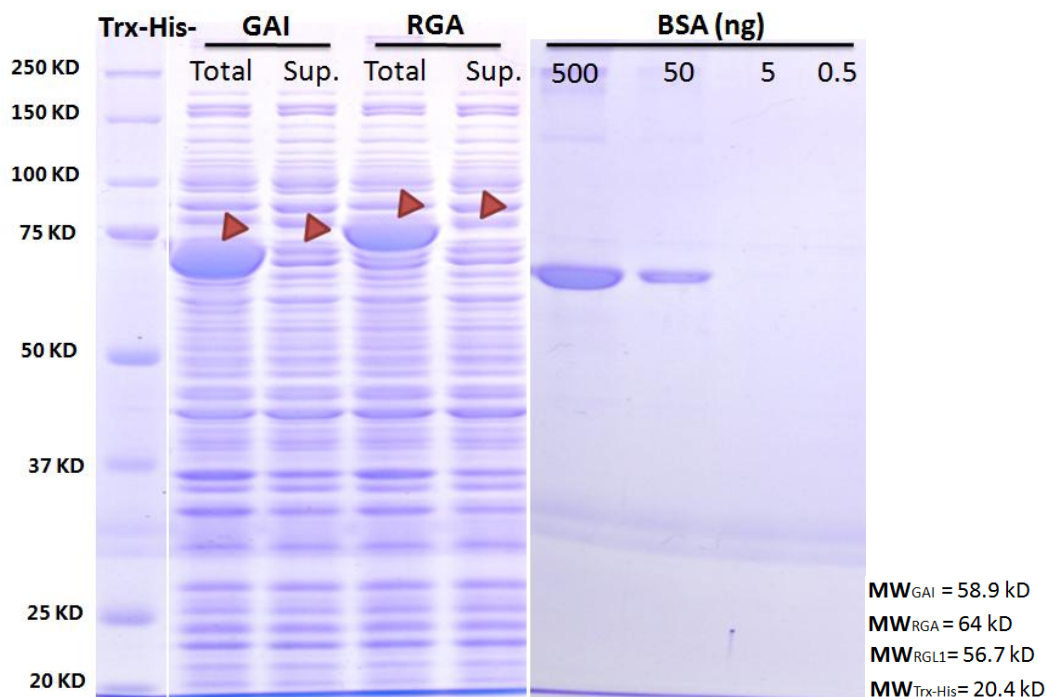


Figure 3-10 Expression of DELLAs fused with Trx-His-tag in vitro.

The full length cDNA of *GAI* and *RGA* were constructed into pET-32a and expressed by Rosetta DE3 producing Trx-His fusion proteins. Expressed proteins were detected by SDS-PAGE and CBB staining. Both Trx-His-GAI and Trx-His-RGA showed poor solubility since few proteins were detected in the supernatant (Sup.) compared to total solution (arrow heads).

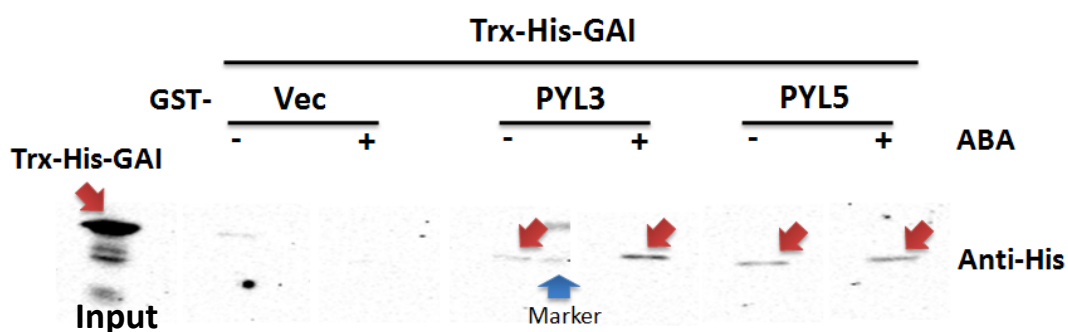


Figure 3-11 pull down assays between GST-PYLs and Trx-His-GAI

The crude proteins of GST-PYLs/vec and Trx-His-GAI were mixed separately and followed by incubation with or without ABA (10 μ M) at 4°C for 90 min. The reaction buffer was loaded into the column prepared by glutathione sepharose 4B beads with affinity for GST tag and incubated at 4°C for 60 min. The column was washed by 10 bed volumes of 1XPBS buffer for three times. Then the column was incubated with elution buffer at 4°C for 30 min. Finally the elution was detected by SDS-PAGE and western blotting with His primary antibody and mouse second antibody. The result indicated that both GST-PYL3 and PYL5 pulled down the Trx-His-GAI successfully (red arrows) although the interactions were weak. These two interactions showed no dependency on ABA. The unspecific bands in the input lane may be degraded Trx-His-GAI since the instability of DELLA.

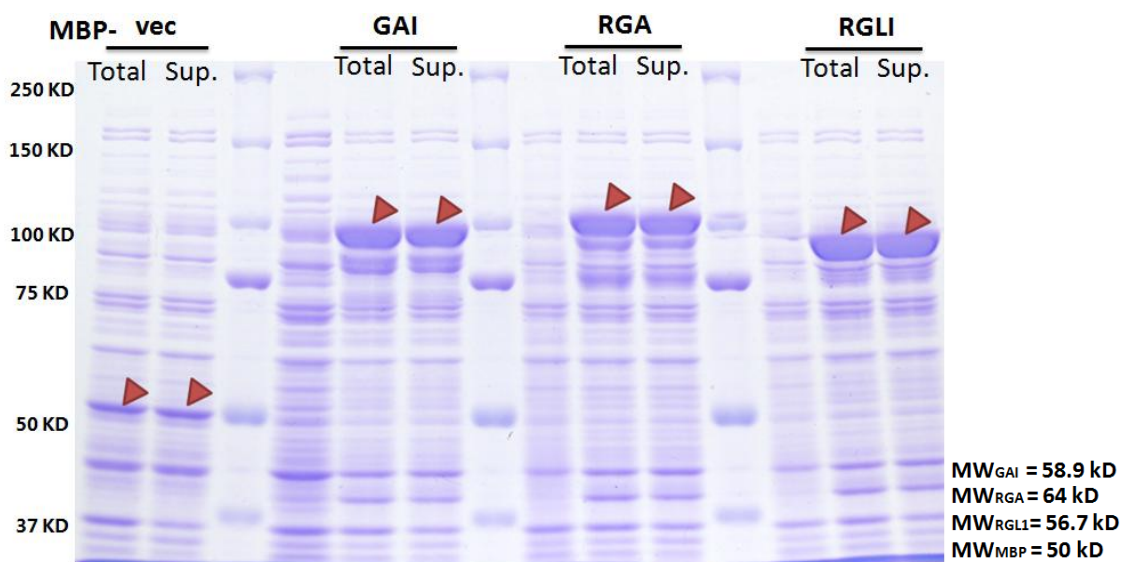


Figure 3-12 Expression of DELLAs fused with MBP-tag *in vitro*

The full length cDNA of *GAI*, *RGA*, and *RGL1* were constructed into pMAL-c2X and expressed by Rosetta DE3 producing MBP-fusion DELLAs. Expressed proteins were detected by SDS-PAGE and CBB staining. All MBP-DELLAs fusion showed good solubility.

control of MBP-DELLAs (Figure 3-13). The Trx-His-GID1a were pulled down by MBP-DELLAs GA dependently (Figure 3-14), which indicated the right conformation of MBP-DELLA. Then the pull down assays between GST-PYLs and MBP-DELLAs were performed. As in the case of the former results, GST-PYL3 and GST-PYL6 (former result was from GST-PYL5) were pulled down by MBP-GAI independent of ABA. Furthermore, ABA promoted the interaction between GST-PYL3 and MBP-GAI (Figure 3-15).

The interactions between PYLs and DELLAs have been verified by Y2H, BiFC and pull down assays but there still remains inconsistency in the ABA dependency for the interaction.

3.2.5 Investigation of the mechanisms of interactions between PYLs and DELLAs

After the confirmation of the interactions between PYLs and DELLAs, next we focused on uncovering the mechanisms of these interactions and expected to understand physiological meanings of these interactions.

Investigation of important amino acids in PYL for the interaction with DELLA by site mutation

It is known that the conformational changes of PYLs induced by ABA are important for PYLs' interaction with PP2C. So I investigated whether these changes are also necessary for the interaction between PYL and DELLA. It was reported that E98 and L91 were two important amino acids of PYL for the interaction with PP2C (Melcher *et al.* 2009). Mutation of E98A in PYL2 caused loss of both ABA and HAB1 binding activity. Mutation of L91A induced much more decreasing in HAB1 binding activity than E98A even though the ABA binding activity was not lost seriously (Figure 3-16) (Melcher *et al.* 2009). E98 and L91 are conservative in all 14 PYLs. I checked whether these two sites were also significant for the interaction

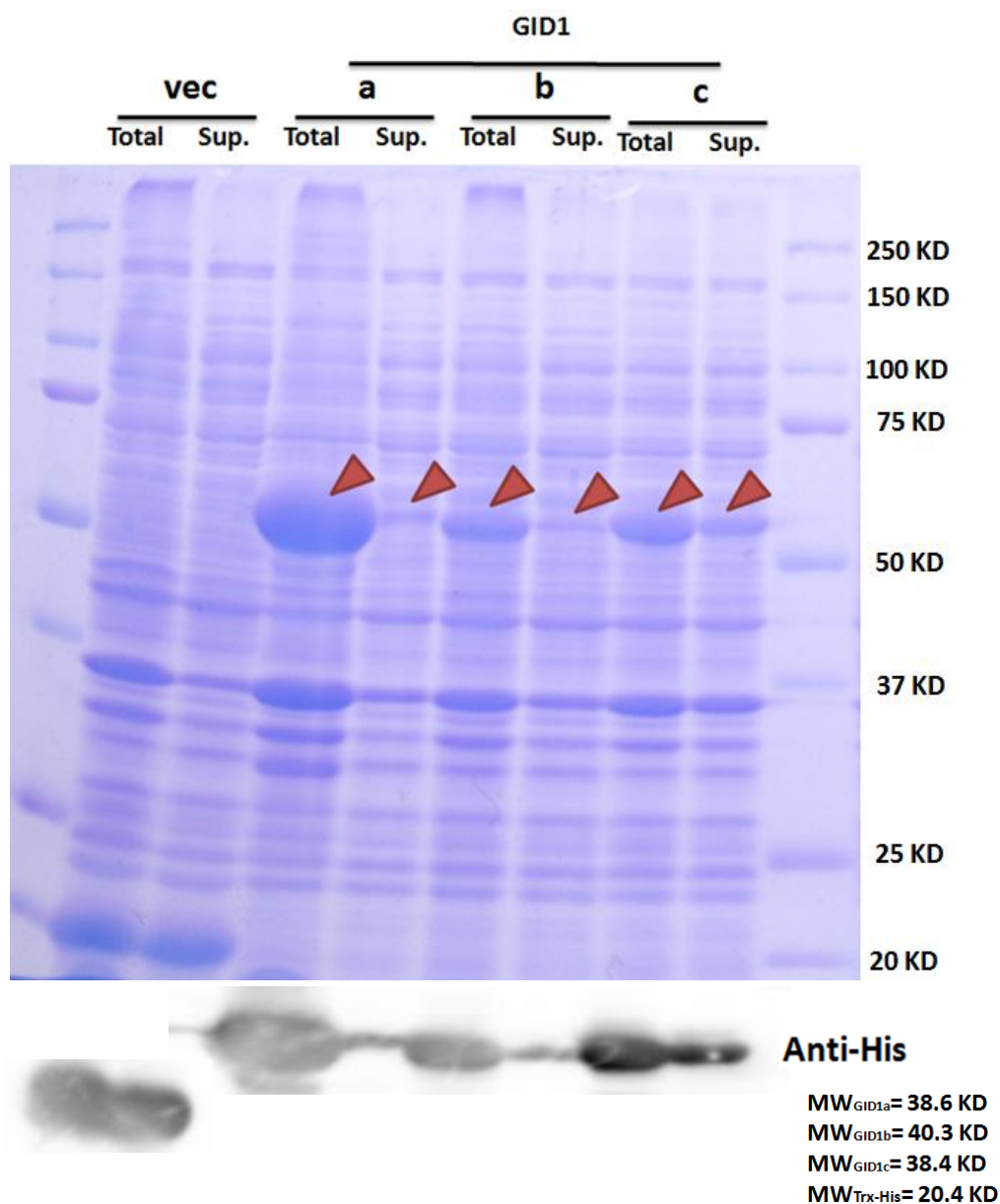


Figure 3-13 Expression of GID1s fused with Trx-His-tag *in vitro*

GID1s (GID1a, GID1b and GID1c) fused with Trx-His-tag were expressed by Rosetta DE3. Expressed proteins were detected by CBB staining and western blotting with His antibody. All MBP-DELLAs fusion showed good solubility (arrow heads).

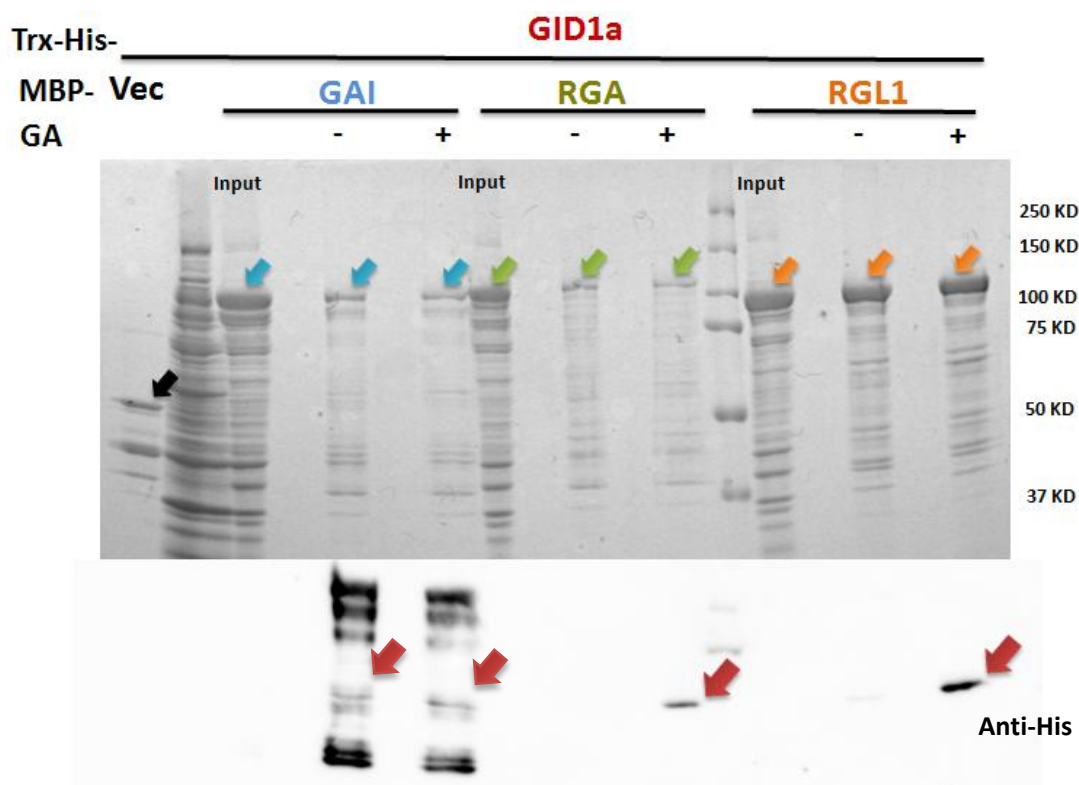


Figure 3-14 pull down assays between MBP-DELLAs and Trx-His-GID1a

MBP-DELLAs were immobilized by incubation with Amylose resin, which possessed affinity for MBP-tag, in the presence or absence of GA₄ (10 μM) at 4°C for 90 min. Trx-His-GID1a was added to the reaction buffer from last step and followed by incubation with resin at 4°C for 60 min. Then the resin was washed by 10 bed volumes of column buffer (refer to method) for three times and incubated with elution buffer at 4°C for 30 min. Finally the elution was tested by SDS-PAGE and both CBB and western blotting with His antibody. The interactions between MBP-RGA/RGL1 and Trx-His-GID1a were strictly dependent on GA. While the interaction between MBP-GAI and Trx-His-GID1a was not dependent on GA.

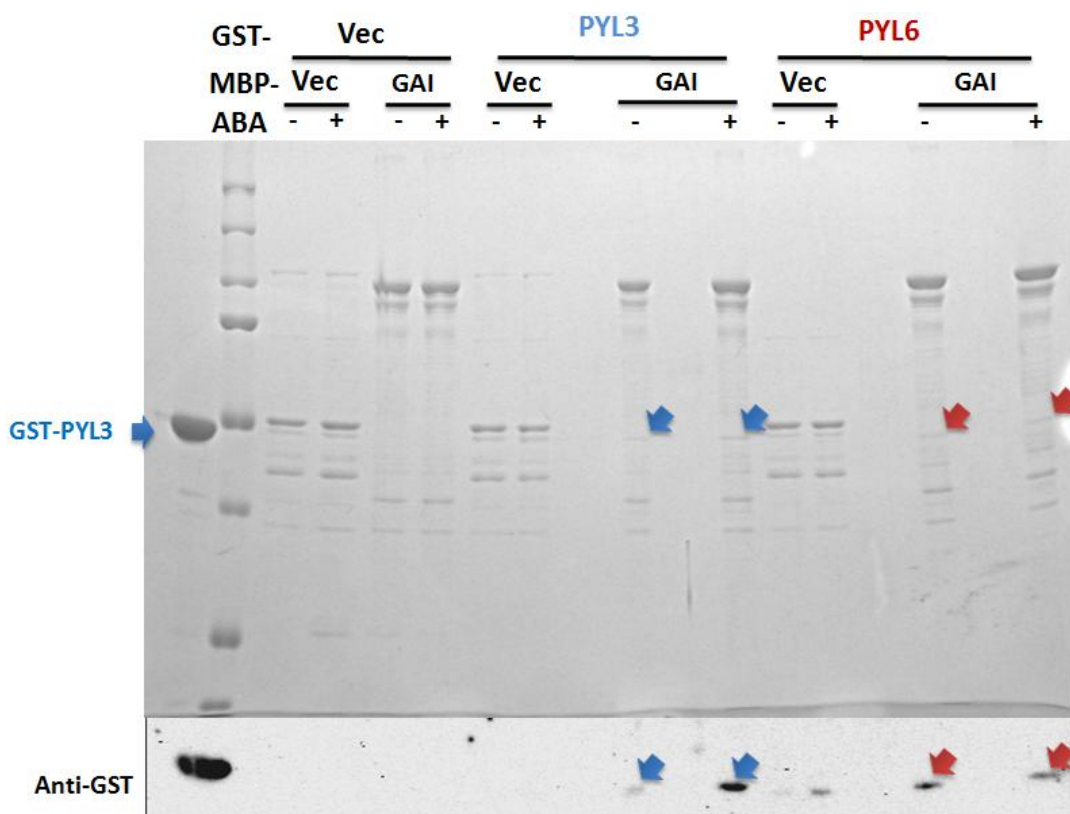


Figure 3-15 pull down assays between MBP-DELLAs and GST-PYLs

GST-PYLs and MBP-DELLAs were individually incubated at 4°C for 120 min containing ABA or not. Incubation buffer was then added into amylose resin, which can immobilize MBP-DELLAs, and followed by incubation on ice for 30min. Then the resin was washed by column buffer for three times and then incubated with elution buffer at 4°C for 15 min. Finally the elution was tested by SDS-PAGE and both CBB and western blotting with GST antibody. The interactions between GST-PYL3/PYL6 and MBP-GAI were observed (blue or red arrows, respectively).

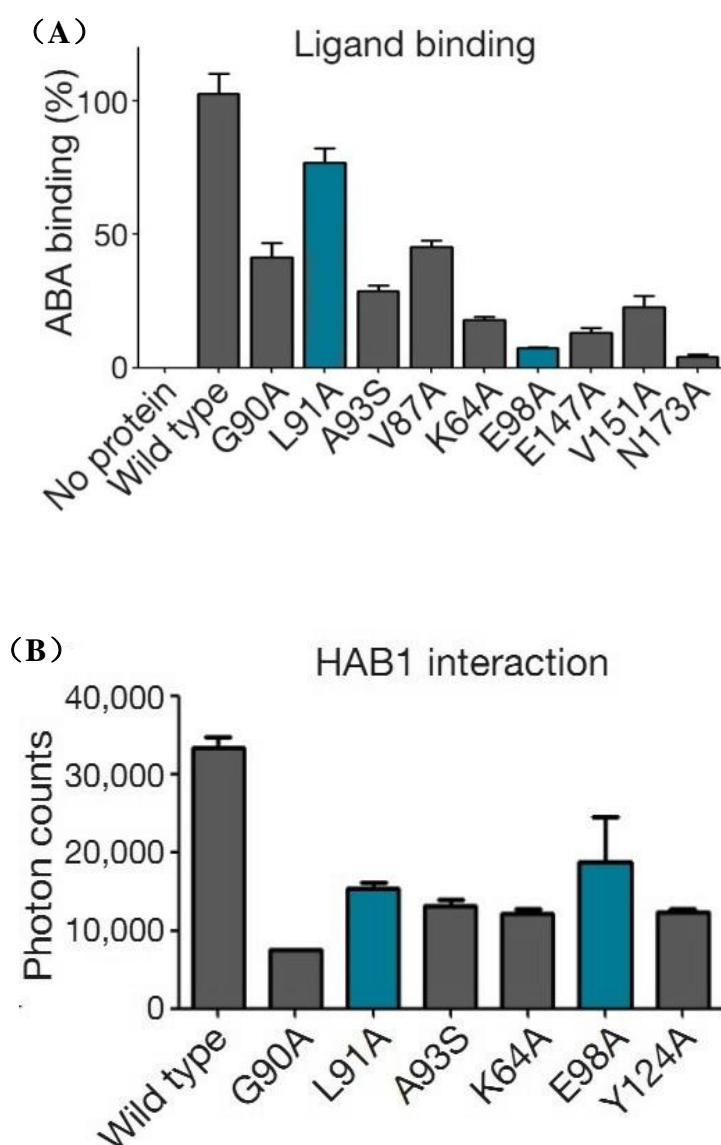


Figure 3-16 Site directed mutagenesis in PYL2

Single amino acid mutation in PYL2 affect the ABA binding activity and the ability for binding to PP2C (HAB1) as showing above. Mutation of E98A in PYL2 impairs both activities of binding to ABA (A) and HAB1 (B). Mutation of L91A in PYL2 decreases the activity of binding to HAB1 (B) although the ABA binding activity decreases moderately. (Figures are extracted and modified from Melcher *et al.*, 2009)

between PYL and DELLA by constructing site mutation in E98 and L91 in PYL3 and PYL5 respectively and detecting their interactions with GAI in Y2H assay. Prior to this experiment, I investigated the interactions between mutants of PYL3/PYL5 and ABI1 and confirmed the reproducibility (Figure 3-17A). Mutation of E98A, which seriously decreased ABA binding activity, disabled the interactions between PYL3/PYL5 and ABI1. However mutation of L91A in either PYL3 or PYL5 did not show the same result as E98A. For the interaction with GAI (Figure 3-17B), mutation of either E98A or L91A in PYL3 diminished the inhibitory effect of ABA (50 μ M) on the interaction with GAI compared to that of WT and even promoted the interaction. On the contrary, mutation of either E98A or L91A in PYL5 impaired the interaction with GAI in the presence of ABA (50 μ M). These results not only show that L91 and E98 are also important sites of PYL for interaction with DELLA, but also further confirmed that the mechanism of interaction of PYL3 with GAI and the interaction of PYL5 with GAI are different.

Recently a structural study showed that PYL3 is a unique type in PYL family by forming *trans*-homodimer during the dissociation induced by ABA (Zhang *et al.* 2012). It is reported that by binding to ABA, apo-PYL3, existing as *cis*-homodimer, undergoes conformational changes and forms a *trans*-homodimer, which more easily dissociates into monomer (Figure 3-18). S195 is a specific site in PYL3. Its mutation (S195L) in PYL3 stabilizes the *cis*-homodimer and prevents the dissociation even in the presence of ABA to some extent. In view of the result that ABA promotes the dissociation of dimeric PYLs into monomer, I hypothesized that the inhibitory effect of ABA on the interaction between PYL3 and GAI may come from its induction of PYL3 dissociation (Figure 3-19A). Thus I constructed the S195L mutation in PYL3 with the anticipation that this mutation will improve the interaction with GAI in Y2H assay. Instead of improving the interaction as expected, the S195L mutation canceled the interaction of PYL3 with GAI. However, S195L mutation did not affect the

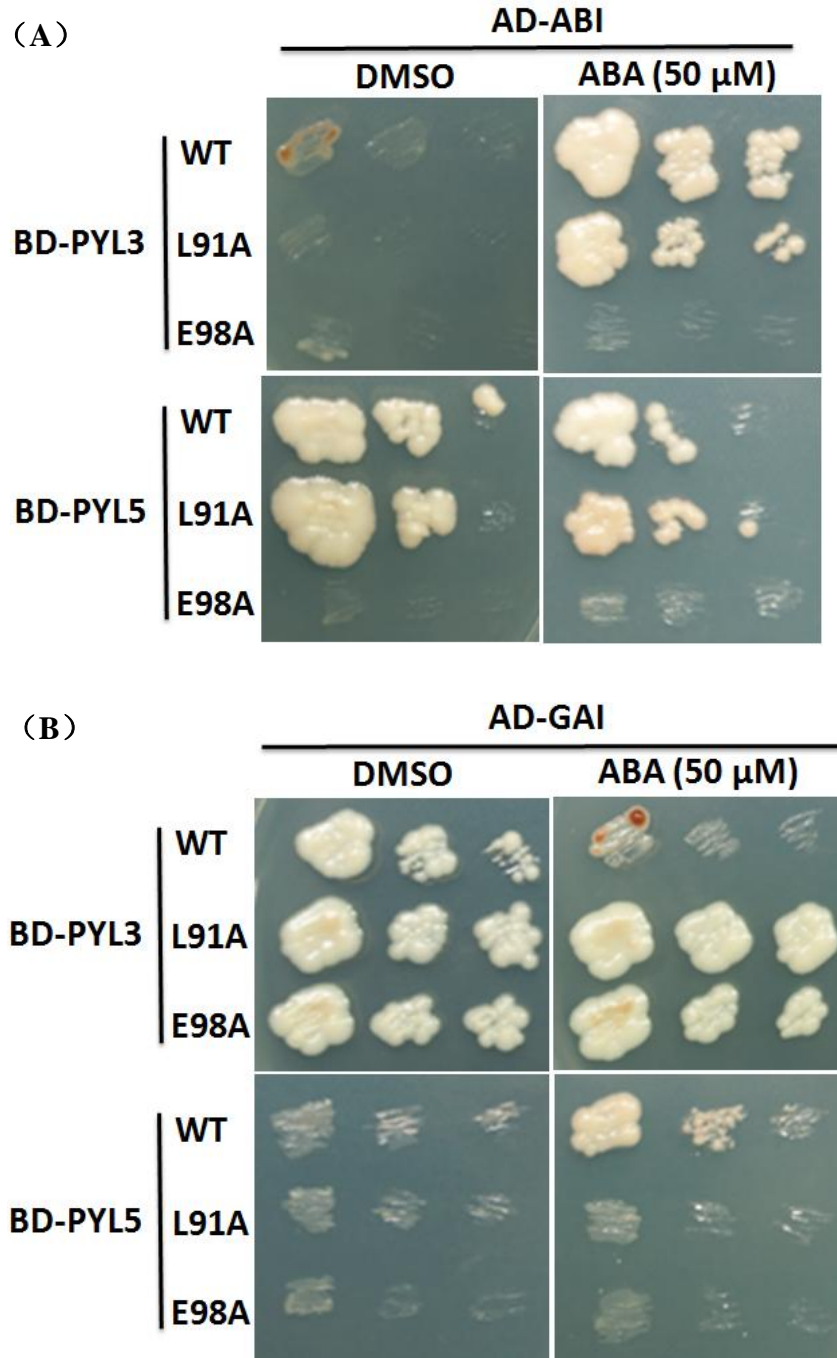


Figure 3-17 Y2H results from site directed mutagenesis in PYL3 and PYL5
 PYL3/PYL5 (WT) and their mutations served as BD. GAI and ABI1 served as AD. The yeasts containing different combinations of AD and BD as indicated were sprayed on SD (-LWHAU) medium with or without ABA. The affects of L91A and E98A on interactions of PYL3/PYL5 with ABI1 (A) and GAI (B) were investigated.

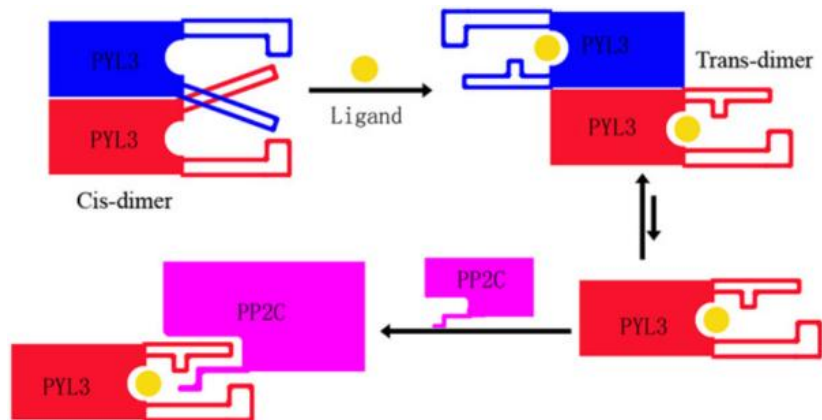


Figure 3-18 The dissociation mechanism of PYL3 upon binding to ABA
 By binding to ligand (ABA), the apo-PYL3 cis-homodimer rotates and forms a trans-homodimer. In turn, the trans-homodimer dissociates to monomer more easily and binds to PP2C more conveniently than the cis-homodimer. The process of transformation from cis-dimer to trans-dimer is prevented by mutation at S195 site, which then decreases the dissociation constant of homodimer. (Figure is extracted from Zhang, X., et al., 2012)

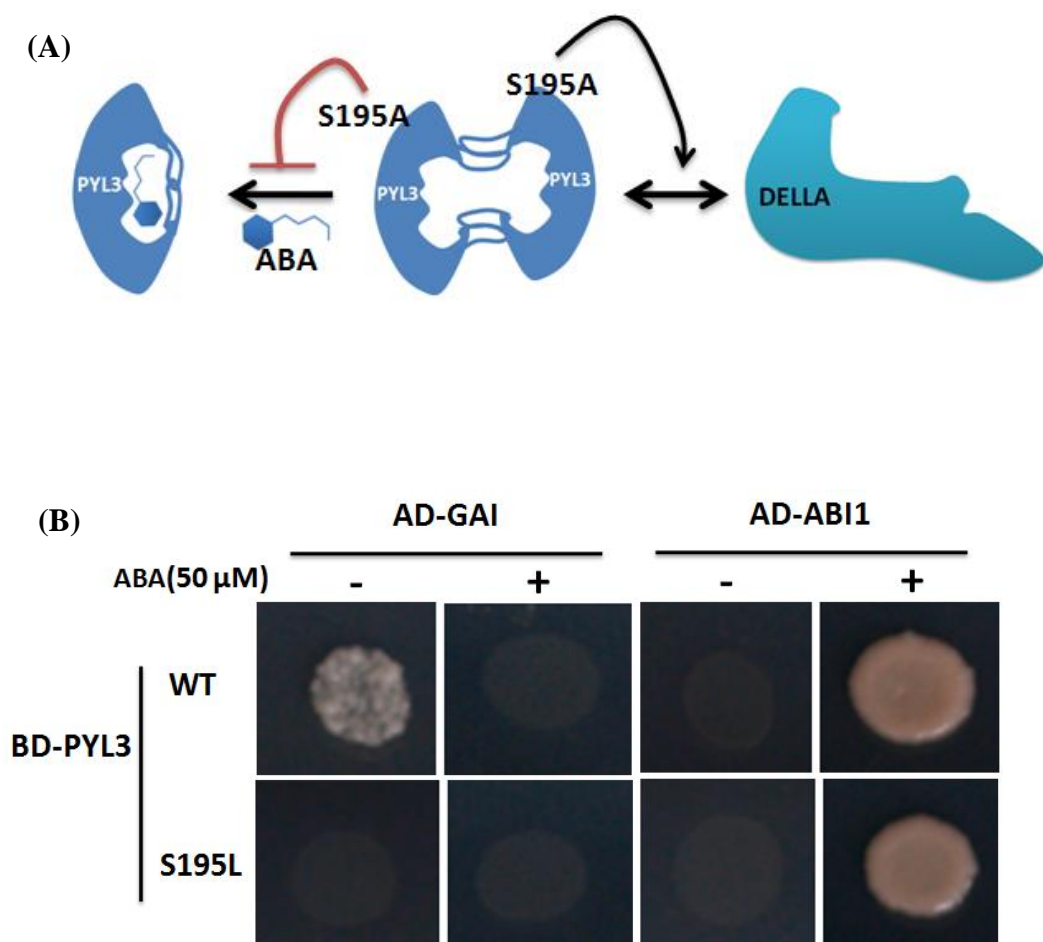


Figure 3-19 Investigation of interaction mechanism between PYL3 and GAI

(A) A postulated schematic for the mechanism of interaction between PYL3 and GAI. The dimeric structure could be necessary for this interaction since the ABA, which induces the dissociation of dimer, prevents the interaction. Site directed mutagenesis at S195 that decreases the dissociation constant of dimer and increases its stability was introduced to elucidate the hypothesis.

(B) The interactions of PYL3 (WT) and its mutation type (S195L) with GAI and ABI1 were detected by Y2H assay on SD (-LWHAU) with or without ABA treatment.

interaction of PYL3 with ABI1 (Figure 3-19B). From these results I infer that S195 may be an important site for the interaction between PYL3 and GAI or *trans*-homodimer of PYL3 is essential for the interaction with GAI, because S195L mutation prevents the transition of *cis*-homodimer into *trans*-homodimer.

Functional motif analysis of DELLA in interaction with PYL

As mentioned before DELLA proteins show a large number of interactions with many factors and mediate various crosstalk in plants. According to the functional difference, the primary structure of DELLA representing by the amino acid sequence can be divided into two parts. Here I named N-terminal part as DELLA domain including DELLA, VHYNP and polyS/T/V motifs, and named C-terminal part as GRAS domain (Figure 3-20). DELLA domain is also called regulatory domain because it is very important for the interaction of DELLA with GA receptor GID1, which induces the binding of DELLA to SCF^{sly1/GID2} that facilitates DELLA degradation by 26S proteasome (Ueguchi-Tanaka *et al.* 2007, Hirano *et al.* 2010). As a result *gai-1* and *rga17* mutants with loss of DELLA motif showed insensitivity to GA (Peng *et al.* 1997, Dill and Sun 2001). A recent report showed that DELLA/VHYNP motif is also necessary for transactivation activity of DELLA (Hirano *et al.* 2012). Loss of polyS/T/V motif increased the GA insensitive responses, which indicated that this motif may regulate the repressive activity of DELLA (Itoh *et al.* 2002). The GRAS domain is considered as the functional domain because there are five motifs including leucine heptad repeats I (LHRI), VHIIID, leucine heptad repeats II (LHRII), PFYRE and SAW and some of these motifs are necessary for the interaction of DELLA with other factors. For instance LHRI is necessary for the interaction between DELLA and PIF (de Lucas *et al.* 2008, Feng *et al.* 2008), the interaction between DELLA and JAZ (Hou *et al.* 2010) and the interaction between DELLA and BZR1 (Gallego-Bartolomé *et al.* 2012, Bai *et al.* 2012). SAW is also important for the interaction between DELLA and BZR1 (Bai *et al.* 2012). Further

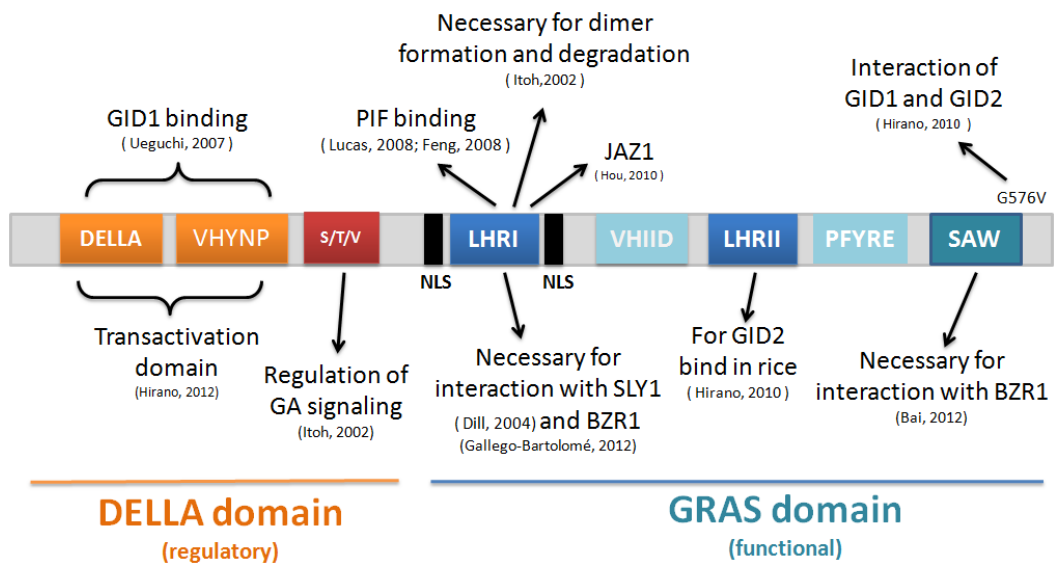


Figure 3-20 A summary of DELLA protein domains and their functions.

more the LHRI, LHRII and the G576 of SLR1 are necessary for the interaction of DELLA with SLY1 or GID2, which induces the degradation of DELLA protein.

Considering the various roles of motifs in DELLA, I constructed several DELLA truncations by deleting different motifs (Figure 3-21) and investigated the roles of the motifs in the interaction with PYL in Y2H assay. The results showed that PYL3 (Figure 3-22A) interacted with the DELLA domain (Δ G) of GAI but not the GRAS domain (Δ D) and the interaction was still suppressed by ABA. In addition, the deletion of SAW motif (Δ S) canceled the interaction between PYL3 and GAI, which indicates that the SAW motif may give a conformational support in full length of GAI for the interaction with PYL3. Moreover, pyrabactin (PYB) neither promoted the interaction between PYL3 and GAI nor inhibited the ABA activity on this interaction. While both AS2 and AS6 mimicked the ABA activity on inhibiting interaction between PYL3 and GAI. For PYL5 (Figure 3-22B), the full length of the GAI was necessary for the interaction, because PYL5 cannot interact with GAI without the DELLA domain (Δ G) nor the GRAS domain. In contrast to PYL3, SAW played a negative role in the interaction between PYL5 and DELLA, since the deletion of SAW not only promoted the interaction between PYL5 and GAI by estimating the growth speed of yeast compared, but also induced the interaction of PYL5 with RGA, which did not appear clearly in the full length of RGA. Furthermore, PYB and AS2 but not AS6 weakly promoted the interaction of PYL5 with both Δ S-GAI and Δ S-RGA. PYL6 and PYL11 showed the similar results as in the case of PYL3 and PYL5. Both of them interacted with the GRAS domain (Δ D) but not the DELLA domain (Δ G) in contrast to the interaction part of PYL3 and the interactions were dependent on ABA (Figure 3-22C, D). As is the case with PYL5, deletion of SAW promoted the interaction of PYL6 and PYL11 with GAI and RGA. PYL11 can even interact with Δ SAW of GAI and RGA in the absence of ABA. Besides, PYL11 can also interact with DELLA domain (Δ G) in an ABA dependent manner. In addition, agonists of ABA showed different effects from ABA on the

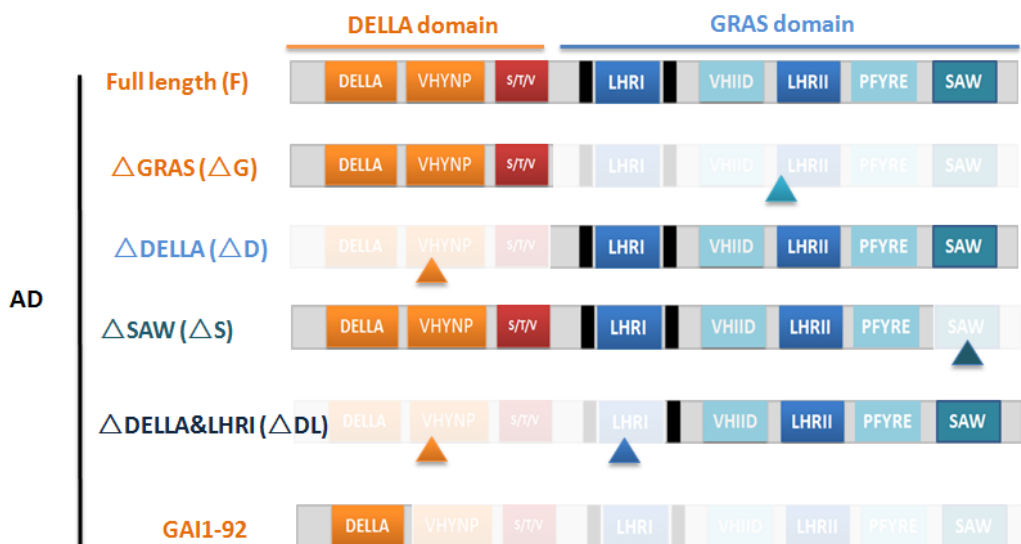


Figure 3-21 A schematic for DELLA truncations constructed to AD of Y2H assay system.

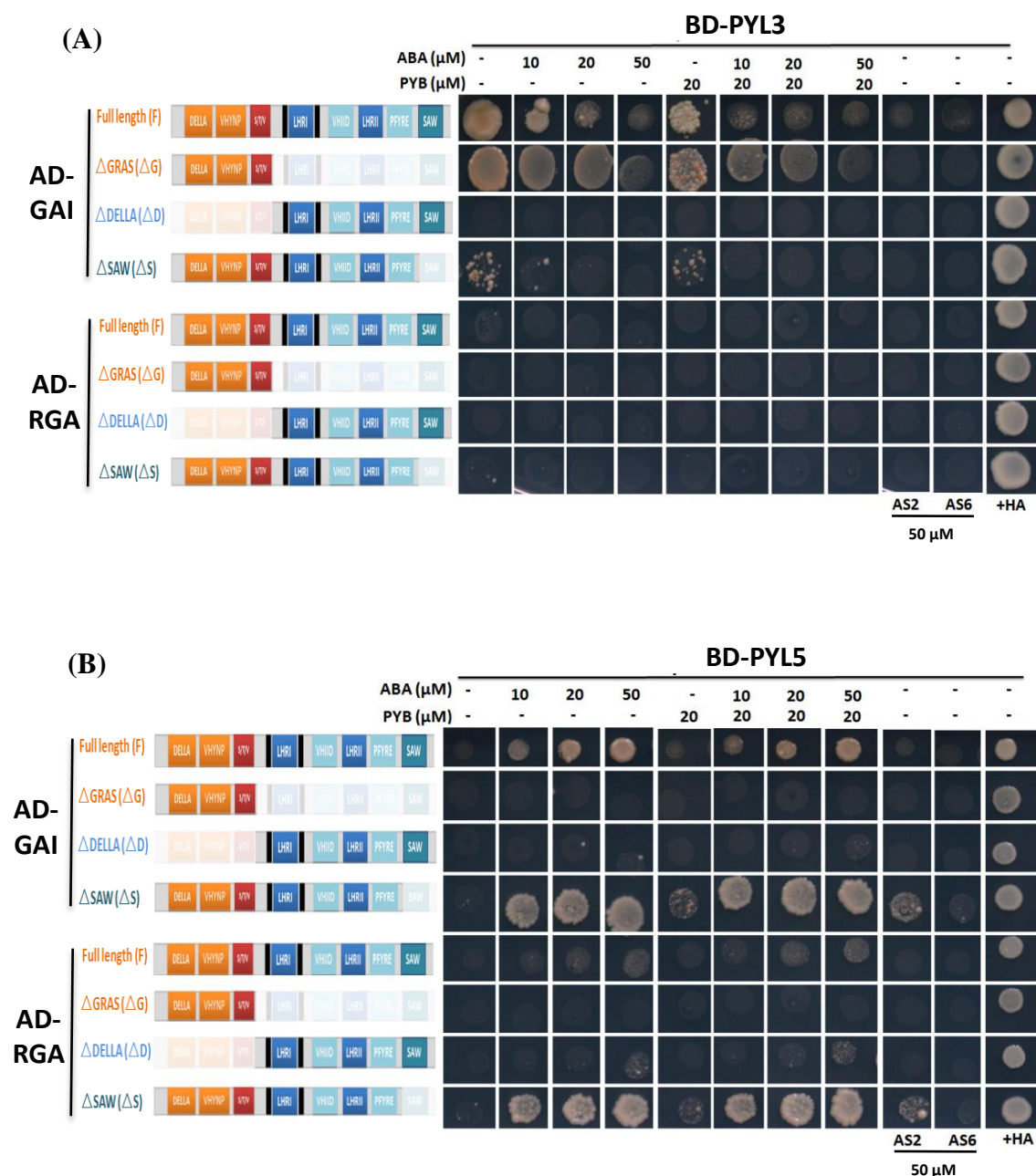


Figure 3-22 (1/2) Y2H assay for interactions between PYLs and DELLA truncations.

PYL3, PYL5, PYL6 and PYL11 served as BD and DELLA truncations served as AD. The yeast liquid SD-LWU pre-culture of each combination between PYL and DELLA was inoculated on SD-LWHAU containing the compounds indicated. (A) Interactions between PYL3 and GAI/RGA. (B) Interactions between PYL5 and GAI/RGA.

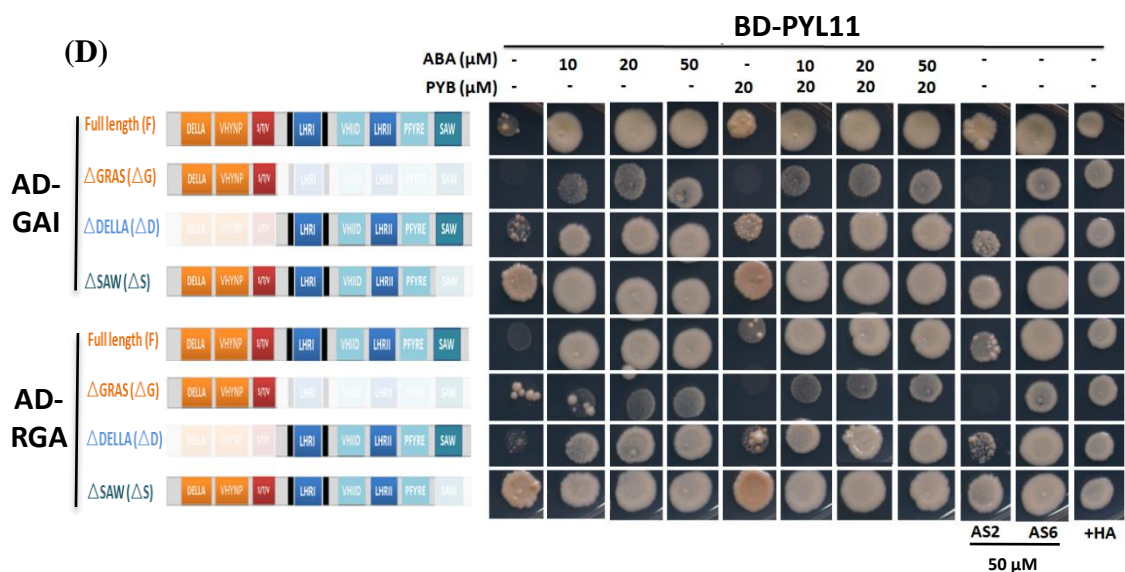
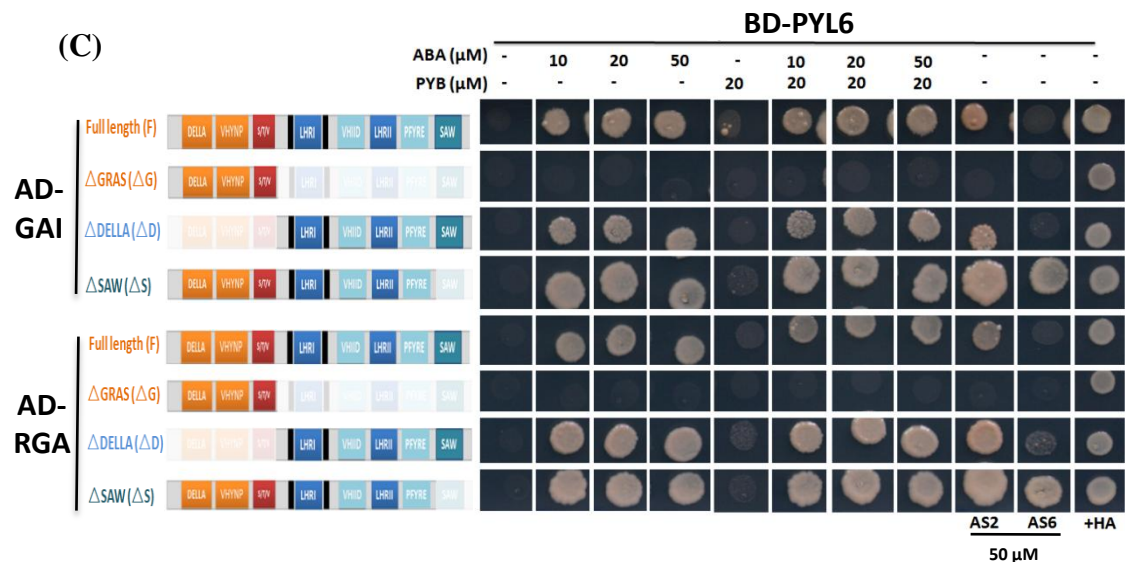


Figure 3-22 (2/2) Y2H assay for interactions between PYLs and DELLA truncations.

PYL3, PYL5, PYL6 and PYL11 served as BD and DELLA truncations served as AD. The yeast liquid SD-LWU pre-culture of each combination between PYL and DELLA was inoculated on SD-LWHAU containing the compounds indicated. (C) Interactions between PYL6 and GAI/RGA. (D) Interactions between PYL11 and GAI/RGA

promotion of the interactions of PYL6/PYL11 with DELLA truncations. AS2 but not PYB acted like ABA in promoting the interaction of PYL6 with Full length, Δ G-GAI, Δ G-RGA, Δ S-GAI and Δ S-RGA. While AS6 only promoted the interaction between PYL6 and Δ S-GAI and Δ S-RGA (Figure 3-22C). On the other hand, all these ABA agonists promoted the interactions of PYL11 with almost all truncations of GAI and RGA showing here with the exception of Δ G (DELLA domain). AS6 but not PYB or AS2 induced the interaction of PYL11 with Δ G of GAI and RGA (Figure 3-22D).

Next, I further investigated in detail the domains in DELLA proteins that different PYL interacts with. Deletion of GRAS domain did not affect the GAI-PYL3 interaction but canceled the effect of ABA on the inhibition of GAI-PYL3 interaction, implying the importance of GRAS domain for ABA dependent inhibition of the GAI-PYL3 interaction. Further deletion of VHYNP and polyS/T/V motifs (GAI1-92) in DELLA domain did not affect its interaction with PYL3 (Figure 3-23), which indicated that DELLA motif alone was enough for the GAI-PYL3 interaction. Both of GAI and RGA with deletion of DELLA domain and LHRI motif can still interact with both of PYL6 and PYL11 (Figure 3-24). Combining the former results that SAW motif was not necessary for the interaction between GAI/RGA and PYL6/PYL11, we suggest that VHIID, LHRII and PFYRE motifs are the interacting domain for PYL6 and PYL11.

Above all, during the interaction with DELLA, different PYLs showed their own binding preference (Figure 3-25). These differences between PYL3, which interacts with DELLA domain, and the other three PYLs, which interact with either full length or GRAS domain, may give a hint that why PYL3 and the other PYLs show different ABA dependency when interact with DELLA. However, the mechanism is still to be uncovered.

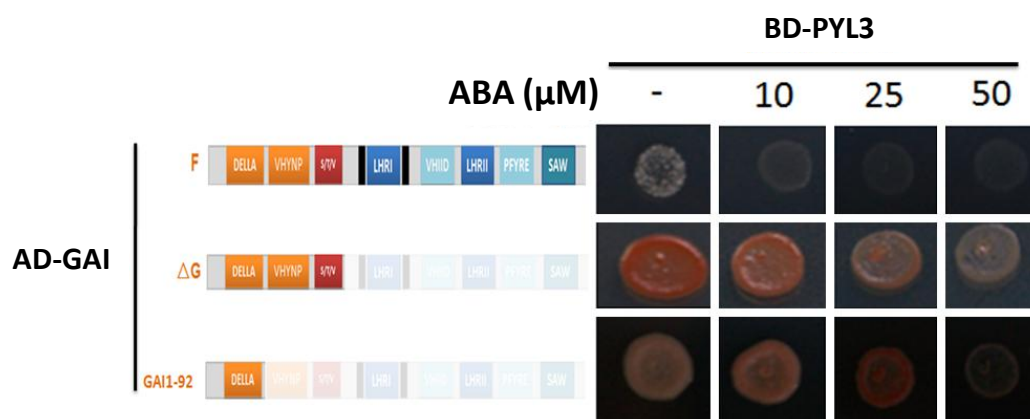


Figure 3-23 Y2H assay for interactions between PYL3 and GAI truncations. PYL3 served as BD and GAI truncations served as AD. The yeast liquid SD-LWU pre-culture of each combination was inoculated on SD-LWHAU containing series concentration of ABA as indicated.

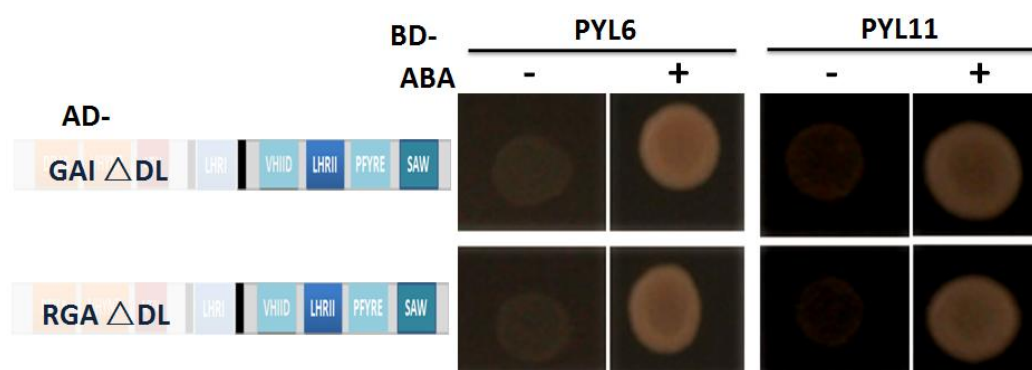


Figure 3-24 Y2H assay for interactions between PYL6/11 and DELLA truncations. PYL6/PYL11 served as BD and GAI/RGA truncations served as AD. The yeast liquid SD-LWU preculture of each combination was inoculated on SD-LWHAU with or without ABA (50 μ M).

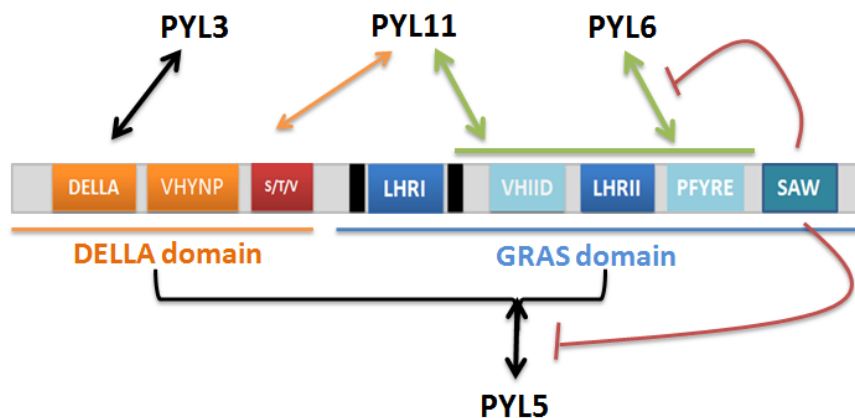


Figure 3-25 PYLs show distinct preference for interacting domains of DELLA.

Double-headed arrows stand for interaction. Suppression symbol indicates the negative roles in regulating the interactions. PYL3 interacts with DELLA domain, PYL6 and PYL11 prefer VHIID-LHR II-PFYRE domain, while the full-length of DELLA is necessary for interaction of PYL5. SAW plays negative roles in the interactions.

3.2.6 Elucidation of the relationship of interactions among PYL, DELLA and GID1 by yeast-three-hybrid

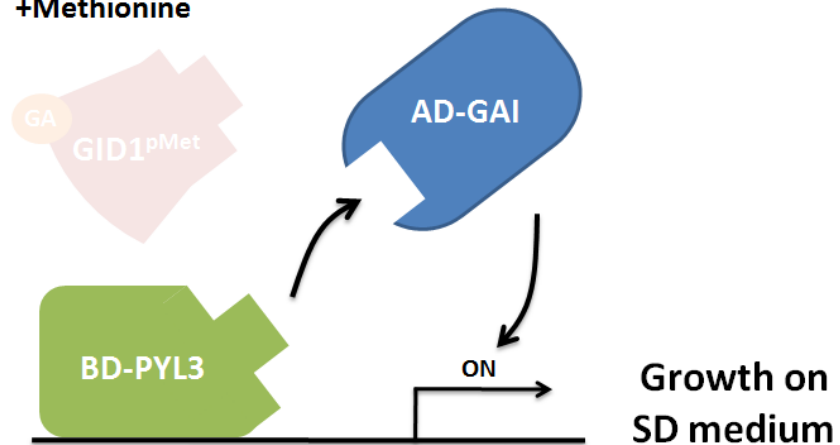
Based on the above results, PYL3 can interact with DELLA domain of GAI, which is also the binding domain of GID1. These results indicated a possibility that PYL3 and GID1 may compete with each other for interaction with GAI. To elucidate whether this competition exists or not, yeast-three-hybrid assay (Y3H) was performed.

The Y3H system used in this experiment is based on the Y2H system used in my research and share the same vector (pGADT7) with the Y2H system to express AD fusion protein. pBridge vector (Clontech), which contains two multiple cloning sites allows the expression of the DNA-BD fusion (MCSI) as well as an additional protein (MCSII) in response to methionine treatment. The interaction result between AD fusion protein and BD fusion protein is determined by the growth of yeast as shown in Y2H assay and the competing, inhibitory or promotion effects of an additional protein introduced by MCSII on interaction between AD and BD is also determined by the growth of yeast.

In this experiment, GAI and RGA protein were fused to AD by pGADT7 vector as mentioned before in Y2H. PYL3 was fused to BD while GID1 was expressed as an additional protein by pBridge vector. In the presence of methionine, if AD-GAI/RGA binds to BD-PYL3, the yeast containing these two fusion proteins survive and grow on SD-Leu,-Trp,-His,-Ade,-Ura (SD-LWHAU) medium (Figure 3-26A). While in the absence of methionine, GID1^{pMet} will be expressed as an additional protein (Figure 3-26B). The competition among PYL3, GAI/RGA and GID1s will be elucidated by observing the growth of yeast.

Yeasts with different combinations were inoculated on the SD-LWHAU medium without methionine for constant expression of the GID1s. The results (Figure 3-27) showed that in the absence of GA, PYL3 can interact with GAI but not RGA despite the existence of GID1b or GID1c which was consist with the results from Y2H

(A) +Methionine



(B) -Methionine

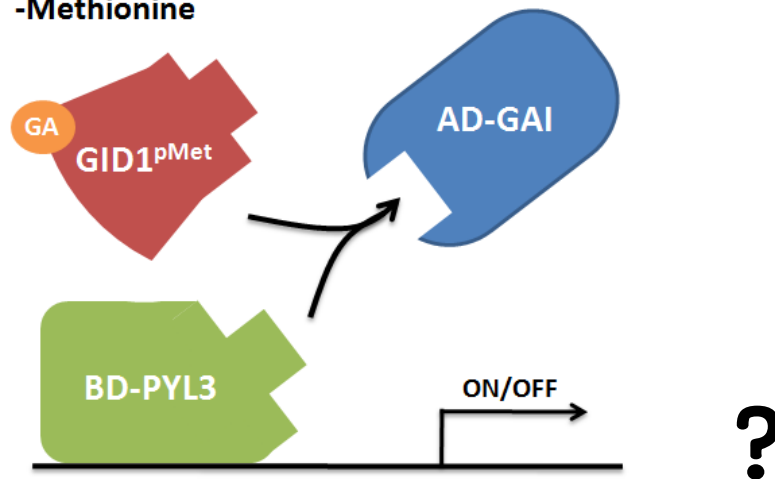


Figure 3-26 Mechanism of Y3H in competence assay between PYL3 and GID1 for interaction with GAI

(A) In the presence of methionine (1 mM), the expression of GID1 were blocked. As a result, Y3H system is the same as Y2H system that the interaction between PYL3 and GAI induced the growth of yeast on SD medium. (B) In the absence of methionine, GID1 as a third protein was expressed in Y3H assay. By adding GA to the SD medium, both PYL3 and GID1 can interact with DELLA domain of GAI. There are two possible results. One is that if the interaction of PYL3 with GAI is stronger than GID1, the yeast will grow. Another is that the yeast will not grow if the interaction of GID1 with GAI is stronger.












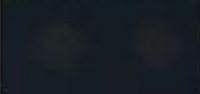
		-GA			+GA		
BD		PYL3			PYL3		
pMET		a	b	c	a	b	c
-LWU -Met	^{AD} GAI	N.D.			N.D.		
	^{AD} RGA	N.D.			N.D.		
-LWHAU -Met	^{AD} GAI	N.D.			N.D.		
	^{AD} RGA	N.D.			N.D.		

Figure 3-27 Y3H assay of interactions among PYL3, GID1 and DELLA
 GAI and RGA served as AD. PYL3 served as BD. GID1b or GID1c as a third protein expressed in the absence of methionine (Met) to constant express GID1s. The yeasts containing each combination were precultured by liquid SD-LWUM and inoculated on SD-LWHAUW with or without GA (10 μ M). a, b and c stands for GID1a, GID1b and GID1c. N.D., not determined.

(Figure 3-2). However, PYL3 lost the interaction with GAI in the presence of GA, which can induce the interaction between GID1b/c and GAI. These results suggest that PYL3 and GID1s should interact with the same domain of GAI. Moreover, the result also indicated that the interaction between GID1s and GAI is stronger than the interaction between PYL3 and GAI. Based on these results, a schematic of interactions among PYL3, GID1 and GAI is postulated that PYL3 interacts with GAI in the absence of GA (Figure 3-28A), while by binding to GA, GID1 undergoes a conformational changes which then competes with PYL3 upon a stronger affinity for GAI (Figure 3-28B).

3.2.7 Investigation of the roles of interactions between PYLs and DELLAs *in vivo*.

The results from the investigation of DELLA truncations confirmed the different interaction mechanisms of PYLs between ABA dependent bind group (PYL5, PYL6 and PYL11) and ABA independent binding group (PYL3). PYL3 interacts with the DELLA domain, which is also the interacting site of GID1. As mentioned former, the DELLA domain is important for the interaction with GID1, which then causes DELLA degradation, and transactivation activity of DELLA. There is a question that whether the binding of PYL3 to DELLA domain inhibits the binding of GID1 on the same site and prevents the degradation of DELLA or affects the transcription activation activity of DELLA. The results of Y3H assay showed that GID1 had a stronger affinity to GAI than PYL3 in the presence of GA (Figure 3-27,28). However, whether the PYL3 antagonizes the GID1 at a low GA concentration in plant or affects the transactivation of DELLA is still to be investigated.

The binding domain of PYL6 and PYL11 is the GRAS domain of DELLA, which involves in the degradation of DELLA and its interaction with other factors. The role of interaction of PYL6 and PYL11 with this part is still a mystery. Achard *et al.* reported that ABA treatment prevented the degradation of DELLA induced by GA

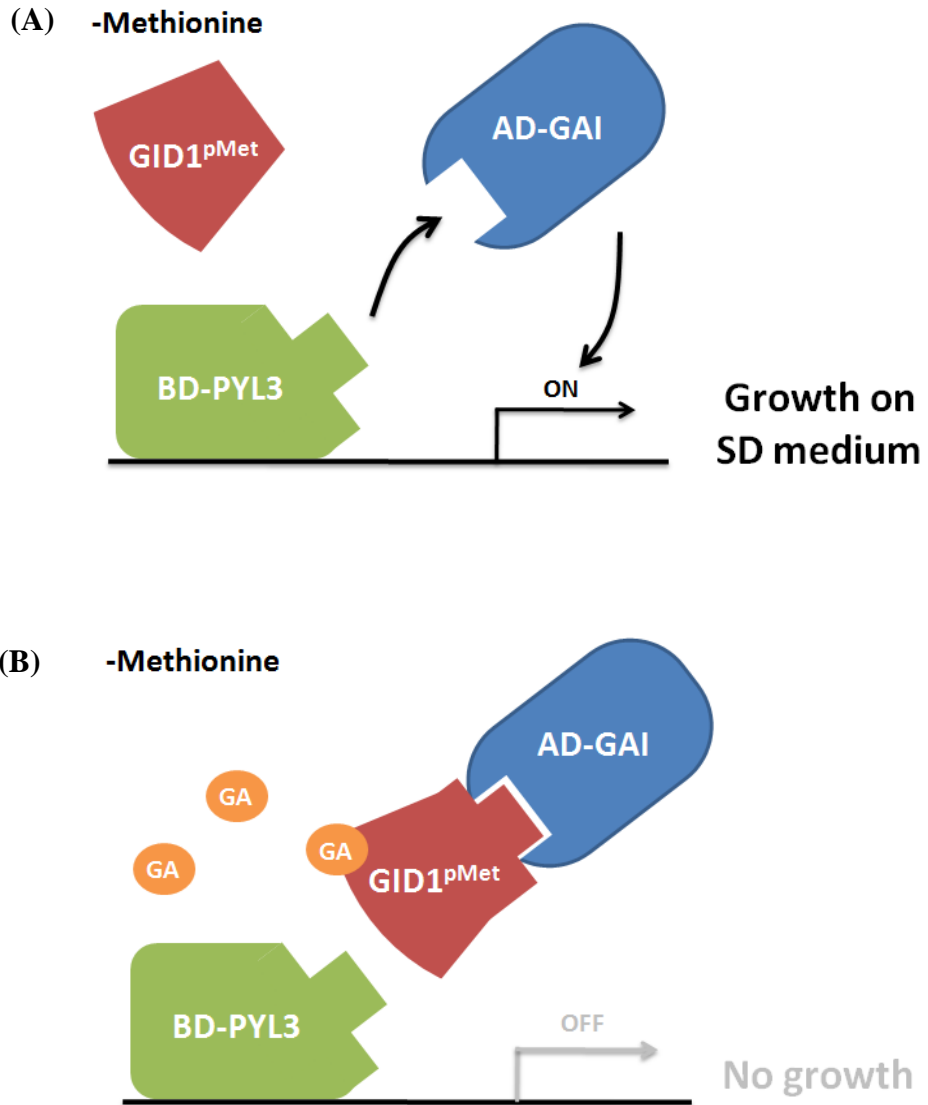


Figure 3-28 A schematic of interactions among PYL3, GID1 and GAI

(A) In the absence of GA, PYL3 interacts with GAI and induces the growth of yeast on SD-LWHAUM medium. (B) In the presence of GA, GID1 interacts with GAI and prevents the interaction of PYL3 with GAI which then result in loss of yeast growth on SD medium.

(Achard *et al.* 2006). This result leads to a possibility that PYL6 and PYL11 stabilize DELLA by antagonizing the binding of SCF^{SLY1/GID2} with DELLA. However, the results from the same paper also verified the stabilization of DELLA was strongly dependent on the ABA-triggered inhibition of ABI1 function since the stabilization of DELLA was lost even by ABA treatment in *abi1-1* background, in which abi1 protein lost the interaction ability with PYL. Pyrabactin (PYB), as ABA agonist, inhibits the ABI1 activity (Yuan *et al.* 2010) while shows no effect on inducing the interaction between PYL and DELLA (Figure 3-3B). I compared the effects of ABA and PYB on preventing the DELLA degradation caused by GA treatment and found that ABA but not PYB stabilized DELLA in the presence of GA (Figure 3-29). This result indicated that the inhibition of ABI1 caused by PYB was not enough to prevent the degradation of DELLA induced by GA. I assumed that in addition to inhibitory activity of PYL on PP2C such as ABI1, the induction of interaction between PYL and DELLA by ABA might be also necessary for the stabilization of DELLA. However, I could not exclude a possibility that the different effects on DELLA stabilization between ABA and PYB were caused by the different absorption and stability, metabolism efficiency of these two compounds in *Arabidopsis*. Further studies are needed.

3.2.8 Discussion

In this chapter the interactions between PYLs and DELLAs were verified both in *Arabidopsis* (Table 3-1) and rice (Figure 3-5). In the view of the better studies of PYL in *Arabidopsis* than in rice, I focused my attention on the research of PYLs from *Arabidopsis* and confirmed the interactions between PYL and DELLA by BiFC and pull down assays.

Even though all PYLs excluding PYL13 show the ability to bind ABA and most of them can interact with and inhibit the PP2C, some of PYLs (including PYL3, PYL5,

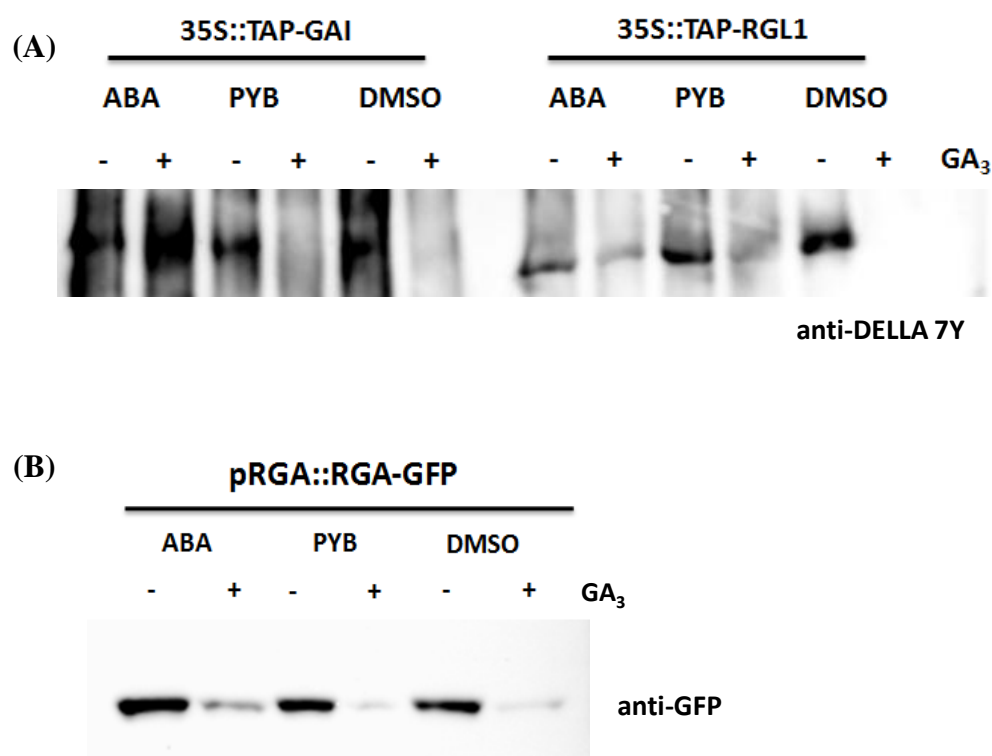


Figure 3-29 Determination of DELLA protein levels in *Arabidopsis* after various treatments by western blotting

15-day-old seedlings of 35S::TAP-GAI, 35S::TAP-RGL1 and pRGA::RGA-GFP were hydroponically treated by DMSO, ABA (20 μ M) or PYB (20 μ M) for 2 hours separately followed by 1 hour GA₃ (10 μ M) or mock treatment. Proteins were extracted and detected by western blotting using (A) DELLA antibody 7Y and (B) GFP antibody.

PYL6 and PYL11) interact with DELLAs. On top of that the type of interactions between PYLs and DELLAs can be divided into two groups according to the results from Y2H assay. One is the ABA dependent group including PYL5, PYL6 and PYL11, the other is ABA independent group uniquely represented by PYL3. Either the promoting function (for PYL5, PYL6 and PYL11) or inhibitory effect (for PYL3) of ABA on the interactions with DELLA is positively correlated with ABA concentration (Figure 3-3A). The mutation in two important amino acid sites (L91A and E98A) for the interaction between PYL and PP2C also affected the interaction between PYL and DELLA, which gives an overhead view that the latter interaction imitates the former one. However, contrary to this view, the responses of the interaction between PYL and DELLA were not always consistent with the interaction between PYL and PP2C since these two types of interactions showed different responses to PYB, AS2 and AS6 (Figure 3-3, Figure 3-22). These results indicated that the conformational changes of PYL induced by ABA or its agonist or antagonist are significant for the interaction with DELLA, which is similar to the interaction between PYL and PP2C but not the same.

The studies of interactions between PYLs and DELLA truncations uncovered the interacting preference of each PYL with DELLAs (Figure 3-25). PYL3 interacts with DELLA domain of GAI, while PYL6 and PYL11 interact with GRAS domain of GAI/RGA. The interacting part of PYL5 could not be DELLA domain, but which is necessary for its structural support. DELLAs with deletion of SAW domain (Δ S) showed increasing interactions with PYL6 and PYL11 and even produced the interaction between PYL5 and RGA which did not exist in the full length of RGA. The different interacting parts may result in the different ABA responses of these PYLs.

Y3H assays of PYL3 further validated that the DELLA domain is the interacting part of PYL3 and elucidated the existence of the competition between PYL3 and GID1 for interaction with GAI. However, the functions of this competition in plant

are still to be studied. In addition, the GRAS domain of DELLA is significant for its regulatory activities and interactions with other factors. Whether PYL6 and PYL11, which can interact with GRAS domain, compete with other factors, which share the same interacting domain deserves further investigation.

Furthermore, much experiments are needed to show the functional evidence of the interactions between PYL and DELLA in plant.

3.3 Materials and methods

3.3.1 Yeast two hybrid (Y2H) screening

The Matchmaker Two-Hybrid System (Clontech) was used for the Y2H assay. pGADT7 (Clontech) and pGBKT7 (Clontech) were used as the expression vectors. PYLs served as the bait (BD) while DELLAs served as prey (AD). *Saccharomyces cerevisiae* AH109 strains were transformed with the combinations between bait and prey plasmids. Plate assays (synthetic defined (SD)-His, Ade) were performed according to the manufacturer's protocol, with the modification that the plate media either did or did not contain ABA.

3.3.1.1 Plasmid construction

(1) Construction of pGAD-DELLAs by restriction enzyme

Full length cDNA of *AtGAI*, *AtRGA*, *AtRGL1*, *AtRGL2* and *AtRGL3* as the insertions were acquired by PCR from cDNA of *A. thaliana* (*Col-1*). The primers with proper restriction enzyme sites are as follows: EcoRI-GAI_FW and XhoI-GAI_RV for GAI, BamHI-RGA_FW and XhoI-RGA_RV for RGA, SmaI-RGL1_FW and SacI-RGL1_RV for RGL1, SmaI-RGL2_FW and XhoI-RGL2_RV for RGL2, SmaI-RGL3_FW and XhoI-RGL3_RV for RGL3. The primer sequences are listed in the Table of Primers. PCR reaction was performed by PCR Thermal Cycler (TaKaRa) using the PCR reaction mixture and PCR program as following.

Table of PCR reaction mixture

Components	Volume (μl) for 20 μl of total reaction mixture
DNA template	1-50 ng
5×PCR buffer	4
2.5mM dNTP mix	1.6
Forward primer (10pM)	0.4
Reverse primer (10pM)	0.4
DNA ploymerase	0.4
Distilled water	Up to 20 μl

Table of PCR reaction program

Cycles	Process	Temperature	Time
1	Pre-denaturation	96 °C	3min
32	Denaturation	96 °C	30sec
	Primer annealing	60 °C*	30sec
	Elongation	72 °C	90sec
1	Final elongation	68 °C	2min
1	Store	10 °C	∞

* 68 °C for RGA

(2) Electrophoresis and purification

The PCR products were separated by electrophoresis (0.8% agarose) and the target bands were extracted by using a razor blade. Then the fragments in the gel were retrieved by Gel/PCR Fragments Extraction Kit (RBCBioscience). Electrophoresis buffer (TAE buffer) is prepared as follows 40 mM Tris-HCl (pH8.0), 1 M acetic acid, 1mM EDTA.

(3) Restriction enzyme digestion

The purified PCR fragments and the target vectors (pGADT7) were double digested by distinct restriction enzymes by the method as follow at proper temperature that depends on different enzymes.

Reaction buffer

DNA	up to 1 µg
10×Buffer*	2 µl
Enzyme*	1 µl
H2O	Up to 20 µl

* It depends on different enzymes. Information is available on the manual book of TaKaRa.

(4) Ligation

The reaction products from last step were purified by Gel/PCR Fragments Extraction Kit (RBCBioscience) and followed by ligation using DNA Ligation

Kit<Mighty Mix>(TaKaRa). The reaction mixture was prepared by adding equal pGADT7 and *AtDELLA* then reaction was performed at 16 °C for 30 min.

(5) Transformation into *E. coli* by heat shock

Competent cell (XL10 gold) stored at -80 °C was taken out and melt on ice for 15 min. 5 µl reaction buffer of ligation from last step and 45 µl competent cell were added into 1.5 ml eppendorf tube and mixed gently by pipetting a few times followed by 30 min cultivation on ice. Then the tube with mixture was cultivated at 42 °C for 45 sec in water bath and immediately returned to the ice for 2min cultivation. After that reaction mixture was added by 950 µl liquid SOC or LB medium and followed by culture at 37 °C for 1 h. Finally the culture broth was sprayed on the LB agar medium containing the antibiotics (50 µM ampicillin) and cultured at 37 °C over-night.

(6) Screening positive clones and plasmid extraction

Over-night culture from last step should not over 16 h since it results in satellite clones that are false positive clones. The positive clones were selected by sterilized toothpick and inoculated both on solid and in liquid LB medium with proper antibiotics (the same as mentioned in last step) then cultured at 37 °C over-night. A shaker is needed for liquid culture with the speed at 160 rpm (round per minute).

The liquid cultures were harvested by centrifugation in a 1.5 ml eppendorf tube at 13,000 rpm for 1min. Subsequent to dispose the supernatant, the plasmids were extracted by using Plasmid Mini Kit (RBCBioscience). The procedure is strictly performed according to the protocol that is available in the product.

The plasmids were submitted to restriction enzyme digestion and checked by electrophoresis using the same methods mentioned before.

(7) Sequencing

The plasmids with correct size of inserts were sequenced by FASMAC Co., Ltd. The sample for sequencing was prepared as follows, 300-600 ng template, 6.4 pmol primer and distilled water that make up the final volume of solution to 14 µl. Primers for sequencing are available in Table of Primers.

(8) Culture medium for *E. coli*

LB (Luria-Bertani Broth) medium: Tryptone 10 g, Yeast Extract 5 g, NaCl 10 g and 1 liter distilled water. For solid medium additional 1.5% agar should also be added. Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, 121 °C for 15 minutes. The antibiotics should be added after cooling the medium to 45-55 °C.

SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10 mM MgSO₄, autoclaving at 15 psi, 121 °C for 15 minutes. 20 mM Glucose should be sterilized separately by 0.2 µm filter and added into the autoclaved mixture after cooling to 45-50 °C.

(9) Construction of pGBK-PYLs by gateway cloning technology

PYR1, *PYL1*, *PYL2*, *PYL3* and *PYL4* constructed in pGBKT7 vector were kindly provided by Dr. Nishimura, N. (National Institute of Agrobiological Sciences). The rest PYLs were constructed into pGBKT7 vector by gateway cloning technology (Invitrogen). PYLs were amplified by two-step adaptor PCR as described by the manufacturer. The following primer sets were used for the first PCR reaction: attB1-PYL5 and attB2-PYL5 for PYL5, attB1-PYL6 and attB2-PYL6 for PYL6, attB1-PYL7 and attB2-PYL7 for PYL7, attB1-PYL8 and attB2-PYL8 for PYL8, attB1-PYL9 and attB2-PYL9 for PYL9, attB1-PYL10 and attB2-PYL10 for PYL10, attB1-PYL11 and attB2-PYL11 for PYL11, attB1-PYL12 and attB2-PYL12 for PYL12, and attB1-PYL13 and attB2-PYL13 for PYL13. For PYL homolog in rice,

the cDNA from rice was amplified by primers as follows: attB1-OsPYL1 and attB2-OsPYL1 for OsPYL1, attB1-OsPYL2 and attB2-OsPYL2 for OsPYL2, attB1-OsPYL3 and attB2-OsPYL3 for OsPYL3, attB1-OsPYL4 and attB2-OsPYL4 for OsPYL4, attB1-OsPYL5 and attB2-OsPYL5 for OsPYL5, attB1-OsPYL6 and attB2-OsPYL6 for OsPYL6. second PCR was performed by adaptor primers attB1_adapter and attB2_adapter. The primer sequences are listed in Table of Primer. The PCR reaction was performed as follows.

Table of PCR reaction buffer

Components	Volume (μl) for 20 μl of total reaction mixture
DNA template	1-50 ng
5×PCR buffer	4
2.5mM dNTP mix	1.6
Forward primer (10 pM)	0.4
Reverse primer (10 pM)	0.4
DNA polymerase	0.4
Distilled water	Up to 20 μl

Table of PCR reaction program

Cycles	Process	Temperature	Time
1	Pre-denaturation	98 °C	1min
30	Denaturation	98 °C	10sec
	Primer annealing	55 °C	15sec
	Elongation	68 °C	*
1	Final elongation	68 °C	2min
1	Store	10 °C	∞

* 1kb/min

The amplified products were then inserted into pDONRTM/zeo via a BP reaction that produced pENTR-PYLs. The reaction mixture (shown in table below) was incubated at 25 °C for 1 h. Then the reaction was ceased by adding 1 μl proteinase K and cultivation at 37 °C for 15 min.

Reaction mixture for BP reaction

Vector (pDONR TM /zeo)	0.5 µl
PCR product	50-100 ng
BP clonase	1 µl
Distilled water	Up to 5 µl

The reaction buffer was transformed into DH5α by the heat shock method as described in method 3.3.1.1(5). The transformation reaction product was sprayed on plate of low salt LB medium (half salt) containing 50 µg/ml ZeocinTM (Invitrogen). The plate was cultivated at 37 °C over-night.

The positive clones containing pENTR-PYLs were inoculated in the liquid low salt LB medium with Zeocin and cultivated at 37 °C over-night by shaking. The plasmid was extracted using Plasmid Mini Kit (RBCBioscience).

The pENTR-PYL plasmids from last step were transferred to pGBKT7 by LR reaction that produced the pGBK-PYLs. The mixture of LR reaction buffer (described in table below) was incubated at 25 °C for 3 h and followed by 75 °C for 10 min. Then the reaction buffer was sprayed on plate of low salt LB medium containing 50 µg/ml kanamycin. Positive clones were selected and inoculated into liquid low salt LB medium with kanamycin. Plasmids were extracted by the methods as described former.

LR reaction buffer

ENTR-PYL	50-100 ng
pGBK vector	150 ng
LR clonase II	0.5 µl
Distilled water	Up to 4 µl

3.1.1.2 Yeast transformation and Y2H screening

(1) Yeast culture medium

YPD (yeast extract peptone dextrose): yeast extract 10g, peptone 20g, and

D-glucose 20 g. For solid medium, 2 g agar should also be added. The mixture was autoclaved at 15 psi, 121 °C for 15 minutes.

SD (synthetic defined) medium: Difco™ Yeast Nitrogen Base w/o amino acids (Becton, Dickinson and Company) 6.6 g, D-glucose 20 g, Synthetic Complete Drop Out Mix 0.83 g, distilled water 1 L. The mixture was autoclaved at 15 psi, 121 °C for 15 minutes. For solid medium, 2 g agar should also be added.

Synthetic Complete Drop Out Mix: adenine hemisulfate 2.0 g, L-arginine HCl 2.0 g, L-histidine 2.0 g, L-isoleucine 2.0 g, L-leucine 4.0 g, L-lysine HCl 2.0 g, L-methionine 2.0 g, L-phenylalanine 3.0 g, L-serine 2.0 g, L-threonine 2.0 g, L-tryptophan 3.0 g, L-tyrosine 2.0 g, uracil 1.2 g, L-valine 9.0 g. Some the components such as L-leucine (L), L-tryptophan (W), L-histidine (H), adenine hemisulfate (A), uracil (U) are omitted (SD-LWHAU) for selection culture.

(2) Transformation by the LiAc/SS Carrier DNA/PEG method

Yeast (strain AH109, CLONTECH) reserved in -80 °C was sprayed and activated on YPD medium at 30 °C for 3 to 5 days before transformation. Preparation of transformation mix in 1.5 ml eppendorf tube is shown in the table below. The yeast from YPD medium was scratched and inoculated into mixture followed by incubation at 42 °C for 1 h.

Transformation mix

Component	Volume (μl)
PEG 3500 50% w/v	60
LiAc 1.0 M	9
Boiled SS-Carrier DNA (2 mg/ml)	12.5
Plasmid DNA (0.1 to 1 μg)	
Distilled water up to total volume	90

Subsequent to incubation in last step, the transformation mix was removed by centrifugation at top speed for 30 sec. Then 1 ml distilled water was added to the tube and mixed vigorously. 50 to 100 μl samples were transferred onto plates of

appropriate SD selection medium and cultured at 30 °C for 3 to 5 days.

In fact the transformation of AD and BD were performed successively. First, pGAD-DELLAs (GAI, RGA, RGL1, RGL2 and RGL3) were transformed into AH109. Then the yeasts containing pGAD-DELLAs were further transformed with pGBK-PYLs (PYR1, PYL1 to PYL13) which should be cultured on the SD-LWU medium.

(3) Screening on SD medium

The yeasts selected by SD-LWU medium were inoculated onto SD-LWHAU medium with or without ABA or other compounds to detect the interaction between BD-PYLs and AD-DELLAs. Two methods were performed for inoculation. One is scribbling the yeasts from solid SD-LWU onto SD-LWHAU by pipette tips directly. The other one is to pre-culture the yeasts in liquid SD-LWU for three days and inoculate 5 µl culture broth onto SD-LWHAU. The growth of yeasts with different combinations among BD-PYLs and AD-DELLAs were observed 3-7 days after inoculation.

3.3.2 BiFC

3.3.2.1 Plasmid construction

To construct vectors for the BiFC assay, the N-terminal (1154 a.a.) and carboxy-terminal (155239 a.a.) fragments of enhanced YFP (eYFP; Clontech) were amplified by standard PCR using the following primer sets: eYFP-N0-f and eYFP-N0-r for eYFP 1-154 (N0 fragment) and eYFP-MC-f and eYFP-MC-r for eYFP 155–238 with methionine at its N terminus (MC fragment). The N0 and MC fragments were inserted into pUGW0 at Aor51HI sites to produce nYFP/pUGW0 and cYFP/pUGW0, respectively.

To construct entry clones, the PYL3 and GAI fragments were amplified by two-step adaptor PCR as described by the manufacturer (Invitrogen). pGBK-PYL3

and pGAD-GAI served as templates, and the following primer sets were used for the first PCR reaction: attB1-PYL3 and attB2-PYL3 for PYL3 and attB1-GAI and attB2-GAI for GAI. The second PCR was performed with the attB1 and attB2 adaptor primers, and the amplified product was then inserted into pDONRTM/zeo via a BP reaction, as described method 3.1.1(9) to construct pENTR-PYL3 and pENTR-GAI. The DNA fragments from pENTR-PYL3 and pENTR-GAI were transferred into cYFP/pUGW0 and nYFP/pUGW0, respectively, via an LR reaction as described method 3.1.1(9).

3.3.2.1.1 Plasmid transformation and amplification

All the above plasmids were transformed into DH5 α by the heat shock method as described in method 3.3.1.1(5).

High concentration of constructed vectors is needed in BiFC. 500 ml culture broth of DH5 α containing BiFC plasmid was harvested by centrifugation at 6000g for 15min. After disposing the supernatant, the plasmid was extracted by Plasmid Maxi Kit (RBCBioscience). The procedure was strictly performed as described in the manual of product.

3.3.2.2 Transformation of plasmid and observation

For the BiFC assay, an Arabidopsis suspension culture was transiently transformed according to methods that were described previously (Ueda *et al.* 2001). The transformed cells were observed with a confocal laser microscopes. Arabidopsis-cultured cells were placed on eight-well multitest glass slides (MP Biomedicals) and covered with a 0.12–0.17-mm-thick coverslip (24-60mm; Matsunami). The acquired images were analysed with the ImageJ software program (National Institutes of Health).

3.3.3 Pull down assay

3.3.3.1 Plasmid construction

(1) Plasmids for PYLs with GST fusion

pGEX-6P-1 vector was used to achieve GST fusion proteins. To insert the PYL3 fragment into *Bam*HI-*Eco*RI site of pGEX-6P-1, the open reading frame (ORF) of PYL3 was amplified by PCR using *Bam*HI-PYL3-FW and *Eco*RI-PYL3-RV as primers and pGBK-PYL3 as a template. The product was digested by *Bam*HI and *Eco*RI and inserted into *Bam*HI-*Eco*RI site of pGEX-6P-1 in-frame to the coding sequence of glutathione S-transferase (GST). All the procedures for PCR, restriction digestion, ligation and transformation were the same as mentioned before. The insertions of PYL5 and PYL6 into *Bam*HI-*Eco*RI site of pGEX-6P-1 separately were the same as described in PYL3 by using *Bam*HI-PYL5-FW and *Eco*RI-PYL5-RV as primers and pGBK-PYL5 as a template for PYL5 and *Bam*HI-PYL6-FW and *Eco*RI-PYL6-RV as primers and pGBK-PYL6 as a template for PYL6. The resultant plasmids were transformed into the *E. coli* strain, Rosetta (DE3) cells (Novagen).

(2) Plasmids for ABI1 with His-Trx fusion

pET-32a(+) vector was used to achieve His-Trx fusion protein. To insert the ABI1 fragment into *Bam*HI-*Hind*III site of pET-32a(+), the open reading frame (ORF) of ABI1 was amplified by PCR using *Bam*HI-ABI1-FW and *Hind*III-ABI1-RV as primers and pGAD-ABI1 as a template. The product was digested by *Bam*HI and *Hind*III and inserted into *Bam*HI-*Hind*III site of pET32a(+) in-frame to the coding sequence of His-Trx. The resultant plasmid was transformed into the *E. coli* strain, Rosetta (DE3) cells (Novagen).

(3) Plasmids for DELLAs with His-Trx fusion

pET-32a(+) vector was used to achieve His-Trx fusion protein. The plasmids

inserted with full length cDNA of *GAI*, *RGA* and *RGL1* separately in-frame to the coding sequence of His-Trx were constructed by Okubo, 2008 using primers with *Bam*HI site for forward primer and *Sal*I site for reverse primer.

(4) Plasmids for DELLAs with MBP fusion

pMAL-c2X vector was used to achieve maltose binding protein (MBP) fusion proteins. In the view of that the frame of fragment inserted in *Bam*HI-*Sal*I sites of pET-32a(+) are similar as in the pMAL-c2, *GAI*, *RGA* and *RGL1* fragments were transferred directly from pET-32a(+) to pMAL-c2X by restriction digestion and ligation reaction. The resultant plasmids were transformed into the *E. coli* strain, Rosetta (DE3) cells (Novagen).

3.3.3.2 Protein preparation

(1) Protein expression

All proteins were expressed using the same method as described as follows. Rosetta (DE3) with plasmid was inoculated in 2 ml liquid LB medium with antibiotics (200 μ M ampicillin and 34 μ g/ml chloramphenicol) and 20 mg/ml glucose and cultivated by shaking (200 rpm) at 37 $^{\circ}$ C over-night. 1 ml pre-culture broth was added into 500 ml flask with 100 ml liquid LB medium containing antibiotics (200 μ M ampicillin and 34 μ g/ml chloramphenicol) and 20 mg/ml glucose and followed by shake cultivation (130 rpm) at 37 $^{\circ}$ C for 3-4 h. By the time of that OD₆₀₀ increased at 0.6-0.8, the strains was harvested in 50ml falcon by centrifugation (3000 rpm) at 4 $^{\circ}$ C for 5 min. 100 ml fresh LB medium containing 200 μ M ampicillin and 34 μ g/ml chloramphenicol was mixed with the strain precipitate by pipette and placed back to 500 ml flask for further shake cultivation (130 rpm) at 18 $^{\circ}$ C for 1h. 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added into the culture broth to induce the expression of target protein at 18 $^{\circ}$ C for 16 h.

The culture broth from last step was harvested into 50 ml falcon (Himac) by 3000

rpm centrifugation at 4 °C for 5 min. For lysis of strains, 500 µl lysozyme was added into precipitate and vortex for 90 sec followed by cultivation at room temperature for 5 min. 9.5 ml 1×PBS was added into the mixture and vortex for 30 sec and followed by centrifugation at 1000 rpm , 4 °C for 1min. The mixture was frozen at -80 °C for 1h. The frozen sample was melt in water bath for 15min. To destroy the cell wall of stains, ultra sonification was performed for 5 min by interval of sonification 1sec and pause 2 sec using Ultrasonic homogenizer (TAITEC VP-5S). 4 µl Benzonase nuclease (Novagen) was added to the sample after ultrasonic performance and mixed for 15 min at room temperature. 500 µl protein mixture (total protein) was sampled for later detection. The rest mixture was precipitated by centrifugation (16,000×g, 20 min, 4 °C). The supernatant was distributed to 1ml eppendorf tube and reserved at -80 °C for later use. The samples including total and supernatant were mixed by 1:1 with SDS-PAGE sample buffer (2×) and boiled for 3 min. Then the samples were performed by SDS-PAGE.

(2) SDS-PAGE

The samples were loaded in SDS-PAGE gel with different concentration of separating gel that depends on the size of protein. The gel was made by pouring the separating gel and stacking gel into glass set for gel according to ingredient description in the table below. SDS-PAGE was performed at 25mA per gel for 70 min.

Preparation of SDS-PAGE gel

	Separating					Stacking
%	5	7.5	10	12.5	15	5
Gel volume (ml)	6	6	6	6	6	3
Distilled water	3.375	2.876	2.378	1.878	1.378	2.062
30% Acrylamide mix	1	1.5	2	2.5	3	0.5
1.5 M Tirs (8.8)	1.5	1.5	1.5	1.5	1.5	
1M Tris (6.8)						0.375
10% SDS	0.06	0.06	0.06	0.06	0.06	0.03

10% APS	0.06	0.06	0.06	0.06	0.06	0.03
TEMED	0.0048	0.0036	0.0024	0.0024	0.0024	0.003

(3) Coomassie Brilliant Blue (CBB)

The gel was stained by Coomassie Brilliant Blue (CBB) (Rapid CBB KANTO) for 10 min and destained by solution containing 750 ml water, 100 ml acetic acid, 150 ml methanol.

(4) Western blotting

After SDS-PAGE from last step, the proteins in separating gel were transferred to Hybond ECL Nitrocellulose Membrane (AmershamTM, GE Healthcare Life Sciences). First a membrane (the same size as separating gel) was soaked in blotting buffer (electrophoresis buffer and methanol in a ration at 3:1) for over 30 min then placed the three layers of absorbent paper (ATTO) (the same size as membrane) soaked by blotting buffer on the transfer apparatus (AE-6677, ATTO). Then separating gel was placed on the membrane followed by placing three layers of absorbent paper soaked by blotting buffer on gel. Then the bubbles in this sandwich were cleared. The transfer was performed at 2 mA/cm² for 1h. The transferred membrane was blocked in blocking buffer [1×TBST (25 mM Tris-HCl, 0.15 M NaCl, 0.1% (v/v) tween 20, pH7.4) with 5% Skim milk (Nacalai tesque)] at room temperature for 30 min at 37 °C or overnight at 4 °C with mild shake. The membrane was incubated with primary antibody buffer (blocking buffer with 1/5000-10000 dilution of primary antibody) for 1 h at 37 °C or over night at 4 °C with mild shake. Then membrane was washed by 1×TBST for three times by shaking for 10 min per time. The membrane was incubated with secondary antibody buffer (blocking buffer with 1/10000 dilution of second antibody) for 1 h at 37 °C or over night at 4 °C with mild shake. Finally the membrane was washed by 1×TBST for three times and followed by incubation of membrane with SuperSignal West Dura Extended Duration Substrate (black and white ECL solutions in a 1:1 ratio, Thermo) for 5 min. After draining the excess reagent and covering blot with clear plastic wrap, the membrane was observed by a

detector Light-Capture II (ATTO).

3.3.3.3 Procedure for pull down assay

There are two methods used in the assays. One is batch purification and the other is gravity flow column purification which are modified from manual for Gllutathions Sepharos 4B (GE Healthcare Life Sciences).

(1) Bath purification

The stock of resin was mixed gently by pipette. 20-50 μ l resin per reaction was transferred to a 1.5 μ l eppendorff tube and followed by 500 \times g centrifugation at room temperature for 5 min and disposing of the supernatant. Equilibration of resin was performed by washing with 10 volumes of 1 \times PBS buffer for 3 times. Then the sample were transferred to the tube with equilibrated resin and mixed gently by pipette. Incubation by an up-and-down rotator with slow speed at room temperature or 4 $^{\circ}$ C for 30-60 min. Sediment the resin by centrifugation at 500 \times g for 5 min. Collect supernatant and name it as flow through (F) for later detection. Wash the resin by 10 \times volume of 1 \times PBS containing proper compounds such as GA for pull down between GID1 and DELLA, ABA for the pull down between PYL and ABI1 or DELLA. Sediment the resin by centrifugation at 500 \times g for 5 min and then collect the supernatant and name it as Wash (W) for later detection. Add the elution buffer into the tube and mix them gently followed by cultivation at room temperature or 4 $^{\circ}$ C for 15 min. Finally, Sediment the resin by centrifugation at 500 \times g for 5 min and collect the supernatant and name it as elute (E) for later detection.

(2) Gravity flow column purification

The resin was equilibrated and cultivated with sample according to the method mentioned in batch purification. At the same time, the TALON Gravity column (Clontech) was equilibrated by 1 \times PBS buffer. After cultivation, the mixture was

added into a column and precipitated by gravity. The flow through (F) was collected for detection. Then the column was washed by 1×PBS buffer containing the compounds for three times by gravity and collected separately. Finally, 100 µl elution buffer was added followed by 15-30 min cultivation at room temperature or 4 °C. The flow through (E) was collected for detection. The details are described in the legend under the figures of pull down.

3.3.4 Site mutation

Site-directed mutagenesis was performed by using the PrimeSTAR mutagenesis basal kit (Takara Bio Inc.) with pGBK-PYL3 and pGBK-PYL5 as a template and the following primer sets: PYL3-L91A_FW and PYL3-L91A_RV for pGBK-PYL3_L91A, PYL3-E98A_FW and PYL3-E98A_RV for pGBK-PYL3_E98A, PYL5-L91A_FW and PYL5-L91A_RV for pGBK-PYL5_L91A, PYL5-E98A_FW and PYL5-E98A_RV for pGBK-PYL5_E98A. PYL3-S195L_FW and PYL3-S195L_RV for pGBK-PYL3S195A. The sequence information is available in the Table of Primers. The experiments were strictly performed according to the product manual. The construction of plasmids and yeast two hybrid assays were the same as Method 3.3.1.

3.3.5 Truncations

The truncations of GAI and RGA were constructed by restriction digestion enzyme using pGAD-GAI and pGAD-RGA as template and primers with proper enzyme as follows: BamHI-GAI-1M_FW and SacI-GAI-92N_RV for pGAD-GAI1-92, GAI1-92FW and GAI1-92RV for pGAD-GAI1-92, BamHI-GAI-1M_FW and SacI-GAI-164N_RV for pGAD-GAI-ΔGRAS , BamHI-GAI-165G_FW and SacI-GAI-532N_RV for pGAD-GAI-ΔDELLA , BamHI-GAI-1M_FW and SacI-GAI-451V_RV for pGAD-GAI-ΔSAW, BamHI-GAI-228S_FW and SacI-GAI-532N_RV for pGAD-GAI-ΔDELLA&LHRI. BamHI-RGA-1M_FW and

SacI-RGA-216E_RV for RGA Δ GRAS, BamHI-RGA-217N_FW and SacI-RGA-587Y_RV for RGA Δ DELLA, BamHI-RGA-1M_FW and SacI-RGA-504V_RV for RGA Δ SAW, BamHI-RGA-281N_FW and SacI-RGA-587Y_RV for RGA Δ DELLA&LHRI. The sequence information is available in the Table of Primers and Appendix. The construction of plasmids and yeast two hybrid assays were the same as Method 3.3.1.

3.3.6 Y3H assay

For the Y3H assay, the yeast strains AH109 and Y187 and the plasmids pGADT7 and pBridge were obtained from Takara Bio Inc. pBridge-BDPYL3-MGID1 was constructed by fusing *PYL3* cDNA with the GAL4-BD domain and inserting *GID1* cDNA into the site downstream of pMET1. Strain Y187 was transformed with the pBridge vector harbouring BDPYL3 and pMET::GID1 and selected on SD media lacking L-tryptophan (SD-W). Strain AH109, harboring pGADT7-GAI, grew on SD media lacking L-leucine and L-methionine (SD-L, M). By mating both strains, the transformants carrying both plasmids (pBridge and pGADT7-GAI) were selected on SD-LWM. For the assay, the transformants were incubated on SD-LWHAM media that lacked L-leucine, L-tryptophan, adenine hemisulphate and L-histidine but contained 3-aminotriazole. The culture medium and condition for yeast are the same as Method 3.1.1.2.

3.3.7 Determination of DELLA protein levels in plant

3.3.7.1 Sample treatments

Hydroponic treatment with separate DMSO, ABA and PYB on 15-day-old *Arabidopsis* seedlings grown under constant light ($18.2\text{--}46.5\ \mu\text{mol/m}^{-2}\ \text{s}^{-1}$) at $22\pm 1\ ^\circ\text{C}$ were performed for 2h. Then the seedlings were transferred to a solution with or without GA₃ and incubated for 1h. The treated samples were harvested by liquid nitrogen and followed by protein extraction.

3.3.7.2 Protein extraction

(1) Extraction buffer

The contents of extraction buffer (TUCT) are described in table below. Fresh extraction buffer is recommended. The TUCT buffer is added with DTT (10 mg/ml) and 1/100 volume of 1mM MG132 immediately before use.

TUCT buffer

	For 50 ml
Tris-HCl (pH8.8)	2 ml 1.5 M Tris-HCl
5 M Urea	15.02 g
1 M Thiourea	3.8 g
1% CHAPS	0.5 g
1% TX-100	0.5 ml
PIC (EDTA free)	0.5 ml

Buffer is valid for 2 months stored at -20 °C

(2) Extraction procedure

The sample reserved at -80 °C was transferred into a special tube with ion beads and mixed with extraction buffer by a weight and volume ratio at 1:2. The mixture was homogenized by homogenizer apparatus immediately. The homogenates were transferred into a new eppendorf tube and followed by 15,000 rpm centrifugation at 4 °C for 30 min. The supernatant was transferred into a new tube and mixed with 1/10 volume of 1 M acrylamide. The mixture was incubated for 10 min at room temperature and centrifuged at 14,000 rpm, 4 °C for 5 min. The supernatant was transferred to a new tube and mixed with 4× volumes of methanol, 1× volume of chloroform and 3× volumes of distilled water in order by vortex at each step. The mixture was centrifuged at 14,000 rpm, 20 °C for 1 min. Following dispose of upper layer, 4× volumes of methanol was mixed with lower layer by vortex and centrifuged at 14,000g, 20 °C for 2 min. The precipitate was dried in vacuo for 10 min. Finally

the dried sample was 1×SDS sample buffer and boiled at 95 °C for 3 min. After centrifugation at 14,000 rpm, 4 °C for 5min, the sample was loaded into SDS-PAGE gel or stored at -80 °C for later use.

3.3.7.3 Western blotting

The procedure is the same as described in Method 3.3.3.2 (4)

CHAPTER 4 Investigation of the interaction of PYLs with GRAS members other than DELLAs

4.1 Introduction

GRAS family is a plant-specific protein family. Its name comes from the first three of members isolated: GAI, RGA and SCR (SCARECROW) (Bolle 2004). All members in GRAS family show a high conservative sequence in their C-terminal part, which contains two leucine rich areas (LRI and LRII) and three main motifs (VHIID, PFYER and SAW), and variable sequences in the N-terminal part (Figure 4-1). Despite the functions of VHIID and its flanking leucine areas (LRI and LRII) are still not well known, LRI-VHIID-LRII sequence has been demonstrated to be the significant domain for the homo or hetero dimerization among GRAS protein family members (Itoh *et al.* 2002, Cui *et al.* 2007). This sequence is also important for its interaction with other interacting partners such as PIF, JAZ1, SLY1/GID2 and BZR1 (Feng *et al.* 2008, Hou *et al.* 2010, Dill *et al.* 2004, Hirano *et al.* 2010, Bai *et al.* 2012, Gallego-Bartolomé *et al.* 2012). PFYRE and SAW motifs are assumed to be required either for the function or for the structural integrity of GRAS proteins (Xiaolin *et al.* 2012). Besides some members contain NLS (nuclear localization signal), which may recruit them into nucleus.

To date, at least 33 members have been identified and some of them may play redundant roles (Lee *et al.* 2008). Based on the phylogenic analysis, GRAS family can be further divided into 10 subfamilies. These proteins play important roles in various aspects of plant growth and development such as regulation in plant hormone signaling (DELLA and SCL3 subfamilies), both root and shoot radial patterning and growth (SCR and SHR subfamilies), stress responses (LISCL and SCL4/7 subfamilies), axillary meristem development (DLT and AtLAS subfamilies), short meristem maintenance (HAM subfamily) and phytochrome signaling (PAT1

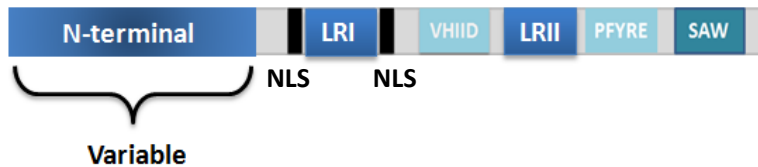


Figure 4-1 The schematic of domains in GRAS protein

N-terminal is variable in different members of GRAS family. C-terminal part can be further divided into five domains. LRI (Leucine-rich region I) is conservative in all GRAS proteins. LRII (Leucine-rich region) exists in half GRAS members. NLS stands for nuclear localization signal that is able to recruit protein into nuclear. VHIID, PFYRE and SAW are named from the most prominent amino acids. VHIID and SAW mediate interactions with some proteins, while the function of PFYRE is not well known.

subfamily) (Table 4-1) (Xiaolin *et al.* 2012).

The former results in this thesis showed that PYL6 and PYL11 interacted with the GRAS domain of DELLA, which is conservative in all GRAS members. These results are reminiscent of the interaction of PYL6 and/or PYL11 with other GRAS members in addition to DELLAs.

4.2 Results

4.2.1 Interaction between PYL and GRAS member

To validate the hypothesis, SCR, SHR and SCL3, which are well studied GRAS proteins, were selected and the interactions of these proteins with PYLs were tested in Y2H assay (Figure 4-2). As expected, in the presence of ABA, both PYL6 and PYL11 interacted with all these three proteins despite the faint interaction between PYL6 and SHR. PYL3 did not interact with any of these proteins which further demonstrated the specific affinity of PYL3 to DELLA domain. Unexpectedly but not surprisingly, PYL5 interacted with SCL3. This result indicated that DELLA domain was not the interacting part for the interaction with PYL5. This result gives us a suggestion regarding a structure required for the interaction with PYL5. That is, other domains such as the N-terminus of SCL3 can replace DELLA domain. As in the case of relationship between PYLs and DELLAs, PYR1, PYL1, PYL2, PYL4 and PYL9 showed no interactions with SCR, SHR nor SCL3.

ABA agonist and antagonist were also tested in the promotion of interactions between PYLs and SCR/SHR/SCL3 (Figure 4-3). PYB only promoted the interaction between PYL11 and SCL3. Another type of ABA agonist, AS2, showed no effect on any interaction. In contrast AS6, which is an antagonist in the interaction between PYL and PP2C, induced the interaction of PYL6 and PYL11 with some GRAS members. These results confirmed the importance of conformational changes induced by compounds and the different mechanisms among interactions between

Table 4-1 Subfamilies in GRAS family and their functions

Subfamily Members		Functions
DELTA	AtGAI, AtRGA, AtRGL1, AtRGL2, AtRGL3, SLR1, StRGA, Rht1, ZmD8, SLN1	Key negative regulators in GA signaling. Negatively regulating plant growth and responses to biotic and abiotic stress. Mediating crosstalk with other signalling pathway by direct binding to factors such as JAZ, PIFs and BZR1.
AtSCR	AtSCR, OsSCR1, ZmSCR	Root radial patterning and root growth, QC identity, asymmetric cell division, sugar response
AtSHR	AtSHR, OsSHR1, OsSHR2, MtNSP1	Root radial patterning and root growth, cell division and endodermis specification, transcription factor for nodule development.
AtSCL3	AtSCL3	Positive regulator of the GA response pathway, integrator of GA/DELLA signalling and the SCR/SHR pathway in root cell elongation.
LISCL	LISCL, AtSCL14, NiGRAS1, CsSCL1, PrSCL1	Transcriptional regulator, transcriptional regulation or activation associated with the plant stress responses, adventitious root formation in response to auxin.
AtSCL4/7	PeSCL7	Transcriptional regulator in response to environmental stresses such as salt, osmotic shock and drought
AtPAT1	AtPAT1, AtSCL13, OsCIGR1, OsCIGR2	PhyA-specific signalling, positive regulator of phyB-dependent red light signalling, hypocotyl elongation, transcriptional regulators in the early stages of plant defence signalling
DLT	Rice semi-dwarf mutant with low-tillering (DLT or Os29)	Modulating BR responses and participating in the control of rice tillering. DLT and OsBZR1 regulate each other for the fine-tuning of BR responses
AtLAS	AtLAS, LeLS, OsMOC1	Axillary shoot formation, initiation of axillary meristems, control of tillering
HAM	HAM, BnSCL1, MtNSP2	Shoot meristem maintenance, transcriptional activator in response to auxin, transcriptional co-activator in nodulation signalling

Modified from Xiaolin, S., et al. (2012)

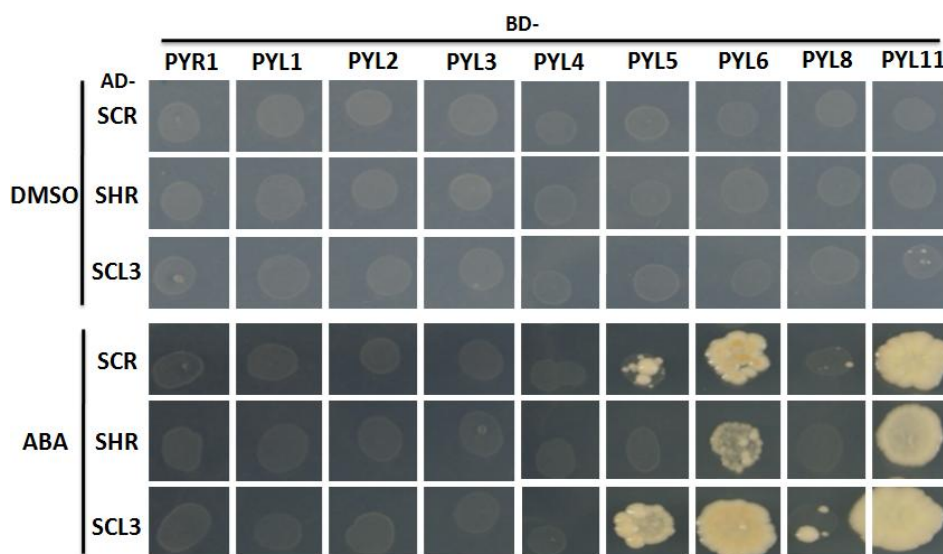


Figure 4-2 Interaction assay between PYLs and GRAS members
 PYLs served as BD and GRAS members (SCR, SHR and SCL3) served as AD. The yeasts containing each combination of AD and BD as indicated were precultured for three days and inoculated on SD-LWHAU medium with or without ABA (50 μ M) and cultured at 30°C for 7 days.

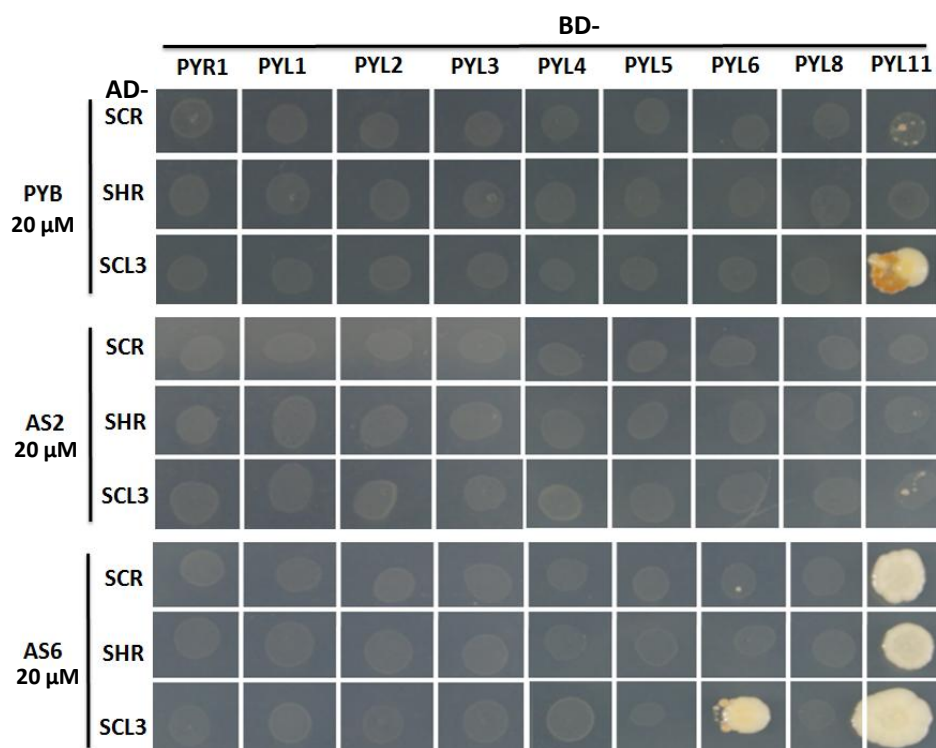


Figure 4-3 Interaction assay between PYLs and GRAS members
 PYLs served as BD and GRAS members (SCR, SHR and SCL3) served as AD. The yeasts containing each combination of AD and BD as indicated were precultured for three days and inoculated on SD-LWHAU medium with ABA agonists such as PYB, AS2 and AS6 and cultured at 30°C for 7 days.

PYL and PP2C and interactions between PYL and GRAS members.

4.2.2 The challenge of new ideas

PYL family belongs to Bet v 1 family, which owns a large cavity that can bind hydrophobic ligands including flavonoids, fatty acids and plant hormones such as brassinosteroids and cytokinins (Radauer *et al.* 2008). Despite the fact that interaction between PYR1 and HAB1 was not promoted by epibrassinolide, GA, MeJA, kinetin or 2,4-D (Park *et al.* 2009), there still remains a possibility that these plant hormones or other molecules should promote the interaction of PYL with other partners such as GRAS members or inhibit the activity of ABA in competition with binding to PYL. I investigated whether the interactions between PYLs and GRAS members (DELLA, SCR, SHR and SCL3) were promoted by plant hormones other than ABA such as GA₄, IAA, BR, GR24, 4-Br debranone (partial agonist of strigolactone) (Fukui *et al.* 2013), SA and PAMD (an inhibitor in SA signaling) (Seo *et al.* 2012) or inhibited by these small molecules due to the competition with ABA for binding to PYL. The results showed that none of these molecules mimicked the function of ABA nor competed with ABA in promoting the interactions (data not shown here) which indicated that these interactions are specific for ABA binding to PYL. However, I could not exclude the existence of other molecules that promote these interactions.

4.3 Discussion

Except for DELLA family, it was demonstrated that PYL could also interact with other GRAS members in an ABA dependent manner. These results may open a brand new knowledge about how PYL family regulates the plant growth and development. That is, PYLs not only function in the traditional pathway of ABA signaling but also may execute their roles in other signaling pathways by interacting with partners other

than PP2C.

Despite the fact that functions of the interactions between PYLs and GRAS members are still to be discovered, there are a number of hints that indicate the possible roles related to these interactions. For instance, SCR and SHR are two interacting transcription factors functioning in regulating root architecture cooperatively (Cui *et al.* 2007, Helariutta *et al.* 2000), leaf growth (Dhondt *et al.* 2010) and sugar responses (Cui *et al.* 2012). It is postulated that SCR sequesters SHR into nucleus and stops its movements by the protein-protein interaction. LRI-VHIID-LRII domain of SCR, which is also the interacting part for some PYLs, is important for the interaction with SHR that show effects on initiation and patterning of lateral root primordia and maintenance of growth of lateral root (Lucas *et al.* 2011). In addition, the lateral root formation and growth is also under regulation of ABA (De Smet *et al.* 2006). It would be valuable to investigate whether this regulation of ABA is dependent on the interaction between PYL and SCR/SHR. Moreover, it is reported recently that SCR regulates stress response genes and sugar response which are also regulated by ABA. The SCR also down-regulates the expression of *ABI4* and *ABI5* (Cui *et al.* 2012). Our data showed that over-expression of *PYL6* increased the expression level of *ABI5* (Figure 2-13). As a result, I suggest that these physiological overlaps between PYL and SCR/SHR should be possibly caused by their interactions.

Moreover, even though no other compounds than ABA has been found to promote the interaction between PYL and GRAS family yet, it is believed that potential candidates should exist because a recent research identified new compounds from plant extracts that inhibit the RCAR13-dependent PP2C activity (Szostkiewicz 2010).

Discussions and future plans

Despite the fact that the PYL family proteins except for PYL13 are canonical receptors of ABA and they play significant roles in ABA signaling pathway through binding to and inhibiting PP2C, there are also a number of reports and communicational results that show new functions of some PYLs. In IPGSA (The International Plant Growth Substance Association) conference 2013, Jian-kang Zhu reported that though PYL13 showed no binding activity to ABA, over-expression of this PYL increased the tolerance of *Arabidopsis* to drought stress which might indicate the function of PYL independent of ABA (Zhao *et al.* 2013). In addition, Jian-kang Zhu also reported the interactions between PYL13 and other PYLs by Y2H assay. The interactions of PYL13 with PYR1, PYL1, PYL2, PYL3, PYL6, PYL11 and PYL12 were independent of ABA while the interaction between PYL13 and PYL4 was dependent on ABA (Zhao *et al.* 2013). Recently a structural study of PYL13 demonstrated the interaction between PYL13 and PYL10 and indicated that PYL13 and PYL10 antagonized each other to bind PP2Cs in an ABA independent manner (Li *et al.* 2013). In addition, the results of mapping protein-protein interactions using HALO-TAG NAPPA microarrays (communication results from Dr. Yazaki J.) showed that PYL6 not only interact with PP2Cs but also can interact with ABI5 that exists in the downstream of ABA signaling pathway. Moreover, PYL6 is able to interact with many factors in other plant hormone signaling pathway such as BZR1 (BR signaling), TGA1 (SA signaling), MYC2 (JA signaling). In this study, the interactions of PYLs with DELLAs and other GRAS members had been validated initially. The above results will open brand new potential roles of PYL family in regulating plant growth and development. Based on these results I suggest the possibility that PYL family not only plays roles in traditional ABA signaling pathway including PP2C, SnRK2 and transcription factors as ABA receptor, but could also mediate the crosstalk with other signaling pathway by interacting with other factors

such as GRAS family. These distinct interacting partners among PYLs may be one of the reasons for the necessity of the existence of so many PYLs in *Arabidopsis*. In addition, as we know that GRAS family plays various roles in plant growth and development, the interactions between PYLs and GRAS members or other unknown factors may be one of the explanations for that why ABA is not only a negative regulator but also necessary for normal growth and development of plant. On the contrary, there is also a possibility that DELLA and other GRAS members affect the ABA signaling pathway by interfering interaction between PYL and PP2C to some extent.

PYL family belongs to Bet v 1 family, which owns a large cavity that can bind hydrophobic ligands. A recent report showed that Fra proteins belonging to PR-10 family which has a structural homology to PYL family, binds natural flavonoid and controls its biosynthesis (Casañal *et al.* 2013). To date although a few experiments have investigated the binding activity of PYL to other ligands (Park *et al.* 2009) based on the interaction between PYL and PP2C, no search for new ligands of PYLs based on their interaction with other partners has been reported. The finding of the interaction between PYL and GRAS family could not only open up the new functions of ABA but also provide means to search for new ligands of PYL besides ABA.

Elucidation of the above questions will broaden the knowledge about the roles of ABA in plant and facilitate the utilization of ABA signaling in agricultural production and plant protection.

Future plans

To determine the mechanisms of interactions between PYLs and DELLAs or other GRAS members better, the crystallization and analysis of these complexes are necessary.

Since PYL6 shows interactions with various partners, next work is focused on

elucidating of their functions at the molecular level and the physiological level. Although the interactions between *PYL6* and *GAI/RGA* were confirmed in my research, whether the inhibitory role of *PYL6* in regulating the hypocotyl growth during photomorphogenesis is related to these interactions is still uncertain. To further elucidate the roles of *PYL6* in plant and the significant roles of its interactions with DELLAs and other GRAS members, the mutants including *PYL6* over-expression lines fused with flag tag and GFP tag, *PYL6* over-expression lines in the pentuple della background and *PYL6*-RNAi lines are under construction.

Chemical biology, an application of small bioactive chemicals to investigate the cellular networks in plants, is a useful technology for understanding the plant hormone biosynthesis, signaling and so on (Hicks and Raikhel 2009). Screening the compounds that induce the interaction between PYLs and GRAS members from a chemical library using Y2H assay will facilitate the finding of new candidate ligands of PYL which in turn could also be used to investigate the new functions of PYL in plant.

Abstract

論文の内容の要旨

応用生命化学	専攻
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論文題目 Physiological and molecular biological studies of PYR/PYL/RCAR
ABA receptors in *Arabidopsis*
(シロイヌナズナ ABA 受容体 PYR/PYL/RCAR の
生理学のおよび分子生物学的研究)

Introduction

ABA is a plant hormone and regulates both plant growth and plant responses to stresses. Uncovering the mechanisms of ABA in these actions will facilitate the regulation of plant growth and alleviation of plant damages caused by various stresses, which then direct the increase in crop production and plant resistance against stresses.

The metabolisms of ABA including its biosynthesis and catabolism have been well studied since its discovery. Although many ABA related factors, such as PP2Cs, SnRK2s, transcription factors and responsive genes were identified successively, the ABA signaling pathway was still incomplete since the loss of information about how

ABA was sensed and transmitted. The discovery of ABA soluble receptors RCAR/PYR/PYL was a hallmark in ABA signaling studies since it coordinated the various factors that were found before and established a canonical pathway from ABA perception and transduction to ABA response in plant physiology.

There are 14 members in *PYL* family in *Arabidopsis*. They show various spatio-temporal expression levels and redundant physiological roles with each other. The mechanisms of PYLs in regulating the ABA signaling pathway and the functions of PYLs in plant growth and development are still to be studied.

Observation of the phenotypes of *PYL* over-expression lines in response to different conditions

The *PYL* over-expression lines of *Arabidopsis* were constructed and the physiological responses of these over-expression lines to ABA and/or other reagent under various conditions were investigated. I confirmed recent published results that PYLs play redundant and distinct roles in regulating the seed germination, root elongation, seedling establishment. By analyzing the over-expression lines of *PYL1*, *PYL2* and *PYL4* I found that these receptors played redundant roles in inhibiting seed germination. However, over-expression lines of *PYL3*, *PYL6*, *PYL11* and *PYL13* showed no increase in or even decrease in the sensitivity to ABA, which indicated that these PYLs could be not involved in regulating the seed germination. Over-expression lines of *PYL1*, *PYL2*, *PYL5* and *PYL7* showed increasing sensitivity to ABA during seedling development. The root of *PYL2*, *PYL5* and *PYL7* over-expression lines were much more suppressed by ABA compared to that of control line. In addition, I first found that *PYL6* functions as negative regulator in hypocotyl elongation during photomorphogenesis and this suppression of hypocotyl by *PYL6* over-expression was restored by GA treatment, indicating that the regulation of hypocotyl elongation via *PYL6* may be related to GA signaling to some extent.

Characterization of the interactions between PYLs and DELLAs and investigation of the mechanisms

Based on the former results that PYL could function as a mediator between ABA and GA signaling and the versatile roles of DELLA protein in interactions with other factors, I hypothesized that the crosstalk between ABA and GA may be at least in part mediated by the direct interactions between PYL and DELLA.

The interactions between PYL (including PYL3, PYL5, PYL6 and PYL11) and DELLA (represented by RGA and GAI) were verified by Y2H assay. The interaction between PYL3 and GAI was independent of and even inhibited by ABA. In contrast, the interactions between PYL5/PYL6/PYL11 and GAI/RGA were dependent on ABA. In addition, the ABA interactions between OsPYLs (AtPYL like proteins in rice) and SLR1 (the only DELLA in rice) were also observed by Y2H assay, which indicated that the interaction between PYL and DELLA is not limited to *Arabidopsis* (dicot), but also exists in rice (monocot). BiFC and pull down assay further confirmed the interactions between PYLs and DELLAs in *Arabidopsis*, but the ABA dependency of these interactions was not consistent with those confirmed in Y2H assay.

By site-directed mutagenesis studies in Y2H assay, L91 and E98, which are important sites for the binding of PYL to ABA and PP2C, are also important for the ABA dependent interactions between PYL3/PYL5 and GAI. ABA agonist and antagonist play different roles in the interaction between PYL and DELLA. These results indicated that conformational changes of PYL induced by ABA or its agonist or antagonist are significant for its interaction with DELLA, which is similar to the interaction between PYL and PP2C but not the same.

As DELLA protein can be divided into two parts including DELLA domain in N-terminal and GRAS domain in C-terminal, I investigated which part of DELLA is required for the interaction with PYLs by Y2H assay. The results indicated that

PYL3 interacted with DELLA domain while PYL6/PYL11 interacted with the GRAS domain. For PYL5, the full length of DELLA was necessary for the interaction. These results gave an explanation that the different ABA dependency of interactions between PYLs and DELLAs may result from different interacting mechanisms. Furthermore, since DELLA domain and GRAS domain are important for the interaction with GID1 or SLY1, and also for other factors such as PIF, JAZ, BZR1 functioning in the plant hormone signaling pathways and so on, I suggest that the binding of PYL to these domains may affect the functions of other plant hormones that still to be uncovered.

PYL interaction with GRAS members

DELLA group is a subfamily of GRAS family with a large number of members that are conservative in GRAS domain. As stated above, PYL6 and PYL11 interact with GRAS domain of DELLA. Then I investigated whether these PYLs can interact with other members of GRAS family in addition to DELLA. Three members of GRAS family, SCR, SHR and SCL3 were selected randomly for the interaction test with PYLs by Y2H assay. The results showed that PYL6 and PYL11 interact with SCR, SHR and SCL3 and these interactions are dependent on ABA. However no interaction was observed between PYL3 and any of these GRAS members. These results further validated the former result that PYL6 and PYL11 interacted with GRAS domain while PYL3 interacted with DELLA domain specifically. However, further investigation is needed to determine the mechanisms and significant roles of these interactions.

Conclusion and future plan

In this study the roles of PYLs in regulating plant growth and stress responses were investigated. In addition the interactions of PYLs with DELLAs and other members of GRAS family were validated firstly, which opened brand new potential

roles of PYL in regulating plant growth and development. Based on these results I suggest the further possibility of PYLs as ABA receptors. That is, PYLs not only play roles in traditional ABA signaling pathway including PP2C, SnRK2 and transcription factors, but also may mediate the crosstalk with other signaling pathway by interacting with other factors in GRAS family. Vice versa, there is also a possibility that DELLA and other GRAS members affect the interaction between PYL and PP2C.

Although the interactions between PYL6 and GAI/RGA were confirmed, whether the inhibitory role of PYL6 in regulating the hypocotyl growth during photomorphogenesis is related to these interactions or not is still unknown. To further elucidate the roles of PYL6 in plant and the significant roles of its interactions with DELLAs and other GRAS members, the mutants including *PYL6* over-expression lines fused with flag tag and GFP tag, *PYL6* over-expression lines in the pentuple della background and *PYL6*-RNAi lines are under construction. Besides, to elucidate the mechanisms of interactions between PYLs and DELLAs better, the complexes will be crystallized and analyzed.

Appendix A-Summary of PYL research progress

(a) Molecular biological and structural biological progress

Name		Apo-type	Ligand	Interacting partners	Reference
PYR1	RCAR11	dimer	ABA, PYB, Quina [1]	ABI1, ABI2, HAB1, AHG3/PP2CA	[2-10]
PYL1	RCAR12		ABA, PYB, Quina	ABI1, ABI2, HAB1, AHG3/PP2CA	[10-12]
PYL2	RCAR14		ABA, Quina	ABI1, HAB1, AHG3/PP2CA,	[9, 10, 12]
PYL3	RCAR13	transdimer	ABA, PYB, Quina	ABI1, HAB1, AHG3/PP2CA, <u>GAI</u>	[10, 13, 14]
PYL4	RCAR10	monomer	ABA	ABI1, HAB1, AHG3/PP2CA	[7, 10, 15]
PYL5	RCAR8		ABA, Quina	ABI1, ABI2, HAB1, HAB2, AHG3/PP2CA, <u>GAI</u> , <u>SCL3</u>	[7, 10, 16, 17]
PYL6	RCAR9		ABA	ABI1, ABI2, HAB1, HAB2, AHG3/PP2CA, <u>GAI, RGA, RGL1, SCR, SHR, SCL3</u>	[7, 10]
PYL7	RCAR2	monomer?	ABA	HAB1, AHG3/PP2CA	[18]
PYL8	RCAR3	monomer	ABA	ABI1, ABI2, HAB1, HAB2, AHG3/PP2CA	[7, 10, 19]
PYL9	RCAR1		ABA	ABI1, ABI2, HAB1, HAB2, AHG3/PP2CA <u>RGL1</u>	[9, 17, 19, 20]
PYL10	RCAR4		ABA	ABI1, HAB1, HAB2, AHG3/PP2CA	[10, 21]
PYL11	RCAR5	unknown	ABA	HAB1, <u>GAI, RGA, RGL1, SCR, SHR, SCL3</u>	
PYL12	RCAR6	unknown	ABA	HAB1, AHG3/PP2CA,	
PYL13	RCAR7	monomer	-	ABI1, HAB1, HAB2, AHG3/PP2CA, PYR1, PYL1, PYL2, PYL3, <u>PYL4</u> , PYL6, PYL10, PYL11, PYL12	[22]

Appendix A-Summary of PYL research progress

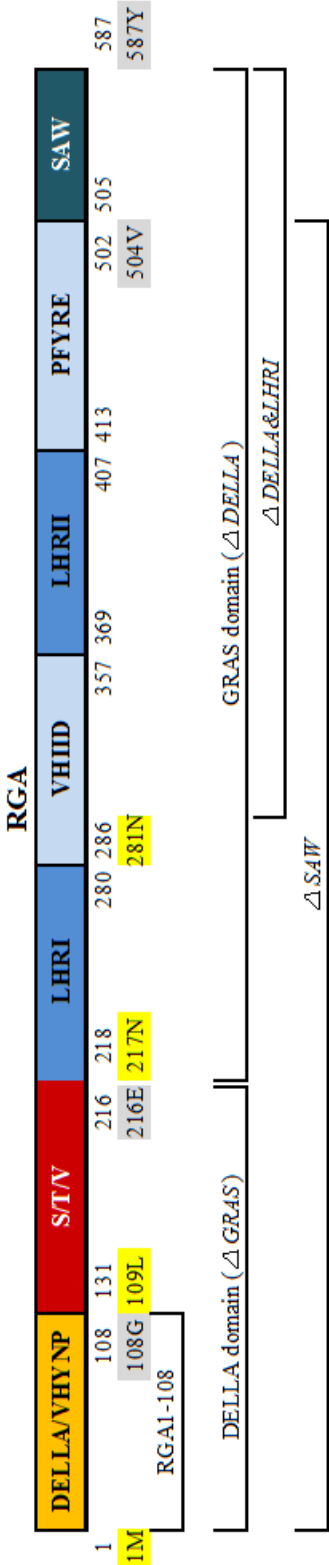
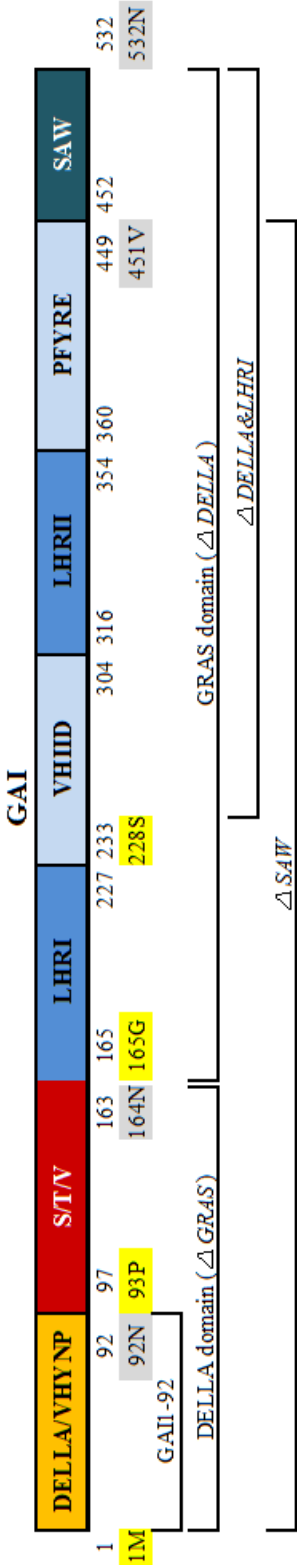
(b) Physiological roles

Name		Physiological roles
PYR1	RCAR11	germination[3], stomatal movement [23, 24]
PYL1	RCAR12	germination [3], stomatal movement [23, 24]
PYL2	RCAR14	germination [3], stomatal movement [23, 24]
PYL3	RCAR13	unknown
PYL4	RCAR10	germination [3], stomatal movement [23, 24]
PYL5	RCAR8	germination [4], root growth [4], stomatal movement [23, 24]
PYL6	RCAR9	hypocotyl growth (This thesis)
PYL7	RCAR2	germination, drought tolerance[18]
PYL8	RCAR3	non-redundant root ABA sensitivity[25], hydrotropic response [25], stomatal movement [23, 24]
PYL9	RCAR1	stomatal aperture , germination [7], root growth [7]
PYL10	RCAR4	unknown
PYL11	RCAR5	unknown
PYL12	RCAR6	unknown
PYL13	RCAR7	drought tolerance[26]

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Amino acid information and primers for DELLA truncations

Domains of DELLAs are divided into seven parts indicated by the number of amino acid at the connection points according to Hirano et al., 2010. Primers for producing truncations are indicated as the amino acid and their number in the color blocks. Primers in the yellow blocks are forward primers with *Bam*HI site. Primers in the gray blocks are reverse primers with *Sac*I site.

Table of Primers

Name	Sequence	Target Vector
attB1-PYL5	5'-AAAAAGCAGGCTccATGAGGTCACCGGTGCAACT-3'	pGBKT7
attB2-PYL5	5'-AGAAAGCTGGGTcTTATTGCCGTTGGTACTTCGA-3'	
attB1-PYL6	5'-AAAAAGCAGGCTccATGCCAACGTCGATACAGTT-3'	
attB2-PYL6	5'-AGAAAGCTGGGTcTTACGAGAATTTAGAAGTGTT-3'	
attB1-PYL7	5'-AAAAAGCAGGCTccATGGAGATGATCGGAGGAGACG-3'	
attB2-PYL7	5'-AGAAAGCTGGGTcTCAAAGGTTGGTTTCTGTATGA-3'	
attB1-PYL8	5'-AAAAAGCAGGCTccATGGAAGCTAACGGGATTGAG-3'	
attB2-PYL8	5'-AGAAAGCTGGGTcTTAGACTCTCGATTCTGTCTGTG-3'	
attB1-PYL9	5'-AAAAAGCAGGCTccATGATGGACGGCGTTGAAGGCG-3'	
attB2-PYL9	5'-AGAAAGCTGGGTcTCACTGAGTAATGTCCTGAG-3'	
attB1-PYL10	5'-AAAAAGCAGGCTccATGAACGGTGACGAAACAAAG-3'	
attB2-PYL10	5'-AGAAAGCTGGGTcTCATATCTTCTTCTCCATAG-3'	
attB1-PYL11	5'-AAAAAGCAGGCTccATGGAAACTTCTCAAAAATATC -3'	
attB2-PYL11	5'-AGAAAGCTGGGTcTTACAACCTTAGATGAGCCAC-3'	
attB1-PYL12	5'-AAAAAGCAGGCTccATGAAAACATCTCAAGAACAG-3'	
attB2-PYL12	5'-AGAAAGCTGGGTcTTAAGTGAGCTCCATCATCTTC-3'	
attB1-PYL13	5'-AAAAAGCAGGCTccATGGAAAGTTCTAAGCAAAAAC-3'	
attB2-PYL13	5'-AGAAAGCTGGGTcTTACTTCATCATTTTCTTTGTG-3'	
attB1-OsPYL1	5'-AAAAAGCAGGCTccATGGAGCAGCAGGAGGAAAGT-3'	
attB2-OsPYL1	5'-AGAAAGCTGGGTcCTATTCCGCCGCCGCCGGTGG-3'	
attB1-OsPYL2	5'-AAAAAGCAGGCTccATGGAGGCGCACGTGGAGAG-3'	
attB2-OsPYL2	5'-AGAAAGCTGGGTcCTAGTCGCGCCGCCGCGAAG-3'	
attB1-OsPYL3	5'-AAAAAGCAGGCTccATGGTGGGGCTTGTGGGAGG-3'	
attB2-OsPYL3	5'-AGAAAGCTGGGTcCTACTGTTCAAGTGGCGAGGTG-3'	
attB1-OsPYL4	5'-AAAAAGCAGGCTccATGAACGGCGCTGGTGGTGC-3'	
attB2-OsPYL4	5'-AGAAAGCTGGGTcTCAAGGATTGGCAAGGCGCTC-3'	
attB1-OsPYL5	5'-AAAAAGCAGGCTccATGCCGTGCATCCCGGCGTC-3'	
attB2-OsPYL5	5'-AGAAAGCTGGGTcTCACGAGCCGGCGGCCCTCG-3'	
attB1-OsPYL6	5'-AAAAAGCAGGCTccATGATGCCGTACACCGCTCC-3'	
attB2-OsPYL6	5'-AGAAAGCTGGGTcCTAGGCGGCGCGGCGCGGC-3'	
attB1_adapter	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'	
attB2_adapter	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'	

To be continued

Table of Primers

Name	Sequence	Target Vector
PYL3-E98A_FW	5'-AGCGTT GCA ATACTCGAAGTTCTTGAC-3'	pGBKT7
PYL3-E98A_RV	5'-GAGTAT TGC AACGCTTGTTGACGCTGG-3'	
PYL3-L91A_FW	5'-TCTGGT GCA CCAGCGTCAACAAGCGTT-3'	
PYL3-L91A_RV	5'-CGCTGG TGC ACCAGAGACCACGCTAAC-3'	
PYL5-E98A_FW	5'-AGCACCG GCA AGGCTCGAGATCTTGAC-3'	
PYL5-E98A_RV	5'-GAGCCT TGC GGTGCTCGAGACCGCCGG-3'	
PYL5-L91A_FW	5'-TCTGGA GCA CCGGCGGTCTCGAGCACC-3'	
PYL5-L91A_RV	5'-CGCCGG TGC TCCAGAGACCACCATGAC-3'	
PYL3-S195L_FW	5'-GTTAAG TTG AATCTACAGAATCTCG-3'	
PYL3-S195L_RV	5'-TAGATT CAA CTTAACGACCGTATCC-3'	
BamHI-GAI-1M_FW	5'- CGGGATCC atATGAAGAGAGATCATCAT-3'	pGADT7
BamHI-GAI-93P_FW	5'- CGGGATCC atCCTCCGTCGTCTAAC-3'	
BamHI-GAI-165G_FW	5'- CGGGATCC atGGTGTGCGTCTCGTTC-3'	
BamHI-GAI-228S_FW	5'- CGGGATCC atAGTCCAATCGACCACTCT-3'	
SacI-GAI-92N_RV	5'- GTGGAGCTC gATTAAGGTGGTGAG-3'	
SacI-GAI-164N_RV	5'- CTCGAGCTC gTTCCTCTGCGAGTCA-3'	
SacI-GAI-451V_RV	5'- CTCGAGCTC gCACAACTGTCAGATCTG-3'	
SacI-GAI-532N_RV	5'- CTCGAGCTC gCTAATTGGTGAGAG-3'	
BamHI-RGA-1M_FW	5'- CGGGATCC atATGAAGAGAGATCATC-3'	
BamHI-RGA-109L_FW	5'- CGGGATCC atCCTCCTCTCTTC-3'	
BamHI-RGA-217N_FW	5'- CGGGATCC agAACGGTGTTCGTTTAGTCC-3'	
BamHI-RGA-281N_FW	5'- CGGGATCC atAATCAGATCGATCATTGTCTC-3'	
SacI-RGA-108G_RV	5'- CTCGAGCTC gATTAAGCTCAGAGAG-3'	
SacI-RGA-216E_RV	5'- CTCGAGCTC gCTCTTGCGAGTCAACC-3'	
SacI-RGA-504V_RV	5'- CTCGAGCTC gCACCAGATTACAAATCTGT-3'	
SacI-RGA-587Y_RV	5'- CTCGAGCTC gTCAGTACGCCGC-3'	
EcoRI-SHR_FW	5'- CCGAATTC ATGGATACTCTCTTTAGACTAG-3'	
BamHI-SHR_RV	5'- CGGATCCC GTTGGCCGCCACGACTA-3'	
EcoRI-SCR_FW	5'- CGGAATTC ATGGCGGAATCCGGCGATTTCAA-3'	
BamHI-SCR_RV	5'- CGGGATCC AGAACGAGGCGTCCAAGCTGA-3'	
EcoRI-SCL3_FW	5'- CGGAATTC ATGGTGGCTATGTTCAAGAAGA-3'	
BamHI-SCL3_RV	5'- CGGGATCCC TTCTGCATCTCC-3'	

Letters in red are mutation sites

Letters underlined are restriction enzyme sites

Letters in lowercase are additional bases to ensure reading frame

Letters in bold are the bases flanking the recognition sequence by restriction enzyme that increase its efficiency

To be continued

Table of Primers

Name	Sequence	Target Vector
EcoRI-GAI_FW	5'- GCGAATTC ATGAAGAGAGATCATCATCTCATCATCAAG-3'	pGADT7
XhoI-GAI_RV	5'- GCCTCGAG CTAATTGGTGGAGAGTTTCCAAGCC-3'	
BamHI-RGA_FW	5'- GCGGATCC ggATGAAGAGAGATCATCACC AATTCCAAGG-3'	
XhoI-RGA_RV	5'- GCCTCGAG TCAGTACGCCGCCGTCG-3'	
SmaI-RGL1_FW	5'- GCCCCGGG cATGAAGAGAGAGCACAACCACCG-3'	
SacI-RGL1_RV	5'- GCGAGCTC TTATTCCACACGATTGATTGCGCCACG-3'	
SmaI-RGL2_FW	5'- GCCCCGGG cATGAAGAGAGGATACGGAGAAACATGG-3'	
XhoI-RGL2_RV	5'- GCCTCGAG TCAGGCGAGTTTCCACGCC-3'	
SmaI-RGL3_FW	5'- GCCCCGGG cATGAAACGAAGCCATCAAGAAACGTCTG-3'	
XhoI-RGL3_RV	5'- GCCTCGAG CTACCGCCGCAACTCCG-3'	
BamHI-PYL3_FW	5'- TCAGGATCC ATGAATCTTGCTCCAA-3'	pGEX-6P-1
EcoRI-PYL3_RV	5'- AGTGGAATTC agTCAGGTCGGAGAAG-3'	
BamHI-PYL5_FW	5'- TCAGGATCC ATGAGGTCACC GGT-3'	
EcoRI-PYL5_RV	5'- AGTGGAATTC agTTATTGCCGGTTGG-3'	
BamHI-PYL6_FW	5'- TAGGATCC ATGCCAACGTCGATACA-3'	
EcoRI-PYL6_RV	5'- CGCGGAATTC agTTACGAGAATTTAGAA-3'	
BamHI-ABI1_FW	5'- CGGGATCC AT GGAGGAAGTATCTCCG-3'	pET-32a
HindIII-ABI1_RV	5'- CCCAAGCTT G TCAGTTCAAGGGTTTG-3'	
PYL3-101bp	5'-TCGTCAACACCATACGGA-3'	Sequencing
PYL5-101bp	5'-ACTAACGGTTTCCACACGCT-3'	
pGEXseq-5'	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	
pGEXseq-3'	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	
pGBKT7-3'	5'-TTTTCGTTTAAAAACCTAAGAGTC-3'	
nEYFP-check-R1	5'-CGGTGGTGCAGATGAACTT-3'	
cEYFP-check-R1	5'-GAACTCCAGCAGGACCAT-3'	
nEYFP-check-F1	5'-GGACGACGGCAACTACAAG-3'	
cEYFP-check-F1	5'-CCGACCACTACCAGCAGAA-3'	
M13_FW	5'-GTAAAACGACGGCCAG-3'	
M13_RV	5'-CAGGAAACAGCTATGAC-3'	

Letters underlined are restriction enzyme sites

Letters in lowercase are additional bases to ensure reading frame

Letters in bold are the bases flanking the recognition sequence by restriction enzyme that increase its efficiency

To be continued

Table of Primers

Name	Sequence	
qABI5_FW	5'-GAAGAGAATGCGCAGCTAAA-3'	Real-time PCR
qABI5_RV	5'-TTGTGCCCTTGACTTCAAAC-3'	
qAct7_FW	5'-GATATTCAGCCACTTGTCTGTGAC-3'	
qAct7_RV	5'-CATGTTGATTGGATACTTCAGAG-3'	
qPYR1_FW	5'-GACGATTCGACAAACCACAA-3'	
qPYR1_RV	5'-GCGCATCTCGAAGTTTGTGTT-3'	
qPYL1_FW	5'-TATGTTGTTGATGTACCGGAAGG-3'	
qPYL1_RV	5'-CCGTATCAGCAAACAATCTCG-3'	
qPYL2_FW	5'-CCTCGAACCGGTTATCAAAA-3'	
qPYL2_RV	5'-GAGAGAAGTGCACGTGGTTG-3'	
qPYL3_FW	5'-AAGGGAAGTTAGCGTGGTCTC-3'	
qPYL3_RV	5'-ACTTCGAGTATCTCAACGCTTGT-3'	
qPYL4_FW	5'-CGATCCGTAACGACCCTTC-3'	
qPYL4_RV	5'-CGACGTAAGACTCGACAACG-3'	
qPYL5_FW	5'-CGTGGTGGTGGAGTCTTACA-3'	
qPYL5_RV	5'-AGAGTTTCCTCCTCCGTGTTT-3'	
qPYL6_FW	5'-GCATGAGTCGGAGGAGGAC-3'	
qPYL6_RV	5'-GACGTATGACTCAACGACACG-3'	
qPYL7_FW	5'-CACCAGTGCAGAGAGAACCA-3'	
qPYL7_RV	5'-CGCACCAGTGACCAAACA-3'	
qPYL8_FW	5'-CAACGCTCCTGTTCATATTGTG-3'	
qPYL8_RV	5'-TTCCTTTCACCACACATCTACTG-3'	
qPYL9_FW	5'-TCGGTGGTGATCACAGACTT-3'	
qPYL9_RV	5'-ACGATTCAATCACCATCGTTC-3'	
qPYL10_FW	5'-GCGAGTACATCAAGAAACACCAT-3'	
qPYL10_RV	5'-CCTCACAATTGACCACACGA-3'	
qPYL11_FW	5'-CCGTGAAGTGACGGTGGT-3'	
qPYL11_RV	5'-TCATCTAATCTCTCTCGGCTGAA-3'	
qPYL12_FW	5'-TTACCAGTCGAAAACACTACGGTGT-3'	
qPYL12_RV	5'-CCACATAACTCTCCACAACCAC-3'	
qPYL13_FW	5'-CGGAGGATATGGCAAAGAAG-3'	
qPYL13_RV	5'-CTAGTTCCTTCCGGCACATC-3'	

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