Technische Universität München

Lehrstuhl für Botanik

Regulation of the abscisic acid response by protein

phosphatase 2C-interacting proteins ABP7 and ABP9 in

Arabidopsis thaliana

Yue Ma-Lauer

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. R. Hückelhoven

Prüfer der Dissertation: 1. Univ.-Prof. Dr. E. Grill
                          2. apl. Prof. Dr. R. A. Torres Ruiz

Die Dissertation wurde am 07.12.2010 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 21.03.2011 angenommen.

Content

Abbreviations

ABA	abscisic acid
ABI	ABA insensitive
ABRE	ABA response element
ACC	1-aminocyclopropane-1-carboxylic acid
Amp	ampicillin
AtHB	homeodomain protein in *Arabidopsis thaliana*
ATP	adenosine 5'-triphosphate
BAP	6-benzylaminopurine
ß-ME	ß-mercaptoethanol
ß-gal	ß-galactosidase
b-HLH-ZIP	basic helix-loop-helix leucine zipper
BiFC	bimolecular fluorescence complementation
bp	base pair
BSA	bovine serum albumin
bZIP	basic-leucine zipper
CaMV	Cauliflower mosaic virus
CE	coupling element
cDNA	complementary deoxyribonucleic acid
CHLH	H subunit of the magnesium-protoporphyrin-IX chelatase
CTAB	cetyltrimethylammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DRE	drought response element

DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
E. coli	*Escherichia coli*
EGFP	enhanced GFP
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtBr	ethidiumbromid
EtOH	ethanol
FT-ICR	Fourier Transform-Ion Cyclotron Resonance
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GTG	GPCR-type G protein
GUS	β-glucuronidase
HAB1	homologue to ABI1
HD-ZIP	homeodomain leucine zipper
hr	hour
IAA	Indole-3-acetic acid
IPTG	isopropyl β-D-thiogalactoside
Kan	kanamycin
kb	kilobase
kDa	kilo Dalton
LB	Luria-Bertani medium
LS	Linsmaier and Skoog medium
Lti	low temperature induced protein
Lti::LUC	luciferase gene under the control of LTI promoter
LUC	luciferase
MES	2-(N-morpholino)ethanesulfonic acid

min	minute
mRNA	messenger RNA
MS	Murashige and Skoog medium
MUG	4- methylumbelliferyl -β-D-glucuronide
MUP	methylumbelliferyl phosphate
NAA	1-naphthaleneacetic acid
NCED	9-*cis*-epoxycarotenoid dioxygenase
Ni-NTA	Ni-Nitrilotriacetic acid
NOS	nopaline synthase
OD	optimal density
Ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PP2C	protein serine/threonine phosphatases of type 2C
PRs	pathogenesis-related proteins
PYL	PYR1-Like
PYR1	PYRABACTIN RESISTANCE1
Rab18	rab-related protein (dehydrin gene family)
Rab18::LUC	luciferase gene under the control of Rab18 promoter
RCAR	regulatory components of ABA receptor
RFU	relative fluorescence units
RLU	relative light units
Rif	rifampicin
RNA	ribonucleic acid
RNAi	RNA interference

rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	N,N,N,N'-tetramethylethylenediamine
TBS	Tris-Buffered Saline
TBST	TBS buffer containing 0.05% Tween-20
Tris	Tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
UTR	untranslated region
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP	yellow fluorescent protein

Amino acids:

A,	Ala	Alanine	M,	Met	Methionine
C,	Cys	Cysteine	N,	Asn	Asparagine
D,	Asp	Aspartic acid	P,	Pro	Proline
E,	Glu	Glutamic acid	Q,	Gln	Glutamine
F,	Phe	Phenylalanine	R,	Arg	Arginine
G,	Gly	Glycine	S,	Ser	Serine
H,	His	Histidine	T,	Thr	Threonine

I,   Ile   Isoleucine   V,   Val   Valine

K,   Lys   Lysine   W,   Trp   Tryptophan

L,   Leu   Leucine   Y,   Tyr   Tyrosine

Bases:

A:Adenine   C:Cytosine   G:Guanine   T:Thymine   U:Uracil

The genes are written in italic capital letters, e.g. *ABI1, ABI2*

The proteins are written in capital letters, e.g. ABI1, ABI2

Mutants are written in italic lowercase letters, e.g. *abi1, abi2*

Summary

The phytohormone abscisic acid (ABA) plays an important role in mediating many aspects of plant growth and development, as well as in response to abiotic stresses (Leung and Giraudat, 1998). The protein phosphatases 2C ABI1 and its homologue ABI2 are key regulators that negatively mediate ABA signalling to a large extent (Schweighofer *et al.*, 2004). The genes *ABP7* (At5g53160.2) and *ABP9* (At1g01360) code for two homologous proteins, which were identified independently as interacting partners of ABI2 in the yeast two-hybrid system by screening the Arabidopsis cDNA library.

ABP7 and ABP9 belong to a 14-member protein family and display similarities to birch allergen Bet v 1 protein. In order to characterize the functions of ABP7 and ABP9, ABP7- and ABP9-deregulated constructs were designed for the ABA sensitivity tests in both the transient expression system and the corresponding transgenic plants. In the transient expression system, overexpression of ABP7 and ABP9 in diverse wild type Arabidopsis protoplasts resulted in dramatic inductions of ABA responses both in the absence and presence of exogenous ABA. Consistently, knockdown of ABP7 and ABP9's expression by RNAi technique resulted in reductions of ABA responses, indicating that ABP7 and ABP9 function as positive regulators in ABA signalling. Overexpression of ABP7 and ABP9 also caused a marked induction of ABA responses in an ABA-deficient mutant *aba2-1*, ABA-insensitive mutant *abi1-1*, and ABA-insensitive mutant *abi2-1*, respectively. The ABP7- and ABP9-mediated stimulation of the ABA responses is antagonized by co-expression of ABI1 and ABI2. *Arabidopsis thaliana* with ectopic expression of ABP9 displayed clear ABA hypersensitivity in the root growth response, seed germination, and stomatal closure. The transgenic Arabidopsis with ectopic expression of ABP7 or with knockdown

expression of the ABP9's family by RNAi technique did not show any phenotype in those physiological responses. Some of the RNAi knockdown transgenic Arabidopsis seedlings exhibited developmental problems, such as only one single cotyledon, extremely short roots, and no primary leaves. All of these developmental problems were ABA-induced. Furthermore, some interesting phenotypes were found in respect to auxin.

ABP9 was located in root tips, root crowns, stipules, stomata, vascular tissues, mesophyll cells, and the centers of anthers, as revealed by the histochemical staining of transgenic Arabidopsis expressing endogenous promoter::genomic ABP9::β-glucuronidase (GUS). RT-PCR analysis indicated that ABP7 and ABP9 were downregulated by ABA, but not regulated by auxin. In addition, ABP9 was upregulated by Agrobacteria when transgenic Arabidopsis seedlings expressing endogenous promoter::genomic ABP9::GUS were inoculated with Agrobacterium GV3101.

The interactions between ABP proteins (ABP7 and ABP9) and ABI proteins (ABI1 and ABI2) were confirmed in Arabidopsis protoplasts by the method of bimolecular fluorescence complementation. ABI1 interacted with both ABP proteins in the nucleus and the cytosol. ABI2 interacted with ABP7 in the nucleus and the cytosol, but it only interacted with ABP9 in the cytosol. The subcellular localization of ABP7- and ABP9-GFP fusion proteins was consistent with the previous result.

According to the similarity of ABP9 to birch allergen Bet v 1, which interacts with a broad spectrum of ligands including cytokinin (Mogensen et al., 2002), it is likely that ABP9 protein possesses ligand(s) binding activity as well. Based on this assumption, the binding assays between ABP9 protein and ^{14}C-ABA or ^{14}C-zeatin were set up. The result showed that ABP9 not detectably interact with ^{14}C-ABA or ^{14}C-zeatin in these assays. The protein phosphatase assays,

however, demonstrated that ABP9 could inhibit the PP2C activities of ABI1 and ABI2 in an ABA-dependent manner.

Zusammenfassung

Das Pflanzenhormon Abscisinsäure (ABA) spielt eine wichtige Rolle bei der Regulierung des Pflanzenwachstums und der Pflanzenentwicklung, sowie bei der Reaktion auf abiotischen Stress (Leung and Giraudat, 1998). Die Proteinphosphatasen 2C ABI1 und das homologe Protein ABI2 sind negative Schlüsselregulatoren der ABA-Signaltransduktion (Schweighofer *et al.*, 2004). Die Gene *ABP7* (At5g53160.2) und *ABP9* (At1g01360) kodieren für zwei homologe Proteine, die unabhängig voneinander als Interaktionspartner von ABI2 im Hefe-Zwei-Hybrid-System durch Screening einer Arabidopsis cDNA Bank identifiziert wurden.

ABP7 und ABP9 gehören einer Proteinfamilie von insgesamt 14 Mitglieder an und zeigen Ähnlichkeiten zum Birkenallergen Bet v 1. Um die Funktionen dieser Arabidopsisproteine zu analysieren, wurden ABP7 und ABP9 Konstrukte sowohl im transienten System in Protoplasten als auch in transgenen Pflanzen exprimiert. Im transienten Expressionssystem zeigte die Überexpression von ABP7 und ABP9 in Protoplasten verschiedener Arabidopsiswildtypen eine drastische Induktion der ABA-Antworten in Abwesenheit und Anwesenheit exogener ABA. Ein Knockdown von ABP7 und ABP9 durch RNAi-Technik führte zu einer Verringerung der ABA-Antworten und zeigte, dass ABP7 und ABP9 positive Regulatoren der ABA-Signaltransduktion sind. Die Expression von ABP7 und von ABP9 zeigte auch eine eindeutige Induktion der ABA-Antwort in Protoplasten der ABA-defizitienten Mutante *aba2-1*, in der ABA-insensitiven Mutante *abi1-1* und der ABA-insensitiven Mutante *abi2-1*. Die ABP7- und ABP9-vermittelte Stimulierung der ABA-Antwort wird durch Ko-Expression von ABI1 und ABI2 antagonisiert. *Arabidopsis thaliana* mit ektopischer Expression von ABP9 zeigte eine ABA-Hypersentitivität in den Tests des Wurzelwachstums, der

Samenkeimung und der Schließung der Stomata. Transgene Arabidopsispflanzen mit ektopischer Expression von ABP7 oder mit Knockdown der ABP9 Familie durch RNAi-Technik zeigten keinen Phänotyp in jenen physiologischen ABA Tests. Diese RNAi-Arabidopsissämlinge wiesen teilweise Entwicklungsprobleme auf, z.B. nur ein einzelnes Keimblatt, extrem kurze Wurzeln oder keine Primärblätter. Diese Änderungen waren ABA-abhängig. Außerdem wurden Auxin-relevante Phänotypen gefunden.

ABP9 war in Wurzelspitzen, Wurzelkronen, Nebenblättern, Stomata, Gefäßgeweben, Mesophyllzellen und in den Antheren lokalisiert. RT-PCR Analyse zeigte, dass ABP7 und ABP9 durch ABA herunterreguliert, aber nicht durch Auxin beeinflusst wurden. Zusätzlich wurde ABP9 nach Agrobakterieninfektion hochreguliert.

Die Interaktionen zwischen ABP Proteinen (ABP7 und ABP9) und ABI Proteinen (ABI1 und ABI2) wurden in den *Arabidopsis*-Protoplasten durch die Bimolekulare Fluoreszenzkomplementations-Methode bestätigt. ABI1 interagierte mit ABP im Kern und im Zytosol. ABI2 Interaktion mit ABP7 konnte im Kern und im Zytosol nachgewiesen werden, mit ABP9 jedoch nur im Zytosol. Die intrazelluläre Lokalisierung von ABP7 und ABP9 als Fusionsprotein mit GFP war im Einklang mit den Ergebnissen der Fluoreszenzkomplementation.

Die Strukturähnlichkeit von ABP9 zum Birkenallergen Bet v 1, das niedermolekulare Liganden einschließlich Cytokinin zu binden vermag (Mogensen *et al.*, 2002), deutete darauf hin, dass ABP9 eine mögliche Liganden-bindende Fähigkeit besitzt. Bindungs-Assays zwischen dem Protein ABP9 und ^{14}C-ABA oder ^{14}C-Zeatin zeigte keine Bindung von ^{14}C-ABA oder ^{14}C-Zeatin. Die biochemische Analyse von ABI1 und ABI2 belegte, dass ABP9 die Proteinphosphataseaktivität von ABI1 und ABI2 auf eine ABA-abhängige

Art und Weise blockieren konnte.

1. <u>Introduction</u>

1.1   ABA signal

The phytohormone abscisic acid (ABA) is widely distributed in plants and some fungi. Its major function is to mediate many aspects of plant growth and development, such as seed maturation, dormancy, and germination. In addition, ABA has a vital function in plant adaptation to abiotic environmental stresses, such as drought, salt, and cold, by regulating stomatal aperture and the expression of stress-responsive genes (Leung and Giraudat, 1998; Finkelstein *et al*., 2002; Himmelbach *et al*., 2003; Christmann *et al.*, 2006). These diverse functions of ABA involve complex regulatory mechanisms that control its production, degradation, signal perception, and transduction.

The molecular formula of ABA is $C_{15}H_{20}O_4$, with a molecular weight of 264.3. It consists of an aliphatic ring with one double bond, two methyl groups, and an unsaturated chain with a terminal carboxyl group (Figure 1). There are *cis* and *trans* isomers of ABA, which is determined by the position of the protons at C-2 and C-4 and the ensuring orientation of carboxyl group at C-2. *cis* ABA also has an asymmetric carbon atom at the ring resulting in the S (+) and R (-) isomers (Milborrow *et al*., 1970; Taylor *et al.*, 1973). (+) *cis* ABA is the most abundant, naturally occurring form.
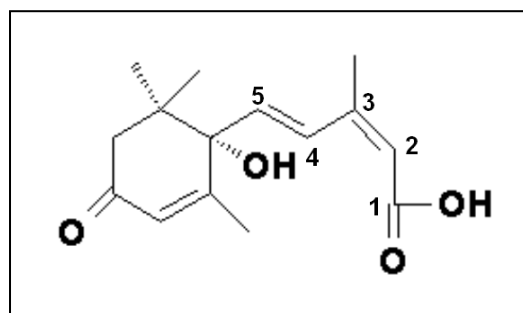
Figure 1. Structure of Abscisic Acid (ABA).

- *ABA biosynthesis*

ABA is synthesized in almost all cells containing chloroplasts or amyloplasts. Since the discovery of ABA in the early 1960s, much effort has been devoted to the study of ABA biosynthesis. In recent years, almost all of the major genes for the enzymes in the biosynthesis pathway have been identified and the pathway for ABA biosynthesis in higher plants is now understood in great detail (Schwarts *et al*., 2003). Two possible routes have been suggested for ABA biosynthesis, one direct and one indirect, in which ABA is derived from the C_{15} compound farnesyl pyrophosphate and a C_{40} carotenoid, respectively (Seo and Koshiba, 2002). The indirect pathway through the cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via abscisic aldehyde, is the main pathway in higher plants.

As shown in Figure 2, the early steps of ABA biosynthesis are the carotenoid precursor synthesis. ABA is synthesized from C_{40} carotenoids (phytoene, ζ-carotene, lycopene and β-carotene). Carotenoids are synthesized from a C_5 compound, isopentenyl pyrophosphate (IPP). In plastids, IPP is synthesized via 1-deoxy-D-xylulose-5-phosphate (DXP) from glyceraldehyde-3-phosphate and pyruvate. The middle steps of ABA biosynthesis are the formation of epoxycarotenoid and its cleavage in plastid, which are shown in the (b) portion of Figure 2. The first step of this part of the pathway is the epoxidation of zeaxanthin to form all-*trans*-violaxanthin catalysed by zeaxanthin epoxidase (ZEP). A reverse reaction occurs in chloroplasts in high light conditions catalysed by violaxanthin de-epoxidase (VDE). The formation of *cis*-isomers of violaxanthin and neoxanthin may require two enzymes, neoxanthin synthase (NSY) and an isomerase. Recent studies show that the ABA4 gene (from Arabidopsis*)* encoding a 17 kDa protein in the proteome of the chloroplast envelope membrane is the corresponding NSY (North *et al.,* 2007). 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyses the oxidative cleavage of a 9-*cis* isomer of epoxycarotenoid such as 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin to

(a) Glyceraldehyde-3-phosphate +
pyruvate

$\downarrow$ **DXS**

DXP

$\downarrow$

IPP (C5)

$\downarrow$

Farnesyl pyrophosphate (C15)

$\downarrow$

GGPP (C20)

$\downarrow$ **PSY**

Phytoene (C40)

$\downarrow$ **PDS**

ζ-Carotene

C40
carotenoids

Lycopene

β-Carotene

(b)

Zeaxanthin

ZEP ↑↓ **VDE**

Antheraxanthin

ZEP ↑↓ **VDE**

all-*trans*-Violaxanthin

NSY   **Isomerase?**

Neoxanthin

Isomerase?   **9-*cis*-Violaxanthin**

9′-*cis*-Neoxanthin

NCED   **NCED**

Xanthoxin   **(c)**

ABA2

Abscisic aldehyde

Xanthoxic   **AAO** **MoCo**   **Abscisic**
acid   **alcohol**

Abscisic acid

Plastid

Cytosol

Figure 2. The ABA biosynthesis pathway (modified from Nambara and Marion-Poll, 2005; Seo and Koshiba, 2002). (a) Carotenoid precursor synthesis in the early steps of ABA biosynthesis. (b) Formation of epoxycarotenoid and its cleavage in plastid. (c) Reactions in the cytosol for the formation of ABA. During this process, xanthoxin is converted by ABA2 into abscisic aldeyhde, which is oxidized into ABA by AAO. Besides this pathway, another two possible pathways from xanthoxin to ABA may also work. They are shown with dashed arrows in orange colour. Abbreviations: AAO, abscisic aldehyde oxidase; DXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DXP synthase; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; MoCo, molybdenum cofactor sulfurase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NSY, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase.

form xanthoxin. The cleavage product xanthoxin is further processed in the cytosol. The later steps of ABA biosynthesis are from xanthoxin to ABA, which are shown in the (c) portion of Figure 2. Xanthoxin is converted by a short-chain alcohol dehydrogenase ABA2 into abscisic aldeyhde, which is oxidized into ABA by abscisic aldehyde oxidase (AAO). AAO contains a molybdenum cofactor activated by a MoCo sulfurase. Besides this pathway, another two possible pathways from xanthoxin to ABA may also work (Seo and Koshiba, 2002). They are indicated by dashed arrows in an orange colour in Figure 2. The possible pathway via xanthoxic acid was suggested because the inhibition of AAO activity by tungstate (a potent inhibitor of the molybdo-enzymes) in ripening avocado fruits results in the accumulation of xanthoxin (Lee and Milborrow, 1997) and some of the Arabidopsis AAO isoforms can oxidize xanthoxin in activity gel staining after native gel electrophoresis (Seo and Koshiba, 2002). These indicate that xanthoxin is a substrate of AAO and AAO might act to convert xanthoxin to xanthoxic acid. The other possible pathway via abscisic alcohol might be activated in some mutants. In *flacca* and *sitiens* tomato mutants, exogenously supplied abscisic aldehyde is converted to abscisic alcohol, showing that abscisic aldehyde is reduced to abscisic alcohol and then oxidized to ABA via a shunt pathway (Rock *et al*., 1991). The shunt pathway appears to be a minor source of ABA in wild-type plants but might play a significant role in mutants impaired in their capacity to oxidize abscisic aldehyde to ABA directly.

- *Regulation of ABA biosynthesis*

ABA biosynthesis can be regulated by seed maturation and germination, drought and salt stress, ABA itself, and circadian rhythm (Bentsink and Koornneef, 2002; Finkelstein *et al.,* 2002; Xiong and Zhu, 2003).

During seed development, there are two peaks of ABA accumulation. The first one is derived from maternal tissues and promotes the synthesis of storage proteins. The second peak is one-third as significant as the first one and is from biosynthesis in the
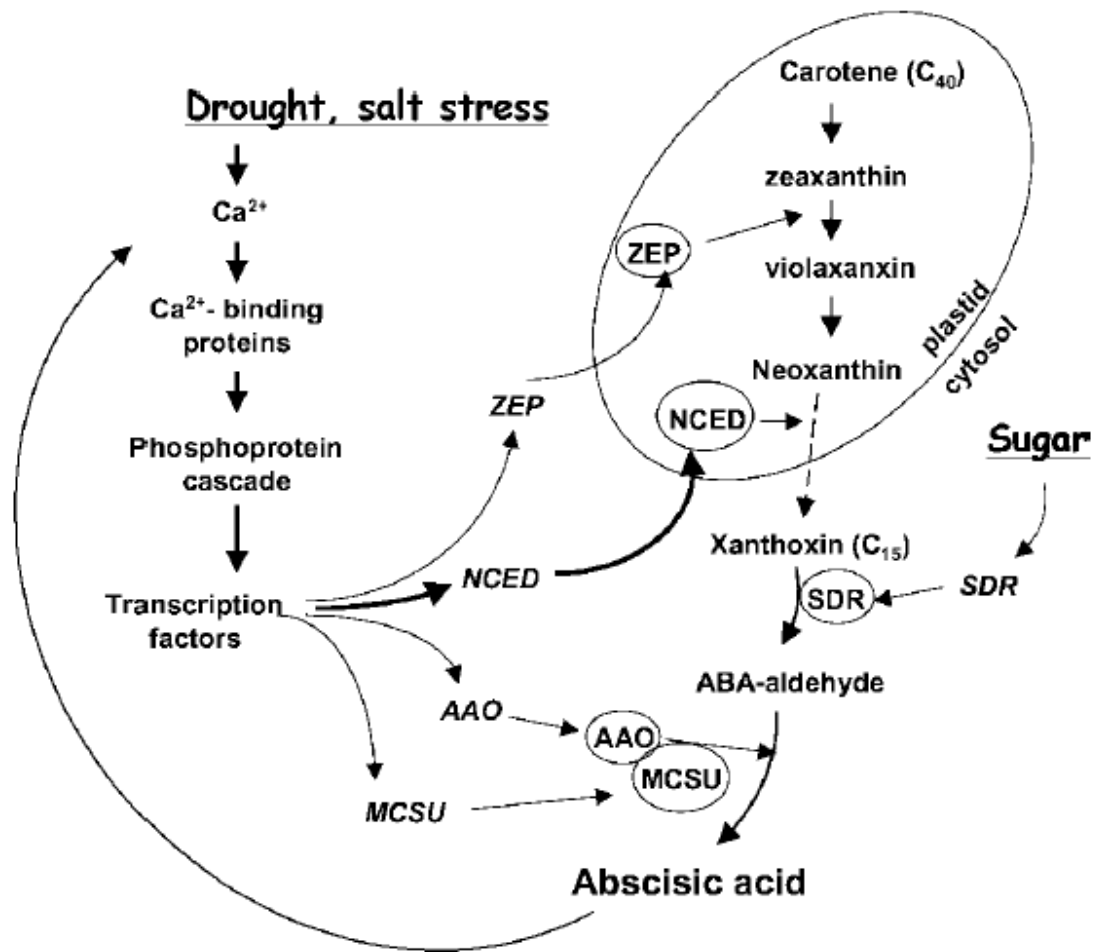
Figure 3. Regulation of ABA biosynthesis (Xiong and Zhu, 2003). Abiotic stresses such as drought and salt activate the biosynthetic genes (italicised), probably through a Ca^{2+}-dependent phosphorelay cascade as shown on the left. ABA feedback stimulates the expression of the biosynthetic genes, which is also likely through a Ca^{2+}-dependent phosphoprotein cascade. Among the biosynthetic genes, NCED is strongly upregulated by stress (indicated with a thick arrow), whereas SDR (ABA2 in Arabidopsis) is regulated by sugar. ABA biosynthetic enzymes are shown in small ovals. The NCED step probably limits ABA biosynthesis in leaves (indicated by a dashed arrow). Abbreviations: AAO, abscisic aldehyde oxidase; MCSU, molybdenum cofactor sulfurase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; SDR, short-chain alcohol dehydrogenase/reductase; ZEP, zeaxanthin epoxidase.

embryo. It may activate the synthesis of late embryogenesis abundant (LEA) proteins and initiate seed dormancy (Bentsink and Koornneef, 2002; Finkelstein *et al.*, 2002). The transcripts for all of the ABA biosynthesis genes are detected in developing seeds. Soluble sugars, osmotic stress, and ABA itself are known to be the signals to regulate ABA biosynthesis in developing seeds (Xiong and Zhu, 2003). The genes of ZEP, AAO, and molybdenum cofactor sulfurase (MoCo sulfurase, or MCSU) are the enzymes participating in ABA biosynthesis. They are induced by sugar level, osmotic stress, and ABA. However, the short-chain alcohol dehydrogenase/reductase SDR (ABA2 in Arabidopsis) is only induced by sugars, not by either osmotic stress or ABA. The NCED gene is induced by both drought stress and ABA, but not by sugars.

The environmental conditions that most dramatically activate ABA synthesis are drought and salt stress. Drought and salt stress induce ABA biosynthesis largely through transcriptional regulation of ABA biosynthesis genes, although regulation of the specific activation of ABA biosynthesis enzymes also exists (Xiong and Zhu, 2003). The regulation of ABA biosynthetic genes by drought and salt stress may vary between different plant parts, developmental stages, and different plant species. The ABA biosynthesis rate limiting gene NCED, which catalyses the cleavage step of xanthophylls, 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin to produce xanthoxin, was reported to be strongly upregulated by drought stress treatment in many plant species, such as maize, tomato, bean, Arabidopsis, and avocado. With the exception of SDR gene, all the other ABA biosynthetic genes are known to be upregulated by drought and salt stress (Seo *et al.*, 2000; Iuchi *et al.*, 2001; Xiong *et al.*, 2001; Xiong *et al.*, 2002).

ABA has long been thought to negatively regulate ABA accumulation by activating its catabolic enzymes. However, Cheng *et al.* (2002) reported that AtZEP, AtNCED3, and AtAAO3 genes can be induced by ABA in an Arabidopsis Landsberg background. This suggests that there is a positive feedback regulation of ABA biosynthesis by ABA. In addition, AtNCED3 transcript levels under drought and salt stress treatments were

significantly reduced in the ABA-deficient mutants los5 and los6 as compared with those in wild-type seedlings, demonstrating that ABA is required for full activation of AtNCED3 by osmotic stress (Xiong *et al.,* 2002). Figure 3 shows the regulation of ABA biosynthesis by drought and salt stress, ABA itself, and sugar.

In tobacco and tomato, ZEP expression was found to fluctuate according to a diurnal rhythm during a time lapse consisting of light and dark periods (Audran *et al.*, 1998; Thompson *et al.*, 2000). In addition, the diurnal fluctuation of NCED expression was observed in tomato leaves (Thompson *et al.*, 2000). However, the peak of NCED expression occurs at the end of the light period, whereas the expression of ZEP peaks in the middle of the light period, indicating the existence of different diurnally regulatory systems for each gene (Xiong and Zhu, 2003).

- *ABA signal transduction--ABA receptors*

The plethora of ABA responses is initiated by ABA perception. ABA perception sites locate on both the "inside" and "outside" of plant cells, which was detected over a decade ago (MacRobbie, 1995; Leung and Giraudat, 1998). This finding indicates the existence of both plasmamembrane-localized ABA receptor(s) and intracellular ABA receptor(s). However, despite a long history of attempts to identify receptor candidates, the receptors of ABA remained completely unknown until the last four years. Since 2006, it has been suggested that several proteins act as ABA receptors. These proteins are FCA, CHLH, GCR2, GTG1 and GTG2 (Razem *et al.*, 2006; Shen *et al.*, 2006; Liu *et al.*, 2007; Pandey et al., 2009). FCA is a nuclear RNA-binding protein and is involved in RNA metabolism and in controlling flowering time (Razem *et al.*, 2006). But FCA was finally demonstrated not to bind ABA, indicating that it is actually not an ABA receptor (Razem *et al.*, 2008; Risk JM *et al.*, 2008). CHLH is the H subunit of the magnesium-protoporphyrin-IX chelatase (Mg-chelatase), which is a key component in both chlorophyll biosynthesis and plastid-to-nucleus signalling. CHLH binds the physiologically active form (+)-ABA in a highly stereospecific manner. It

positively regulates ABA signalling in all facets including stomatal closure, seed germination, vegetative growth, and gene expression (Shen *et al.*, 2006). GCR2 is a debatable ABA receptor candidate. It was incipiently reported as a G protein-coupled receptor (GPCR) and a plasma membrane receptor for ABA (Liu *et al.*, 2007). Nevertheless, several independent studies have shown that GCR2 is not a GPCR, it is unlikely to be a transmembrane protein, and it is not required for ABA response in seed germination and early seedling development (Gao *et al.*, 2007; Guo *et al.*, 2008). GTG1 and GTG2 are two plasmamembrane proteins with homology to G protein-coupled receptors (GPCRs). These two proteins directly bind ABA in vitro in a stereoselective manner. And the loss of both GTG1 and GTG2 caused ABA hyposensitivity (Pandey *et al.*, 2009; Christmann and Grill, 2009). Thus, the GTGs are two promising candidates of the plasmamembrane-localized ABA receptors.

- *ABA signal transduction—phosphorylation and dephosphorylation*

Reversible protein phosphorylation is an early and central event in ABA signal transduction. A number of protein phosphatases such as PP2Cs, and protein kinases such as OST1, play important roles in ABA-induced stomatal closure and gene expression (Christmann *et al.*, 2006; Fedoroff, 2002; Wasilewska *et al.*, 2008).

The PP2Cs (ABI1, ABI2, AtPP2CA, HAB1) in Arabidopsis are defined as negative regulators of ABA responses and are transcriptionally upregulated by ABA (Schweighofer *et al.*, 2004; Christmann *et al.*, 2006). ABI1 and its homologue ABI2 are partly redundant key regulators of diverse ABA-mediated responses including seed germination, stomatal closure, and vegetative growth. ABI1 and ABI2 also interact with several cellular targets. One of the direct targets of ABI1 is the homeodomain transcription factor AtHB6, which is upregulated in dependence on both ABA and ABI1 (Himmelbach *et al.*, 2002; Christmann *et al.*, 2006). There is also an interaction between ABI1 and protein kinase OST1/SRK2E/SnRK2.6 (Yoshida *et al.*, 2006 a). OST1 can be activated by ABA and by hyperosmotic stress independently of ABA

(Yoshida *et al.*, 2002). And the interaction between ABI1 and OST1 is supposed to activate the kinase thereby promoting stomatal closure (Yoshida *et al.*, 2006 a; Christmann *et al.*, 2006). Phosphatic acid (PA) is another interaction partner of ABI1. PA is hypothesized to prevent ABI1's negative regulation to ABA responses by sequestering ABI1 to the plasma membrane, thereby decreasing the translocation of ABI1 from the cytosol to the nucleus (Mishra *et al.*, 2006). ABI2, the homologue of ABI1, physically interacts with a calcium sensor SCaBP5 and its associated protein kinase PKS3/CIPK15 (Guo *et al.*, 2002). ABI2 also interacts with another protein kinase Salt Overly Sensitive 2 (SOS2) which is required for salt tolerance in *Arabidopsis thaliana* (Ohta *et al.*, 2003). Moreover, the precursor protein of fibrillin is also an interacting partner of ABI2 (Yang *et al.*, 2006). The other PP2Cs that function as negative regulators in ABA signalling are AtPP2CA and HAB1. They mainly play their roles in the ABA responses in seeds rather than stomatal closure and root growth (Kuhn *et al.*, 2006; Saez *et al.*, 2004; Yoshida *et al.*, 2006 b).

The protein kinase OPEN STOMATA1 (OST1) is activated by ABA and is one of the ten members belonging to the Sucrose Non-Fermenting Related Kinase2 (SnRK2) family. The *ost1* mutations impede stomatal response to ABA, leading to hypersensitivity to mild drought conditions (Mustilli *et al.*, 2002). Besides OST1, another two family members, SnRK2.2 and SnRK2.3, are also highly inducible by exogenous ABA (Boudsocq *et al.*, 2004). The Arabidopsis OST1, SnRK2.2, SnRK2.3, and SnRK2.8 (responding to hyperosmotic stress independently of ABA), all phosphorylate in vitro a motif in the constant subdomains existing among basic-leucine zipper (bZIP) transcription factors, including ABA Response Element Binding protein (AREB)1, AREB2, and ABI5 (Furihata *et al.*, 2006; Wasilewska *et al.*, 2008).

To induce stomatal closure, ABA first triggers cytosolic calcium ($[Ca^{2+}]_{cyt}$) increases in guard cells. Then, the $[Ca^{2+}]_{cyt}$ elevation activates S-type (Slow-activating sustained) anion channels via reversible phosphorylation events (Maeser *et al.*, 2003; Schmidt *et al.*, 1995). Calcium-dependent protein kinases (CDPKs) play important roles in this

step. Two insertion mutants of CDPKs, *cpk3* and *cpk6,* were tested and both of them showed impairment of the upregulation of anion currents by ABA (Mori *et al.*, 2006).

- *ABA signal transduction—transcriptional and post-transcriptional regulation*

ABA can mediate expression of many genes such as ABI1, ABI2, AtHB6, RD29A, RD29B, RD22, Late Embryogenesis Abundant (LEA) and so on (Hoth *et al.*, 2002; Himmelbach *et al.*, 2002; Soederman *et al.*, 1999; Nakashima *et al.*, 2006; Abe *et al*., 1997). Regulators of the ABA-responsive genes expression are a number of transcription factors of different types. For example, AtHB6 is a HD-ZIP (homeodomain leucine zipper) transcription factor, which targets an AT-rich *cis* element (CAATTATTA) also in its own promoter (Himmelbach *et al*., 2002). ABI3 and ABI5 encode transcription factors and appear to act in combination to control embryonic gene expression and seed sensitivity to ABA in Arabidopsis (Nakashima *et al.*, 2006). ABI3 has a B3 domain. It encodes a transcription factor homologous to maize VP1 and has been demonstrated to be an important regulator of LEA protein (Nakashima *et al.*, 2006). ABI5 encodes a bZIP transcription factor and binds ABREs (ABA-responsive element; ACGTGG/TC) in vitro (Bensmihen *et al.*, 2002). In addition to the ABREs, a coupling element (CE1) has been demonstrated to be involved in ABA included expression. The AP2-type transcription factor *maize* ABI4 homolog has been demonstrated to bind to the CE1 element in a number of ABA-responsive genes (Niu *et al.*, 2002). The dehydration- and ABA-responsive gene RD22 is targeted by the MYB transcription factor and the b-HLH-ZIP (basic helix-loop-helix leucine zipper) MYC transcription factor (Abe *et al.*, 1997). The MYB/MYC system regulates slow adaptive responses to dehydration stress.

In addition to transcriptional regulation, ABA responses can also be regulated post-transcriptionally by mRNA processing and degradation. The ABH1 gene, which has been identified as a mutant with increased ABA sensitivity based on seed germination, encodes the Arabidopsis homolog of a nuclear mRNA cap-binding protein

and functions in a heterodimeric complex to bind the mRNA cap structure (Hugouvieux *et al.*, 2001). Interestingly, the loss of function of another cap-binding protein CBP20 also confers hypersensitivity to ABA during germination (Papp *et al.*, 2004). The gene ABA HYPERSENSITIVE GERMINATION 2 (AHG2), which is induced by ABA, encodes a poly(A)-specific ribonuclease and is hypothesized to function in mRNA degradation. The *ahg2-1* mutant showed ABA hypersensitivity not only in germination, but also at later developmental stages (Nishimura *et al.*, 2005). It is conceivable that these RNA-binding proteins mediate ABA responses by influencing the decay rates of the transcripts of certain ABA regulators. Besides RNA binding proteins, microRNA has been demonstrated as another pathway to regulate ABA-associated transcripts. MicroRNAs (miRNA) are single-stranded RNA molecules of 20-24 nucleotides in length. They can result in certain mRNA degradation by pairing the partially complementary mRNAs (Sunkar and Zhu, 2004). One recent study showed that ABA induces the accumulation of microRNA159 in an ABI3-dependent fashion in germinating Arabidopsis seeds, and microRNA159 mediates cleavage of MYB101 and MYB33 transcripts in vitro and in vivo (Reyes and Chua, 2007).

- *ABA signal transduction—secondary messengers*

Ca^{2+}, cyclic ADP-ribose (cADPR), inositol 1,4,5 trisphosphate ($InsP_3$), inositol hexakisphosphate ($InsP_6$), diacylglycerol pyrophosphate or H_2O_2 have been identified as "secondary messengers" of ABA signalling (Schroeder *et al.*, 2001; Zalejski *et al.*, 2005; Christmann *et al.*, 2006). The elevation of $[Ca^{2+}]_{cyt}$ in guard cells during ABA responses frequently follows Ca^{2+} oscillations, then calcium sensors such as CPK3 and CPK6 transduce the Ca^{2+} signal further downstream (Mori *et al.*, 2006). cADPR is an endogenous Ca^{2+}-mobilizing second messenger found in cells of animals, plants, and protozoans (Guse, 2008). In plants, cADPR not only induces the increases in $[Ca^{2+}]_{cyt}$ with consequent decreases in stomatal aperture, but is also intimately involved in ABA-turgor signalling in guard cells (Leckie *et al*, 1998). Both ABA and pathogen attacks can trigger the formation of the secondary messenger hydrogen

peroxide which is dependent on the NADPH oxidases *RbohD* and *RbohF* (Kwak *et al.*, 2003; Torres and Dangl, 2005). In addition, ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis (Bright *et al.*, 2006). H_2O_2 can also rapidly inactivate the ABA negative key regulators ABI1 and ABI2 (Meinhard and Grill, 2001; Meinhard *et al.*, 2002).

- *The role of ABA in pathogen response*

Salicylic acid (SA), jasmonic acid (JA), and ethylene are the major plant hormones in regulating pathogen resistance responses. This regulation is not achieved through the isolated activation of each single hormonal pathway but rather through a complex regulatory network (Adie *et al.*, 2007). ABA plays an important role in response to abiotic stress and in plant development, as well as having an antagonistic effect to these three hormones on biotic stress signalling (Mauch-Mani and Mauch, 2005). ABA has been considered a negative regulator of disease resistance with few exceptions. Marta de Torres-Zabala and her colleagues found that the ABA signalling pathway is a major target for the effectors secreted by *Pseudomonas syringae.* The exogenous application of ABA enhanced the susceptibility to both virulent and non-virulent *P. syringae*. Meanwhile, the mutant plants with reduced or increased ABA sensitivity showed restriction or enhanced multiplication of bacteria, respectively (de Torres-Zabala *et al.*, 2007). These data suggest that ABA negatively regulates pathogen resistance of plants. However, the role of ABA in pathogen resistance responses appears to be more complex. In some cases, ABA seems to have a positive effect in activating the pathogen defense system. For example, ABA showed a positive function on callose deposition (Mauch-Mani and Mauch, 2005). Another example is that the ABA deficient mutants (*aba2-12* and *aao3-2*) and the ABA insensitive mutant (*abi4-1*) had dramatically increased susceptibility to the fungal pathogen *Pythium irregulare* when these mutants were compared with wild-type Arabidopsis (Adie *et al.*, 2007). Collectively, these results implicate that ABA as a positive regulator to pathogen resistance. The contradictory conclusions drawn from different experiments might be

explained in the following way. The final response output to a specific pathogen may be influenced by mutually synergistic or antagonistic interactions with other hormones, or the attack from a specific pathogen may trigger a relatively specific signalling. A recent study shows that ABA is required for *Leptosphaeria maculans* resistance via ABI1- and ABI4-dependent signalling (Kaliff *et al.*, 2007). The ABA insensitive mutant *abi1-1* displayed susceptibility to *L. maculans,* while the other ABA insensitive mutant *abi2-1* remained resistant. Interestingly, the mutant *abi4-1*, which appeared highly susceptible to *P. irregulare* (Adie *et al*., 2007), also remained resistant to *L. maculans* (Kaliff *et al.*, 2007). This indicates that ABI4 plays different roles in the resistance signalling to *P. irregulare* and to *L. maculans*.

Another connection between pathogen resistance and ABA signalling is stomata. Stomata have been suggested to be critical in innate defense against bacterial invasion (Melotto *et al.*, 2006) and stomatal closure is regulated by ABA. Melotto and his colleagues have observed that virulent bacteria *Pseudomonas syringae* selectively move only towards open stomata when inoculated at the leaf surface, and although the deposition of the bacteria provokes transient stomatal closure, the stomata re-open in three hours (Melotto *et al.*, 2006). The *P. syringae* mutants defective in producing coronatine (the virulence factor responsible for suppressing stomatal defense) fail to cause disease when deposited on the leaf surface, but remain virulent when delivered directly into the leaves by injection. The phytotoxin coronatine can effectively counteract ABA-induced stomatal closure. The Arabidopsis mutant *ost1-2* impeding stomatal response to ABA and the ABA deficient mutant *aba3-1* appeared susceptible to *P. syringae*, regardless of whether it can produce coronatine (Melotto *et al.*, 2006).

1.2 Pathogenesis-Related Proteins

- *Overview of pathogenesis-related proteins*

Pathogenesis-related proteins (PRs) are expressed by host plants following infections

(by fungi, bacteria or viruses) or parasitic attacks (by nematodes, insects or herbivores), or after induction by abiotic stress factors such as chemicals (salicylic, polyacrylic, and fatty acids, inorganic salts) and physical stimuli (wounding, UV-B radiation, osmotic shock, low temperature, water deficit and excess). In some plant tissues, however, PRs are constitutively expressed, e.g. in pollens or fruits, tissues that are more likely to be attacked. PR proteins display multiple effects within the plant and possess antimicrobial activity, and can thus be regarded as a part of the plant's defense system. They comprise a wide range of forms, such as hydrolases, transcription factors, protease inhibitors, enzymes associated with various metabolic pathways, and allergenic products (Edreva, 2005; Scherer *et al.*, 2006; Ebner *et al.*, 2001).

PRs are distinguished by specific biochemical properties. They are low-molecular proteins of 6-43 kDa. Many of them are stable at low pH ($<$3), thermostable, and display considerable resistance to proteases (Edreva, 2005). Therefore, they can act as food allergens (Ebner *et al.*, 2001). PRs are both extracellular and intracellular. They are present in the primary and secondary cell walls of infected plants. They were also found in cell wall appositions (papillae) deposited at the inner side of the cell wall in response to fungal attack (Edreva, 2005). For their intracellular localization, they are present in vacuoles and apoplasts, and the apoplasts are the main site of their accumulation (Van Loon and Van Strien, 1999; Christensen *et al.*, 2002). Up until now, both acidic and basic PRs have been identified. They are established in all plant organs such as leaves, stems, roots, and flowers, being particularly abundant in leaves (Edreva, 2005). According to sequence characteristics and their enzymatic or biologic activities, PRs can be divided into 17 families (Edreva, 2005; Park *et al.*, 2004 a; Christensen *et al.*, 2002). The properties of these 17 families are shown in Table 1 (Edreva, 2005; http://www.bio.uu.nl/~fytopath/PR-families.htm).

A significant constitutive expression of PRs in transgenic plants overexpressing PR genes sometimes accompanies increased resistance to pathogens (Edreva, 2005; Liu

and Ekramoddoullah, 2006). This might be due to PRs' toxicity, which can be generally accounted for by their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability. Hydrolytic enzymes such as ß-1,3-glucanases (PR-2), chitinases (PR-3, PR-4, PR-8 and PR-11), and proteinases (PR-7) can act as tools to weaken and decompose fungal cell walls containing glucans, chitin and proteins (Edreva, 2005; Selitrennikoff, 2001). Proteinase-inhibitors (PR-6) can inactivate the proteins secreted by invading insects or nematodes. The peroxidase activity of PR-9 can contribute to rigidify and strengthen plant cell walls in response to pathogen attack (Edreva, 2005). The ribonuclease function of PR-10 isolated from hot pepper is to cleave invading viral RNAs (Park et al., 2004 b). Plasma membrane-permeabilizing ability proper to PR-5, PR-12, PR-13 and PR-14 contributes to plasmolysis and damage of fungal and bacterial pathogens, inhibiting their growth and development (Edreva, 2005; Selitrennikoff, 2001).

Tabel 1. Recognized and proposed families of pathogenesis-related proteins.

Families	Type member	Properties
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	b-1,3-glucanase
PR-3	Tobacco P,Q	Chitinase type I,II,IV,V,VI,VII
PR-4	Tobacco `R´	Chitinase type I,II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tabacco `lignin-forming peroxidase´	Peroxidase
PR-10	Parsley `PR1´	`ribonuclease-like´
PR-11	Tobacco `class V´ chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (german)	Oxalate oxidase
PR-16	Barley OxOLP	`oxalate oxidase-like´
PR-17	Tobacco PRp27	unknown

- *PR-10 family*

Among 17 families of PRs, the PR-10 family is of the most interest to this study. PR-10 proteins are widely distributed in seed plants such as white birch, pea, rice, and sugar pine (Liu and Ekramoddoullah, 2006). Up until now, more than 100 PR-10 or PR-10-related sequences have been identified. They comprise two groups according to their amino acid sequences, subcellular localization and putative function: intracellular pathogenesis-related proteins (IPR) with homology to ribonuclease, and (S)-norcoclaurine synthases (NCS). PR-10 proteins are identified mainly as IPR proteins, including tree pollen allergens and major food allergens (Liu and Ekramoddoullah, 2006; Samanani *et al.*, 2004; Markovic-Housley *et al.*, 2003). Members of the IPR group share several conserved features: they have small molecular masses (15-18 kDa), acidic isoelectric point (pI), cytosolic localization, and a similar three-dimensional (3D) structure (Liu and Ekramoddoullah, 2006). A 30-Å-deep, Y-shaped, hydrophobic cavity functioning as a ligand-binding site has been identified in the crystal structures of some IPR proteins, such as Bet v 1l (a naturally occurring hypoallergenic isoform of the major birch pollen allergen Bet v 1), and proteins LlPR10-1A and LlPR10-1B from yellow lupine (Markovic-Housley *et al.*, 2003; Biesiadka *et al*., 2002). However, members of the NCS group are distinctive in the PR-10 family due to oligopeptide extension at both their C- and N-terminals. And the presence of putative N-terminal signal peptides in NCS proteins suggests that they have an association with a subcellular compartment other than the cytosol (Liu and Ekramoddoullah, 2006).

The expression pattern of PR-10 proteins is not distinctive in pathogenesis-related proteins. Constitutive accumulation of PR-10 proteins has been detected during plant growth and development in plant flower organs, pollen grain, fruits, seeds, vegetative organs of roots, stems, and leaves. Meanwhile, both biotic stress (attacks by viruses, bacteria, and fungus) and abiotic stress (wounding treatment, cold hardiness, salinity,

drought stress, copper stress, oxidative stress, ultraviolet radiation, and some plant hormones) can induce the expression of PR-10 proteins (Liu and Ekramoddoullah, 2006; Utriainen *et al.*, 1998; Koistinen *et al.*, 2002; Wang *et al.*, 1999). Interestingly, ABA has been demonstrated to be able to induce the expressions of lily PR-10 genes and pea ABR17 (abscisic acid-responsive 17) which is also a PR-10 protein (Wang *et al.*, 1999; Srivastava *et al.*, 2006 a).

PR-10 proteins have multiple biological activities, including ribonuclease activity, (S)-norcoclaurine synthase activity, ligand-binding activity, antimicrobial activity, and activity to enhance plant tolerance under abiotic stress (Park *et al.*, 2004 b; Samanani *et al.*, 2004; Markovic-Housley et al., 2003; Flores *et al.*, 2002; Srivastava *et al.*, 2006 a). Two ribonucleases (RNase 1 and 2) from ginseng share high structural homology with PR-10 proteins (Moiseyev *et al.*, 1997). Meanwhile, ribonuclease activity has been confirmed for some recombinant and natural PR-10 proteins, such as Bet v 1 from birch (*Betula verrucosa*) pollens, LaPR-10 from lupine (*Lupinus albus*) roots, PR-10c from birch (*Betula pendula*), GaPR-10 from *Gossypium arboreum*, SPE-16 from *Pachyrrhizus erosus*, CaPR-10 from hot pepper (*Capsicum annuum*), PR10.1 from *Brassica napus*, and pea ABR17 (Liu and Ekramoddoullah, 2006; Srivastava *et al.*2006 b; Srivastava *et al.*, 2007). However, not all of the IPR proteins in the PR-10 family possess this catalytic property. For example, LIPR10-1A from yellow lupine has no RNase activity at all, although its homologous protein LIPR10-1B from the same species has displayed RNase activity at some level (Biesiadka *et al.*, 2002). Some NCS proteins in PR-10 family are capable of catalyzing the formation of (S)-norcoclaurine from the condensation of 3,4-dihydroxyphenylethylamine (dopamine) and 4-hydroxyphenylacetaldehyde in the biosynthesis of benzylisoquinoline alkaloids, while other NCS proteins such as VrCSBP (a cytokinin-specific binding protein from mung bean *Vigna radiata*) do not have any NCS activity at detectable levels (Samanani *et al.*, 2004; Liscombe *et al.*, 2005). Ligand-binding ability is possessed by some PR-10 proteins from both IPR and NCS groups. VrCSBP, the NCS protein purified from mung bean, has shown cytokinin-binding ability (Fujimoto *et al.*, 1998; Liu

and Ekramoddoullah, 2006). In the IPR group, there are more ligand-interacting proteins. Bet v 1, the major birch allergen, has shown affinity for a broad spectrum of physical ligands, including fatty acids, flavonoids, and cytokinins (Mogensen et al., 2002). Bet v 1l, the hypoallergenic isoform of Bet v 1, has been confirmed to bind brassinolide and 24-epicastasterone by a specific non-covalent interaction (Markovic-Housley *et al.*, 2003). Pru av 1, a cherry allergen PR-10 protein, is able to bind the phytosteroid homocastasteron (Neudecker *et al.*, 2001). Birch PR-10c has been demonstrated by saturation transfer difference NMR to interact with cytokinin, flavonoid glycosides, sterols, and emodin (Koistinen *et al*., 2005). Some PR-10 proteins were confirmed to be with antimicrobial activity, although over-expression of PR-10 proteins does not always accompany enhanced resistance against pathogens (Flores *et al.*, 2002; Liu *et al.*, 2006; Constabel *et al.*, 1993). Ocatin, a PR-10 protein from the Andean crop oca (*Oxalis tuberosa*), can inhibit the growth of several phytopathogenic bacteria and fungi (Flores *et al.*, 2002). SsPR10, a PR10 protein from yellow-fruit nightshade (*Solanum surattense*), has exhibited inhibition ability against hyphal growth of *Pyricularia oryzae* (Liu *et al.*, 2006). Some PR-10 proteins also have functions that enhance plant tolerance under abiotic stresses. A constitutive expression of pea ABR17, the PR-10 protein which has RNase activity and can be induced by ABA can result in better germination of the corresponding transgenic Arabidopsis under salt and cold stress (Srivastava *et al.*, 2006 a; Srivastava *et al.*, 2006 b).

In conclusion, diverse PR-10 proteins exhibit a complex expression pattern and display various biological activities in different species, under different environmental stress, or at different developmental stages. Therefore, it is not reasonable to assign a unique function to PR-10 proteins throughout the plant kingdom (Liu and Ekramoddoullah, 2006). The interactions between some PR-10 proteins and plant hormones suggest that PR-10 proteins might be involved in plant hormone-mediated signal transductions. Further experiments and data are expected to illustrate their relations to phytohormones.

1.3   The family of ABP7 and ABP9 in *Arabidopsis thaliana*

ABI1 and its homologue ABI2 are two key negative regulators of ABA signalling. *ABP7* (At5g53160) and *ABP9* (At1g01360) express ABI2-interacting protein candidates, which were identified from an Arabidopsis cDNA library using the yeast two-hybrid system by Dr. Yi Yang (Yang, 2003). Subsequent study revealed that ABP7 and ABP9 can also interact with ABI2's point-mutated form *abi2* (ABI2[Gly168Asp]), N-terminal truncated ABI1 (dNABI1, a.a. 123-434), and the point-mutated form dN*abi1* (dNABI1[Gly180Asp]) in the yeast two-hybrid system (Data shown in Figure 4). The full length ABI1 and *abi1* were not tested, due to their property to auto-activate the expression of LacZ reporter in this experiment (Zhang, 2004).

The sequences of ABP7 and ABP9 share 70% identity at the amino acid level. BLAST (Basic Local Alignment Tool) study has shown that they belong to a 14-member family in *Arabidopsis thaliana* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Among this family, two members (At2g40330 and At2g38310) were suggested to be downregulated by ABA in microarrays (Hruz *et al.*, 2008).

The 14 proteins in this family share three conservative domains (the names of their genes are shown in Figure 5). All of the 14 proteins except the one from At4g17870 show similarity to birch allergen Bet v 1, which is a PR-10 protein with ligand-binding activity and ribonuclease activity (www.arabidopsis.org; Mogensen *et al.*, 2002; Bufe *et al.*, 1995). This indicates that the protein members from this family might also display PR-10 functions such as binding with ligand(s) and so on. Interestingly, the expressions of *ABP7* and *ABP9* have been suggested to be transcriptionally upregulated by the attack of *Agrobacterium tumefaciens* (Hruz *et al.*, 2008).
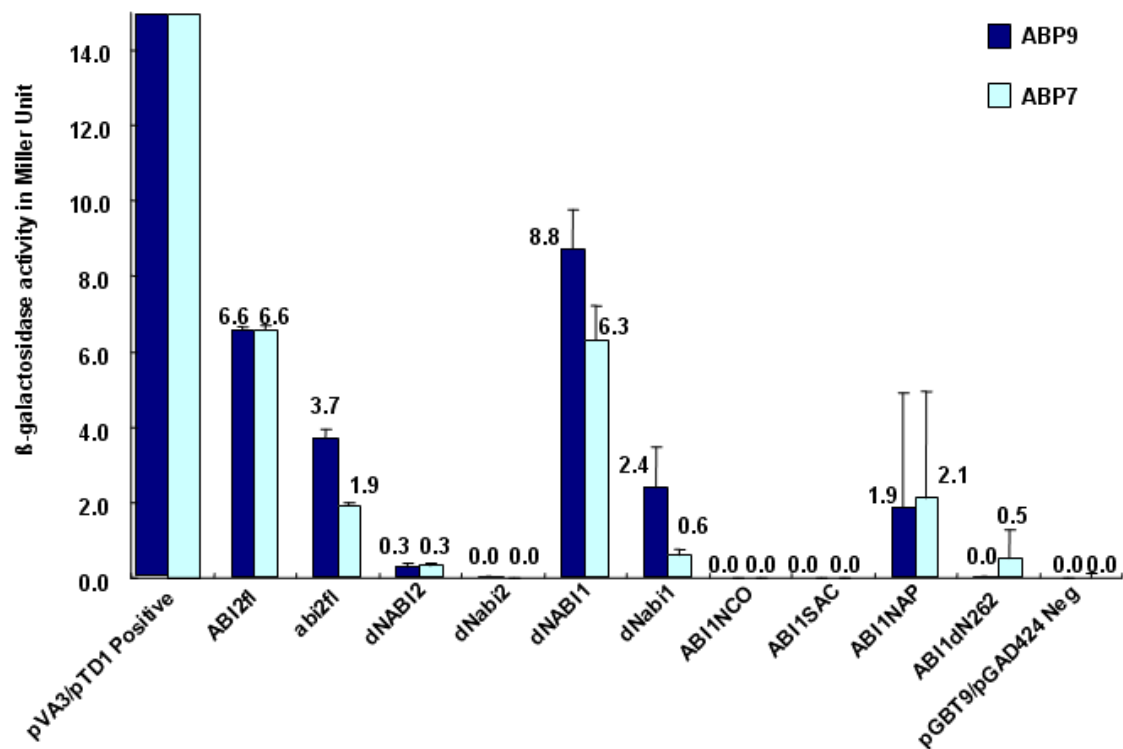
Figure 4. Two-hybrid interactions of ABP9/7 with ABI2, dNABI1, and their mutated versions. pVA3/pTD1 positive: positive control; ABI2fl: ABI2 full length, a.a. 1-423; abi2fl: full length ABI2^{Gly168Asp}; dNABI2: N-terminal deleted ABI2, a.a. 95-423; dNabi2: dNABI2^{Gly168Asp}; dNABI1: N-terminal deleted ABI1, a.a.123-434; dNabi1: dNABI1^{Gly180Asp}; ABI1NCO: C-terminal truncated from Ncol1 site in ABI1, a.a. 1-180; ABI1SAC: N-terminal truncated from Sac1 site, a.a. 1-268; ABI1NAP: non-activie PP2C, truncated ABI1^{Asp177Ala} (a.a. 121-434); ABI1dN262: N-terminal deleted ABI1, a.a. 262-434; pGBT9/pGAD424Neg: negative control.

1.4   The aim of this work

ABA signal transduction has been studied for decades. However, many steps in ABA signalling still remain unknown or unclear. It has been suggested that CHLH, GTG1 and GTG2 act as ABA receptors (Shen *et al*., 2006; Pandey *et al*., 2009). However, they are unlikely to be the only receptors of ABA. More receptors and regulators are expected to be discovered and characterized to subsequently better illustrate the ABA

31

signal pathway.
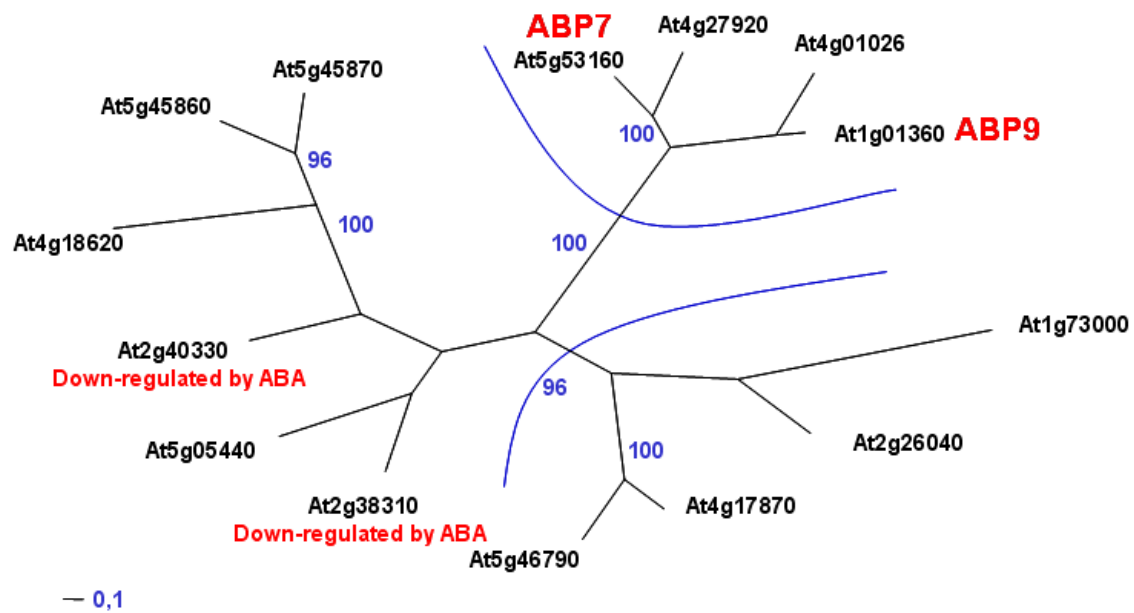
Figure 5. The unrooted parsimony tree of ABP7 and ABP9's family. The tree was generated based on the amino acid sequences of the family members, by the programs of PHYLIP Protpars and PHYLIP Drawtree (Felsenstein, 2004). Bootstrap support percentages (bootstrap values) ＞90% is shown in blue numbers next to the branches. Branch length of 0.1 is indicated in the left lower corner. This parsimony tree suggests that ABP7 and ABP9 belong to the same small sub-family.

ABI1 and ABI2 are two key upstream regulators which are involved in many ABA responses (Himmelbach *et al.*, 1998; Leung and Giraudat, 1998). ABP9 and its homologue ABP7 have been identified independently as ABI2's interacting partners in the yeast two-hybrid system by screening the Arabidopsis cDNA library (Yang, 2003). These two proteins were further demonstrated to interact with N-terminal deleted ABI1 as well (Zhang, 2004). In addition, two members in ABP7 and ABP9's family have been predicted to be downregulated by ABA. Therefore, it is quite likely that ABP7 and ABP9 also participate in ABA signal transduction and display their certain functions, such as positively or negatively regulating ABA responses. On the other hand, ABP7 and ABP9

might play roles in interacting with small ligand(s) or in pathogen attack responses, due to their similarity to birch PR-10 protein Bet v 1. Thus, the aim of this work is to characterize ABP7 and ABP9. This aim would be realized by testing the ABA responses in ABP7 and ABP9 deregulated protoplasts and transgenic plants, by examining ABP7 and ABP9's expression pattern in plants, by studying their gene regulation, by detecting the subcellular localization of ABP7 and ABP9, by confirming their interactions with ABI proteins (ABI1 and ABI2) in protoplasts, and by searching for their interacting ligand(s) such as phytohormones and other small molecules.

2. <u>Materials and methods</u>

2.1 Materials

2.1.1 Vectors and primers (refer to Appendix)

2.1.2 Plant materials

Arabidopsis thaliana

Reschiev (RLD) was used for generation of all the transgenic plants and as a source for protoplast isolation. Landsberg *erecta* (La-er) and Columbia (Col-0) were used as the source for protoplast isolation as well. All accessions were received from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA.

Mutant plants

ABA deficient mutant *aba2-1*, ABA-insensitive mutant *abi1-1* and *abi2-1*, and *gca2-1* mutant were used for transient assay.   Auxin resistant mutant *axr1-12*, ethylene insensitive mutant *ein2-1* were used for physiological analysis. All of the mutants are obtained from ABRC, Ohio, USA, except that *gca2-1* mutant was generated by Dr. M. Iten from Prof. Dr. E. Grill's lab in Zurich (Iten, 1992).

2.1.3 Micro-organisms

The *Agrobacterium tumefaciens* stain C58 harboring Ti-plasmid pGV3101 (Koncz and Schell, 1986) was used for plant transformation by floral dipping (Clough, 2004).   For cloning procedure, plasmids were propagated in *E. coli*

(*Escherichia coli*) stains DH5α (Stratagene GmbH, Heidelberg) and XL1 Blue (Stratagene GmbH, Heidelberg).

2.1.4 Reagents

DNA ladders were purchased from MBI Fermentas GMBH. The prestained protein marker was obtained from New England Biolabs, GmbH.

The chemicals used in this work were purchased in p.a. quality from Fulka/Sigma (Munich), Merck AG (Darmstadt), Roth GmbH & Co. (Karlsruhe), Serva Feinbiochemica (Heidelberg), and Qiagen (Hilden). The detergent Silvet L-77 was purchased from Lehle Seeds (Round Rock, Texas, USA. Oligonucleotides were obtained from MWG-Biotech GmbH (Ebersberg). The anti-Histag antibody is from QIAGEN (Hilden) and the anti-ABP7 and anti-ABP9 anti-serum were produced by Eurogentec (Belgium).

Enzymes used in the work:

Cellulase R-10: Yakult Honshia Co. Ltd. Japan

Macerozym R-10: Yakult Honshia Co. Ltd. Japan

Lysozym: Fula Sigma-Aldrich GmbH, Deisenhofen

Ribonuclease A: Serva GmbH, Heidelberg

Klenow-fragment polymerase: MBI Fermentas GmbH, St. Leon-Rot

M-MuLV reverse transcriptase: MBI Fermentas GmbH, St. Leon-Rot

Pwo-polymerase: Peqlab Biotechnology GmbH, Erlangen

Taq-polymerase: MBI Fermentas GmbH, St. Leon-Rot

Alkaline phosphatase, shrimp: Roche Applied Science, Germany

T4-ligase: MBI Fermentas GmbH, St. Leon-Rot

Restriction enzymes: MBI Fermentas GmbH, St. Leon-Rot

2.1.5 Media

Medium for bacteria culture:

LB medium:

10.0 g/l	Bacto Tryptone
5.0 g/l	Yeast extract
10.0 g/l	NaCl
18 g/l	Agar (No agar for LB liquid medium)
pH 7.0	

The medium was autoclaved to get sterile.

Antibiotics used in LB medium (stock solutions of the antibiotics are dissolved in H_2O and filtrated through 0.22 μm filter):

Ampicillin:50 mg/l or 100 mg/l (50 mg/ml for the stock solution)

Kanamycin:25 mg/l (10 mg/ml for the stock solution)

Tetracycline: 50 mg/l (5 mg/ml in ethanol for the stock solution)

Rifampicin:50 mg/l (50 mg/ml in methanol for the stock solution)

Medium for Arabidopsis seedlings:

MS medium (Murashige and Skoog, 1962):

100 ml/l	10x Macrosalts stock
2.5 ml/l	Vitamin stock (400x)
2.5 ml/l	Microsalts stock (400x)
1 g/l	MES
10 g/l	Sucrose
9 g/l	Agar (No agar for MS liquid medium)
pH 5.8	

The medium was autoclaved to get sterile.

Macrosalts stock (10x):

16.5 g/l	NH_4NO_3
19.0 g/l	KNO_3
4.4 g/l	$CaCl_2·2H_2O$
1.7 g/l	KH_2PO_4
3.7 g/l	$MgSO_4·7H_2O$

Microsalts stock (400x):

0.01 g/l	$CoCl_2·6H_2O$
0.01 g/l	$CuSO_4·5H_2O$
14.60 g/l	Na_2EDTA
1.20 g/l	H_3BO_4
0.30 g/l	KI
4.00 g/l	$MnSO_4·H_2O$
0.10 g/l	$Na_2MoO_4·2H_2O$
0.80 g/l	$ZnSO_4·7H_2O$
11.12 g/l	$FeSO_4·7H_2O$

pH 4.5

Vitamin stock (400x):

0.40 g/l	Nicotinic acid
0.40 g/l	Pyridoxine
4.00 g/l	Thiamine
40.00 g/l	Myo-Inositol

Medium for Arabidopsis cell culture:

Linsmaier-Skoog (LS) medium (Linsmaier and Skoog,1965):

1650.0 mg/l	NH_4NO_3
1900.0 mg/l	KNO_3
370.0 mg/l	$MgSO_4 \cdot 7H_2O$
170.0 mg/l	KH_2PO_4
332.0 mg/l	$CaCl_2$
37.3 mg/l	Na_2EDTA
27.8 mg/l	$FeSO_4 \cdot 7H_2O$
6.2 mg/l	H_3BO_3
22.3 mg/l	$MnSO_4 \cdot 4H_2O$
8.6 mg/l	$ZnSO_4 \cdot 4H_2O$
0.83 mg/l	KI
0.25 mg/l	$Na_2MoO_4 \cdot 2H_2O$
0.025 mg/l	$CuSO_4 \cdot 5H_2O$
0.025 mg/l	$CoCl_2 \cdot 6H_2O$
0.4 mg/l	Thiaminiumdichlorid
100.0 mg/l	Myo-Inositol

pH 6.0

After autoclaving, NAA to the final concentration of 0.5 mg/l and kinetin to the final concentraion of 0.1 mg/l were added to the medium.

MSCol medium for *Columbia-0* root cell cultures (Mathur and Koncz, 1998):

10 ml of cell cultures were cultivated with 50 ml of medium in a 250 ml flask. The cell cultures were shaken with 120 rpm at 26 °C in the dark.

MSCol medium (5 liters):

21.5 g	MS salts (4.3 g/l)
5 ml	NPT-Vitamine (1 ml/l):

5 mg	Nicotin acid (1 mg/l)
5 mg	Pyridoxin-HCl (1 mg/l)
50 mg	Thiamin-HCl (10 mg/l)
500 mg	myo-Inositol (100 mg/l)
150 g	Sucrose (30 g/l)

pH = 5.8

After autoclaving, 5 mg 2,4-D (1 mg/l) was added into the medium. The medium was stored at 4 °C.

The NPT-Vitamine stock (40 ml):

40 mg	Nicotin acid
40 mg	Pyridox-HCl
400 mg	Thiamin-HCl

The stock was filtrated to get sterile and stored at –20°C.

2.1.6 Buffers

Sodium Phosphate Buffer (0.1 M):

pH	Volume of 1M Na_2HPO_4 (ml)	Volume of 1M NaH_2PO_4 (ml)
7.2	68.4	31.6
7.4	77.4	22.6
7.8	89.6	10.4

TAE buffer (50x):	10x Tris EDTA (TE) buffer, pH 8.0:
242 g Tris base	100 ml Tris-Cl (pH8.0)
57.1 ml glacial acetic acid	10 mM EDTA (pH8.0)
100 ml 0.5M EDTA (pH8.0)	

Tris-Cl buffer, pH 8.0 (1 M):

121.1 g Tris base in 800 ml of H_2O

42 ml HCl

H_2O was added up to 1 liter.

Tris-buffered saline (TBS) buffer:

8 g of NaCl, 0.2 g of KCl, and 3 g of Tris base in 800 ml of H_2O

0.015 g phenol red

The pH value was adjusted to 7.4 with HCl. H_2O was added up to 1 liter.

2.1.7 Antibodies

Polyclonal goat anti-rabbit IgG HRP (Horseradish Peroxidase) conjugated antibodies were purchased from PIERCE (Perbio Science Deutschland GmbH). Anti-His HRP Conjugate was purchased from QIAGEN (Hilden). Anti-ABP7 antisera and anti-ABP9 antisera were supplied by Eurogentec (Liège, Belgium).

2.2 Methods

2.2.1 Standard molecular biology methods

2.2.1.1 DNA analysis

The methods used are as described by the protocols provided by the manufacturers or according to the standard protocols (Sambrook and Russell, 2001):

- Isolation of plasmid DNA

- Electrophoresis of DNA in agarose gels

- Polymerase chain reaction (PCR)

- Phenol chloroform extraction and ethanol precipitation

- Digestion of DNA with restriction enzymes

- "Fill in" reaction with Klenow polymerase

- Dephosphorylation with alkaline phosphatase

- Ligation with T4 DNA ligase

- Competent cells preparation for *E.coli* strain DH5α by the CaCl$_2$ method

- Competent cells preparation of *E.coli* strain XL1-Blue (Holt, 1990, http://www.protocol-online.org)

- Competent cells preparation of agrobacteria GV3101

- Plasmid transformation to chemical competent *E. coli* by "Heat Shock"

- Plasmid transformation to competent argrobacteria by electroporation

- CTAB extraction of genomic DNA from *Arabidopsis* (http://carnegiedpb.stanford.edu/methods/ppsuppl.html)

2.2.1.2 RNA analysis

RNA isolation:

5-day-old RLD wild-type seedlings grown on MS agarose medium were transferred to MS agarose medium, MS agarose medium containing 10 μM ABA, or MS agarose medium containing 10 μM 2,4-D. 6 hours later, total RNA was isolated from the seedlings. The protocol is as following:

- Grind 100 mg of the frozen (liquid nitrogen) seedlings in an Eppendorf tube by using a micro pestle.

- Add 1 ml of TRItidy G solution (AppliChem), continue homogenisation, and incubate on ice for 5 min.

- Add 200 μl of chloroform, mix well and incubate for 10 min on ice.

- Centrifuge at 12,000 g for 15 min at 4°C.

- Transfer the upper aqueous phase into a new tube and determine the transferred volume.

- Add the same volume of isopropanol and incubate for 15 min on ice.

- Pellet the precipitated RNA by centrifugation at 12,000 g for 15 min at 4°C. Wash the pellet with 500 μl ice-cold (-20°C) ethanol and then wash the pellet with 500 μl ice-cold 80% ethanol.

- Air-dry the pellet and finally dissolve the pellet in 20 μl DEPC-treated water. Store the total RNA at -80°C.

- All of the Eppendorf tubes and pipette tips used here should be RNAse-free.

Synthesis of the first strand cDNA:

- Add 2 μg of RNA template into an Eppendorf tube.

- Add 1 μl 0.5 μg/μl of oligo(dT)$_{18}$ primer, then add distilled DEPC water up to 11 μl.

- Incubate at 70°C for 5 min.

- Supplement the mixture with 4 μl of 5x reaction buffer, 1 μl (20 U) of ribonuclease inhibitor and 2 μl of 10 mM dNTP.

- After incubation at 37°C for 5 min, add 2 μl (40 U) of M-MuLV reverse transcriptase.

- Incubate the reaction at 37°C for 1 hour.

- Stop the reaction by heating at 70°C for 10 min.

2.2.2 Protein analysis

2.2.2.1 Expression and purification of proteins

pQE30 plasmid (QIAGEN) was used to express 6xHis-tagged ABP7, ABP9,
N-terminal truncated ABP9 (ΔN-ABP9, a.a. 70-187, without N-terminal
domain) and C-terminus truncated ABP9 (ΔC-ABP9, a.a. 1-137, without
C-terminal domain) in $E.coli$ strain XL1-Blue. When the OD_{600} of the $E.coli$
culture reached 0.5-0.7, IPTG (isopropyl-β-D-thiogalactoside) was added to a
final concentration of 0.1mM for overnight induction. Harvest the cells in the
next day and purify the 6xHis-tagged proteins under the denaturing condition
by using Ni-NTA affinity columns. The protocol used is modified from the
protocol in QIAexpress protein purification system (QIAGEN). The details are
as following:

- Harvest the cells from 6 liters of $E.\ coli$ culture after over-night IPTG
  induction.
- Freeze the cells in liquid nitrogen and thaw the cell pellet for 15 min
  on ice.
- Resuspend the cell pellet in the denaturing buffer (100 mM
  NaH_2PO_4, 10 mM Tris·Cl, 8 M urea, pH 8.0) at 5 ml per gram wet
  weight.
- Stir cells for 15-60 min at room temperature or lyse them by gentle
  vortexing until the solution becomes translucent, taking care to
  avoid foaming.
- Centrifuge lysate at 10,000 x g for 20-30 min at room temperature to
  pellet the cellular debris. Save the supernatant (total protein).
- Slowly mix 3 volumes of the supernatant (total protein) with 1
  volume of buffer (pH 8.0) containing 100 mM NaH_2PO_4 and 10 mM
  Tris·Cl.
- Filter and degas the sample.
- Prepare a clean and empty column with a diameter about 1 cm. Fill
  in 4 ml Ni-NTA into this column in the cold room.
- Equilibrate the Ni-NTA column with 40 ml ddH_2O.

- Then equilibrate the Ni-NTA column with 40 ml buffer B.

- Load the filtered and degassed sample into the column.

- After loading the sample, use buffer B to run the column until the photometric detector finds no protein in the outlet stream.

- Wash the column with 40 ml buffer C.

- Then wash the column with 40 ml buffer D.

- Elute the 6xHis-tagged protein from the column with buffer E until the photometric detector finds no protein in the outlet stream. Carefully collect the outlet stream when the photometric detector shows a peak.

- Clean the column with 40 ml ddH$_2$O, then with 40 ml 20% ethanol.

Buffer B:

100 mM NaH$_2$PO$_4$

10 mM Tris·Cl

6 M urea

pH 8.0 (with NaOH)

Buffer C:

100 mM NaH$_2$PO$_4$

10 mM Tris·Cl

6 M urea

pH 6.3 (with HCl)

Buffer D:

100 mM NaH$_2$PO$_4$

10 mM Tris·Cl

6 M urea

pH 5.9 (with HCl)

Buffer E:

100 mM NaH$_2$PO$_4$

10 mM Tris·Cl

6 M urea

pH 4.5 (with HCl)

2.2.2.2 Protein extraction from *Arabidopsis thaliana*

20 to 300 mg of plant tissue was ground in an equal amount of buffer A (50 mM sodium phosphate with pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na$_2$EDTA, and 0.1% Triton X-100) in an Eppendorf tube by using a pestle. Then the sample was centrifuged at 8,000 g for 5 min at 4°C. The supernatant was

transferred to a fresh Eppendorf tube. This supernatant should be taken for an immediate measurement or be stored in 50% (v/v) glycerol and freeze in liquid nitrogen.

2.2.2.3 Determination of protein concentrations by Bradford assay

Reagents:

Dye stock: Coomassie Blue G 100 mg was dissolved in 50 ml of methanol. If turbid, the solution should be treated with 100 mg Norit (activated charcoal powder) and filtered through a glass-fiber filter. Then the solution was added to 100 ml of 85% H_3PO_4 and diluted to 200 ml with water. The solution should be dark red and have a pH of –0.01. The final reagent concentrations were 0.5 mg/ml Coomassie Blue G, 25% methanol, and 42.5% H_3PO_4. This solution is stable in a dark bottle at 4°C.

Assay reagent: The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O. The solution should appear brown, and have a pH of 1.1. It is stable for weeks in a dark bottle at 4°C.

Protein standards: Protein standards should be prepared in the same buffers as the samples to be assayed. For a Bradfold standard assay, a convenient standard curve was made by using bovine serum albumin (BSA) with concentrations of 0, 250, 500, 1000, 1500, and 2000 μg/ml. For a Bradford microassay, a standard curve was made by using BSA with concentrations of 0, 10, 20, 30, 40, and 50 μg/ml.

Assay procedure: 40 μl sample was mixed with 200 μl assay reagent and 760 μl distilled H_2O in a 1 ml cuvette. The absorbance at 595 nm was measured in

a spectrophotometer (HTS 7000 Plus Bio Assay Reader from Perkin Elmer). Pprotein concentrations were calculated according to the BSA standards.

2.2.2.4 Protein detection by Western blot

SDS-PAGE electrophoresis is one of the standard methods for protein detection. In this work, 15% SDS gel was used for running 6xHis-tagged proteins. After SDS-PAGE, coomassie blue staining, silver staining, and Western blot were used to detect proteins.

Coomassie blue staining:

After electrophoresis, the SDS-PAGE gel was soaked into coomassie staining solution and incubated over-night at RT with gentle shaking. Then the gel was transferred into destaining solution and incubated with gentle shaking until the protein bands were clear.

Coomassie staining solution: Destaining solution:

0.1% coomassie R-250   7.5% acetic acid

45% ethanol   5% ethanol

10% acetic acid

Silver staining (Blum *et al.,* 1987):

● Fix the gel in the fixing buffer for 60 min.

● Wash with 50% ethanol for three times. Every time 20 min.

● Pretreat the gel with 0.2 g/l $Na_2S_2O_3 \cdot 5H_2O$ for 1 min.

● Rinse the gel with H_2O for 3 times, every time 20 sec.

● Impregnate the gel in 2 g/l $AgNO_3$ for 20 min.

● Rinse the gel with H_2O for 3 times, every time 20 sec.

- Soak the gel in the developing solution until the protein bands show up clearly.

- Add stopping buffer and wait until bubbles stop.

- Take photos.

Fixing buffer:

50% ethanol

12% acetic acid

0.5 ml/l 37% formaldehyde

Developing buffer:

60 g/l Na_2CO_3

4 mg/l $Na_2S_2O_3 \cdot 5H_2O$

0.5 ml/l 37% formaldehyde

Stopping buffer:

35% methanol

10% acetic acid

Western blot protocol (http://www.westernblotting.org):

- After SDS-PAGE electrophoresis, soak the gel in electro-transfer buffer (25 mM Tris·Cl, pH 8.3, 192 mM glycin, 20% methanol) for 5 min.

- Blot the gel to the Nitrocellulose Transfer Membrane (Whatman, Dassel Germany) by using the Semi-Dry Transfer Cell (Biorad) at 25 V for 1 hour.

- Block the membrane in TBS buffer containing 0.05% Tween-20 and 5% milk at room temperature (RT) for 1 hour with gentle shaking.

- Incubate the blot with the primary antibody (5,000-fold diluted anti-ABP7 or ani-ABP9 serum) at RT for 1 hour with shaking and wash the blot by suspending it in TBST buffer (TBS buffer containing 0.05% Tween-20) for 5 min.

- Wash the gel with TBST buffer for 5 min with agitating.

- Repeat the washing step 5 times.

- Then incubate the blot with the second antibody (100,000-fold diluted goat anti-rabbit IgG, HRP conjugate) at RT for 1 hour with shaking.

- Wash the gel with TBST buffer for 5 min with agitating and repeat the washing step 5 times.

- Prepare the working solution (substrate) by mix equal parts of the Stable Peroxide Solution and the Luminol Solution (PIERCE, Perbio Science Deutschland GmbH).

- Incubate the blot with working solution for 5 min in the dark.

- Remove the working solution and take photos by CCD camera.

2.2.2.5 β-Glucuronidase activity assay

Protein from Arabidopsis was extracted with buffer A (see Materials and Methods 2.2.2.2 for details). Then the protein concentrations were checked with a Bradford assay. The protein samples were subsequently diluted to 200 μg/ml with buffer A. 50 μl protein sample was mixed with 50 μl 0.2 mM 4-MUG (0.2 mM 4-methylumbelliferyl β-D-glucuronide in buffer A) in a 96-well microtiter plate and a GUS assay was performed in the plate reader using a kinetic program. The conversion of 4-MUG to 4-methylumbelliferone was followed by measuring fluorescence emission at 447 nm and using excitation at 363 nm.

2.2.2.6 Protein phosphatase 2C enzyme activity assay

PP2C activity of ABI2 can be assayed by its ability to dephosphorylate casein in the presence of Mg^{2+} or to dephosphorylate MUP (methylumbelliferylphosphate) in the presence of Mn^{2+} (Meinhard et al., 2002). In this work, the PP2C activity assay was carried out in the solution containing 100 mM Tris-HCl (pH 8.0), 1 mM MUP, and 1 mM $MnCl_2$. Catalysis was monitored by the formation of the fluorescent product methylumbelliferone in a microplate reader. The fluorescence reaction was measured by a kinetic

program with the excitation wavelength of 360 nm and emission wavelength of 465 nm.

2.2.2.7 Assays to identify binding ligand(s) of ABP7 and ABP9

2.2.2.7.1 Refolding of the denatured protein

Due to the formation of inclusion bodies, the 6xHis-tagged proteins (full-length ABP7, full-length ABP9, C-terminal truncated ABP9, and N-terminal truncated ABP9) were purified under a denaturing condition. Subsequently, the native proteins were obtained by refolding the denatured proteins with the following protocol:

- Harvest the cells from 6 liters of *E. coli* culture after over-night IPTG induction.
- Freeze the cells in liquid nitrogen and thaw the cell pellet for 15 min on ice.
- Resuspend the cell pellet in the resuspending buffer (10 mM Tris·Cl, 500 mM NaCl, 20% glycerol, 8 M urea, pH 7.4) at 5 ml per gram wet weight.
- Stir cells for 15-60 min at room temperature or lyse them by gentle vortexing until the solution becomes translucent, taking care to avoid foaming.
- Centrifuge lysate at 10,000 x g for 20-30 min at room temperature to pellet the cellular debris. Save the supernatant (total protein).
- Slowly mix 3 volumes of the supernatant (total protein) with 1 volume of buffer (pH 7.4) containing 10 mM Tris·Cl, 500 mM NaCl, 20% glycerol.
- Filter and degas the sample.

- Prepare a clean and empty column with a diameter about 1 cm. Fill in 4 ml Ni-NTA into this column in the cold room. And do all of the following steps only in the cold room.

- Equilibrate the Ni-NTA column with 40 ml ddH$_2$O.

- Then equilibrate the Ni-NTA column with 40 ml binding buffer.

- Load the sample into the column.

- After loading the sample, use at least 40 ml of the binding buffer to run the column until the photometric detector finds no protein in the outlet stream.

- Slowly run a buffer gradient from the binding buffer to the refolding buffer (40 ml of either buffer).

- Wash the column with the refolding buffer until the photometric detector finds no protein in the outlet stream.

- Wash the column with 40 ml washing buffer.

- Elute the 6xHis-tagged protein from the column with 40 ml elution buffer. Carefully collect the outlet stream when the photometric detector shows a peak.

- Make a western blot to check whether the eluted protein is correct.

- Then switch the eluted 6xHis-tagged protein from the elution buffer to storage buffer by using a buffer exchange NAP-25 Column (Amersharm Biosciences).

Binding Buffer:

10 mM Tris·Cl

500 mM NaCl

20% glycerol

6 M urea

pH 7.4

Refolding Buffer (pH 7.4):

10 mM Tris·Cl

500 mM NaCl

20% glycerol

2 mM   2mM β-ME

0.02% NaN$_3$

Washing Buffer (pH 7.4):

Elution Buffer (pH 7.4):

10 mM Tris·Cl   10 mM Tris·Cl

150 mM NaCl   150 mM NaCl

20% glycerol   20% glycerol

2 mM   2mM β-ME   2 mM   2mM β-ME

0.02% NaN$_3$ 0.02% NaN$_3$

25 mM imidazole   250 mM imidazole

Storage Buffer (pH 7.4):

10 mM Tris·Cl

150 mM NaCl

20% glycerol

2 mM β-ME

0.02% NaN$_3$

2.2.2.7.2 Binding test between ABP9 and phytohormones

The binding tests between refolded 6xHis-tagged ABP9 and ^{14}C-ABA or ^{14}C-zeatin were set up in the buffer (pH 7.4) containing 150 mM NaCl, 20% glycerol, 10 mM Tris·Cl, 2 mM β-ME, 0.02% NaN$_3$, and 1 M azide. The instrument of the binding tests and experimental details were described in Result 3.3.3.

2.2.3 Transient gene expression in protoplasts

2.2.3.1 Protoplasts preparation

2.2.3.1.1 Isolation of protoplasts from leaves of *Arabidopsis thaliana*

● Cut 40 leaves from 4-week-old Arabidopsis seedlings and incubate them in 15 ml enzyme solution for 4 hours with very gentle shaking.

- Filtrate through a 150 μm mesh nylon net and collect the protoplasts in a 50 ml Falcon tube.

- Add 5 ml WIMK to this Falcon tube and filtrate through the nylon net again. Collect the protoplasts in the same Falcon tube.

- Pellet protoplasts by 2 min centrifugation at 60 g and RT.

- Carefully remove the supernatant and discard.

- Add 5 ml of WIMK and carefully resuspend the pellet by rolling the tube with a hand.

- Pellet the protoplasts by centrifugation at 60 g for 2 min at room temperature.

- Add 3-5 ml MaMg and carefully resuspend the pellet by rolling the tube with a hand. The final concentration of protoplasts should be $0.5\text{-}1.0\text{x}10^6$/ml.

- Put the protoplasts at 4°C for 30 min before the subsequent transformation.

Enzyme solution:

1% Cellulase

0.25% Macerozyme

400 mM Mannitol

8 mM $CaCl_2$

0.1% BSA (Fluka 05488)

5 mM MES-KOH

pH 5.6

The solution was filtrated to get sterile.

WIMK:

500 mM mannitol

5 mM MES-Tris

pH 5.8-6.0

The solution was autoclaved.

MaMg:

400 mM mannitol

15 mM $MgCl_2$

5 mM MES-KOH

pH 5.8

The solution was autoclaved to get sterile.

2.2.3.1.2 Protoplasts preparation from Arabidopsis cell cultures

- Use cell culture growing in the dark.

- 3 days after sub-cultivation of the cell culture, split 10 ml cell culture and spin at 400 g for 5 min at RT.

- Resuspend the cell pellet in 10 ml digestion buffer without enzyme.

- Spin the cells at 400 g for 5 min at RT.

- Resuspend the cell pellet in 10 ml digestion solution and dispense into a Petri dish.

- Incubate at 26°C in the dark for 6 hours with shaking at 50 rpm.

- Filtrate the solution through a mesh nylon net (150 μm) and collect the protoplasts in a 50 ml Falcon tube by centrifugation at 60 g for 3 min.

- Carefully remove the supernatant and discard.

- Add 10 ml of WIMK and carefully resuspend the pellet by rolling the tube with a hand.

- Pellet the protoplasts by centrifugation at 60 g for 3 min at room teperature.

- Add 3-5 ml MaMg and carefully resuspend the pellet by rolling the tube with a hand. The final concentration of protoplasts should be $0.5\text{-}1.0\text{x}10^6$/ml.

- Put the protoplasts at 4°C for 30 min before the subsequent transformation.

Digestion solution:

1% Cellulase (Yakult, Japan)

0.25% Macerozyme (Yakult, Japan)

400 mM Mannitol

Digestion buffer without enzyme:

400 mM Mannitol

8 mM $CaCl_2$

pH 5.6

8 mM CaCl$_2$ The solution was filtrated to get

0.1% BSA (Fluka 05488)   sterile.

5 mM MES-KOH

pH 5.6

The solution was filtrated to get sterile.

WIMK: MaMg:

500 mM mannitol   400 mM mannitol

5 mM MES-Tris   15 mM MgCl$_2$

pH 5.8-6.0   5 mM MES-KOH

The solution was autoclaved.   pH 5.8

                                         The solution was autoclaved.

2.2.3.2 Protoplast transfection

- Mix 10-20 μg reporter DNA and effecter DNA into a 2 ml Eppendorf tube, then add the same volume of 1M mannitol. The total volume should not exceed 30 μl.
- Add 100 μl of protoplasts suspension.
- Calculate the total volume of protoplasts, DNA, and mannitol.
- Add the equal volume of 40% PEG 4000, 300 mM CaCl2, 0.5% MES-KOH, pH 5.8. Immediately mix by inverting the tube 3-4 times.
- Incubate for 3-5 min.
- Add 750 μl of sterile WIMK buffer. Invert 3 times to mix well.
- Pellet the protoplasts by centrifugation for 2 min at 2500 rpm.
- Carefully remove the supernatant.
- Resuspend the pellet in 100 μl WIMK buffer.
- Incubate the protoplasts at 22-26°C overnight with shaking at 50 rpm.

2.2.3.3 Activity assay of the reporter genes

- Transfer 50 μl of protoplasts suspension to the black microtiterplate.

- Add 100 μl MUG (4-methylumbelliferyl β-D-glucuronide) substrate working solution to each well.

- Measure GUS activity with a kinetic program.

- Transfer 100 μl of the probe to luminometer tubes.

- Measure LUC activity with program "protisfirefly QUICK" in the luminometer (Berthold Technologies GmbH & Co. KG).

<u>20 x MUG substrate stock:</u>

1 M Na_2HPO_4/NaH_2PO_4 (pH 7.0)

200 mM Na_2EDTA

2% Triton X100

20 mM DTT

4 mM 4-methylumbelliferyl β-D-glucuronide

The solution was stored at -20°C.

<u>MUG substrate working solution:</u>

Dilute the MUG substrate stock 20 folds with CCLR solution

The solution was stored at -20°C.

<u>CCLR solution:</u>

25 mM Tris-phosphate, pH 7.8

2 mM Dithiothreitol

2 mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacidic acid

10% (v/v) glycerin

The aliquots were stored at -20°C.

<u>LAR (Luciferase assay reagent):</u>

20 mM Tricine/NaOH pH 7.8

2.7 mM $MgSO_4$

0.5 mM EDTA

33.3 mM DTT

0.53 mM ATP

0.52 g/l $(MgCO_3)_4Mg(OH)_2$

0.2 g/l Coenzyme A

0.15 g/l Luciferin

2.2.3.4 Signal detection by fluorescence microscopy and confocal microscopy

GFP and YFP signal from the protoplasts was observed under a fluorescence microscope (Axioskop, ZEISS). The excitation wavelength was 500 nm. The emission wavelength was 535 nm. A confocal microscope Fluoview FV1000 (Olympus) was used in this study.

2.2.4 Analysis of plants

2.2.4.1 Transformation of *Arabidopsis*

Floral dip is a simple method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* (Steven *et al*., 1998). 400 ml of overnight *Agrobacteria* culture and 4-week-old RLD plants were prepared for transformation. The detailed protocol is as following:

- Harvest the Agrobacteria by centrifugation at 5,000x g for 10 min.
- Resuspend the cell pellet in 200 ml of inoculation medium containing 5% glucose and 0.05% Silwet L-77.
- Dip the 4-week-old RLD plants in the resuspended Agrobacteria for 30 sec.
- Cover the plants with a plastic lid so that the plants can stay in a humid environment.
- Water the plants with 0.1x Macrosalt solution.
- After 4 days, do the transformation again with the same protocol above.

2.2.4.2 Physiological assays of transgenic plants

Root growth tests:

- Sterilize Arabidopsis seeds in an Eppendorf tube with the 1m of solution containing 80% ethanol and 0.1% Triton X-100 for 30 min with shaking.
- Centrifuge for 5 sec and remove the supernatant.
- Add 1ml of 3% NaOCl solution and vortex. Incubate for 3 min.
- Wash the seeds 5 times with sterile miiliQ H_2O in the sterile bench.
- Distribute the seeds on MS medium and incubate at 4°C in the dark for 2 days to break dormancy.
- Transfer them to a culture room (22-24°C) and grow them for 4 days.
- Transfer the 4-day-old seedlings parallelly to MS and MS containing phytohormone medium. There should be around 20 seedlings on each plate. Seal the plates with Soehngen Pore tape (W.Soehngen GmbH, Taunusstein) and mark the roots.
- Grow the seedlings vertically in the culture room for 24 or 48 hours.
- Mark the roots again and measure the root growth.
- Statistically analyse the data.

Seeds germination tests:

- Sterilize seeds like above.
- Evenly distribute the seeds on MS and MS containing phytohormone medium, at least 50 seeds per plate.
- Incubate at 4°C in the dark for 2 days to break seed dormancy.
- Transfer the plates to a culture room.
- After 2-6 days, observe the seeds under a microscope (Stemi SV 6, ZEISS) and calculate the germination percentages.

Stomatal closure tests:

- Sterilize seeds like above.

- Distribute the seeds on MS medium and incubate at 4°C in the dark for 2 days to break dormancy.

- Transfer them to a culture room (22-24°C) and grow them for 4 days.

- Transfer the 4-day-old seedlings to MS and MS containing phytohormone plates. There should be around 20 seedlings on each plate.

- Grow the seedlings in the culture room for 24 hours.

- Take pictures of lower surfaces of the cotyledons under a microscope (Axioskop, ZEISS) and measure stomatal apertures.

- Statistically analyse the data.

2.2.4.3 Histochemical staining of transgenic Arabidopsis

- Fix the GUS-protein expressing transgenic seedlings or plant tissues in 90% acetone for 15-20 min on ice.

- Remove the acetone and soak the plant materials with a pre-solution for 1 min.

- Remove the pre-solution and add the staining solution. Incubate at 37°C for several hours to overnight, until the blue GUS staining is visible.

- Remove the staining solution and incubate the plant materials in 15%, 30%, 50%, and 70% ethanol (step gradient). For every step, the incubation time is 20 min.

- Heat the materials in 70% ethanol up to 65°C until the chlorophyll is completely extracted. Change the 70% ethanol several times in between, when it is necessary.

- Incubate the materials in 50%, 30%, 15%, and 0% ethanol (step gradient). For every step, the incubation time is 20 min.

- Soak the materials in a 2.67 g/ml chloralhydrate solution and store at 4°C.

Pre-solution: Staining solution:

50 mM Na_2HPO_4/NaH_2PO_4 (pH 7,2)   50 mM Na_2HPO_4/NaH_2PO_4 (pH 7,2)

0.5 mM $K_3Fe(CN)_6$ 0.5 mM $K_3Fe(CN)_6$

0.5 mM $K_4Fe(CN)_6$ 0.5 mM $K_4Fe(CN)_6$

                                           2 mM X-Gluc

                                           (5-bromo-4-chloro-3-indolyl-b-D-

                                           glucuronic acid)

2.2.5 Computer analysis of DNA sequences, data, and pictures

DNA sequences were obtained from TAIR (The Arabidopsis Information Resource at the Carnegie Institution of Washington, Stanford, USA). Multialin (Corpet, 1988) and Align (Pearson et al., 1997) softwares were used for DNA alignment. DNA blast was accomplished by NCBI website (National Centre for Biotechnology Information). The analysis and modification of vector sequences were achieved by using BioEdit (Hall, 1999) and vector NTI (Invitrogen, Germany) softwares. The parsimony trees based on the DNA sequences or amino acid sequences of ABP9 gene family were generated by PHYLIP program (Department of Genome Sciences, University of Washington, USA).

3. Results

3.1 Functional characterization of ABP7/9's role in ABA signal transduction in the transient expression system
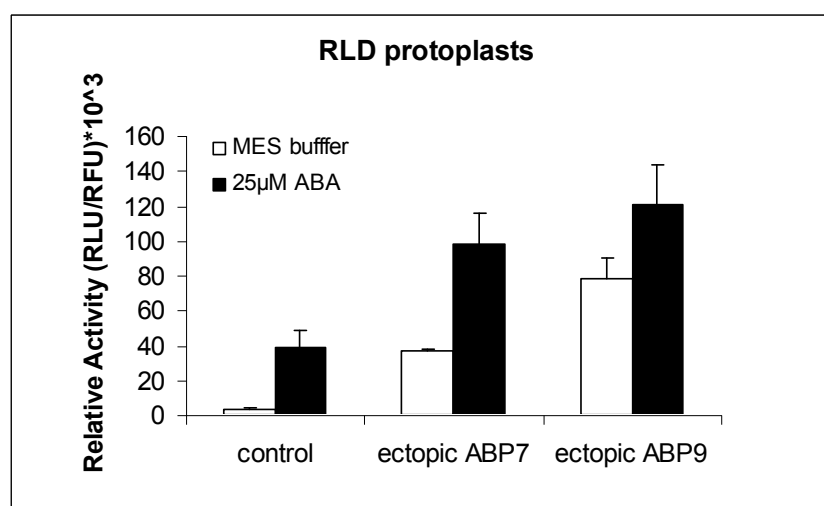
The transient expression system is a very sensitive and fast system well suited to check the roles of effectors in ABA signalling. This process is performed by studying their influence on the response of ABA reporters such as Rab18::LUC (Lang and Palva, 1992). In order to characterize the functions of ABP7 and ABP9 in ABA signal transduction, the corresponding ectopic expression constructs and RNAi knockdown expression constructs were generated. They were subsequently tested as effectors in Arabidopsis protoplasts. The plasmid pSK Rab18::LUC was co-transfected with the effectors to analyze the ABA response. In addition, the plasmid pSK 35S::GUS was co-transfected to correct for differences in transfection efficiency.

3.1.1 ABP7 and ABP9 overexpressors and ABA signal transfer

To overexpress ABP7 and ABP9, expression cassettes consisting of CaMV 35S promoter::ABP7cDNA::NOS terminator or CaMV 35S promoter::ABP9cDNA::NOS terminator were cloned into the vector pBI221 delta GUS, a modified pBI221 vector from which the GUS-cassette had been removed (see Appendix for details). These constructs were first tested as possible effectors in ABA signalling in RLD and La-er wt leaf cell protoplasts (Figure 6 and Figure 7). For this, 2.5 μg effector plasmid DNA, 3 μg pSK Rab18::LUC plasmid DNA, and 3 μg pSK 35S::GUS plasmid DNA were co-transferred in each sample. ABA treatment (diluted in MES buffer) and treatment with MES buffer alone started 6 hours after transformation. The final

concentration of ABA was adjusted to 25 μM ABA in RLD protoplasts but to10 μM in La-er protoplasts to account for the different ABA sensitivities of these two Arabidopsis ecotypes. GUS activity (presented as RFU/sec) and LUC activity (presented as RLU/sec) was measured after another 12 hours. ABA signalling presented as the mean of relative activity (RLU/RFU) ±SD of two or three independent transfections.

Figure 6 and Figure 7 show that in both RLD and La-er wt Arabidopsis, ABA induced the expression of Rab18::LUC. Meanwhile, overexpression of ABP7 and ABP9 resulted in a strong induction of Rab18::LUC expression in the absence or presence of exogenous ABA. The expression of 35S::GUS was not influenced by ectopic ABP7, ectopic ABP9, or ABA. The Rab18 promoter consists of both the ABA response element (ABRE) and drought response element (Iuchi *et al.*, 2001). In order to discover whether the signal induction effects of ABP7 and ABP9 overexpression are Rab18-promoter specific, another ABA response reporter Iti65::LUC (RD29B::LUC) was tested as well (Figure 8). Compared with the Rab18 promoter, the Iti65 promoter (Uno *et al.*, 2000) contains ABRE but not the drought response element. Data in Figure 8 show that the expression of Iti65::LUC was also strongly induced by ectopic ABP7 and ectopic ABP9. The ectopic expression of ABP7 and ABP9 could induce ABA reporters (Rab18::LUC and Iti65::LUC) much more strongly in the absence of ABA, but the induction was less pronounced when the protoplasts were treated with ABA. The reason for this may be that the combined effects of ABA plus the effectors have resulted in the maximum induction of the reporters.
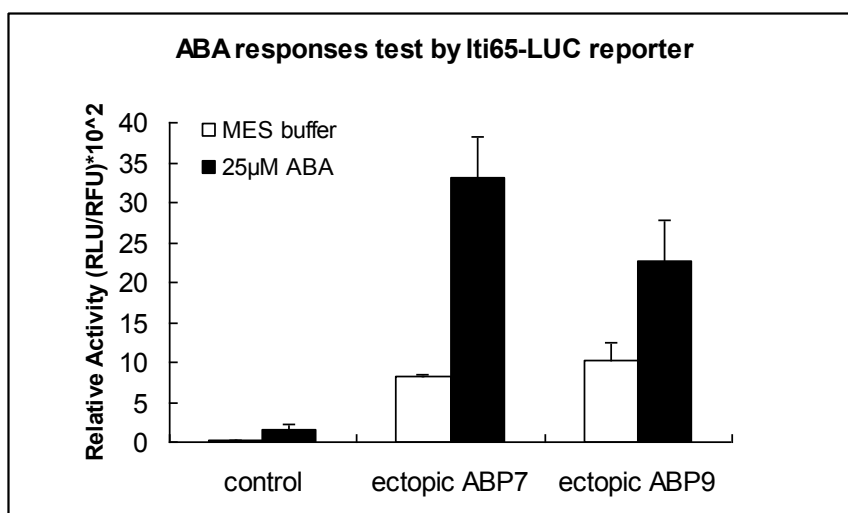
LUC (RLU/sec)*10^3	Control-1	Control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2	ectopic ABP9-3
MES buffer	12.3	3.6	72.4	75.3	120.9	118.3	143.4
25 μM ABA	96.1	94.4	186.6	150.5	228.0	97.1	286.9
GUS (RFU/sec)	Control-1	Control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2	ectopic ABP9-3
MES buffer	3.1	1.2	1.9	2.1	1.9	1.4	1.7
25 μM ABA	3.0	2.1	1.7	1.8	1.8	1.0	2.1

Figure 6. The effects of overexpression of ABP7 and ABP9 on ABA signalling in RLD wt protoplasts. Protoplasts were isolated from leaves of 4-week-old Arabidopsis seedlings. 2.5 μg effector plasmid DNA, 3 μg pSK Rab18::LUC plasmid DNA, and 3 μg pSK 35S::GUS plasmid DNA were co-transfected in each sample. GUS-deleted pBI221 vector was used as an internal control for ectopic ABP7 and ectopic ABP9 effectors. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two or three independent transfections in the graph. LUC and GUS activity from each sample is shown in the table underneath the graph. In the RLD protoplasts system, overexpression of ABP7 resulted in a 10-fold signal induction in the absence of exogenous ABA and in a 2.5-fold signal induction in the presence of exogenous ABA. Overexpression of ABP9 resulted in a 22-fold ABA signal induction in the absence of exogenous ABA and in a 3-fold ABA signal induction in the presence of exogenous ABA.
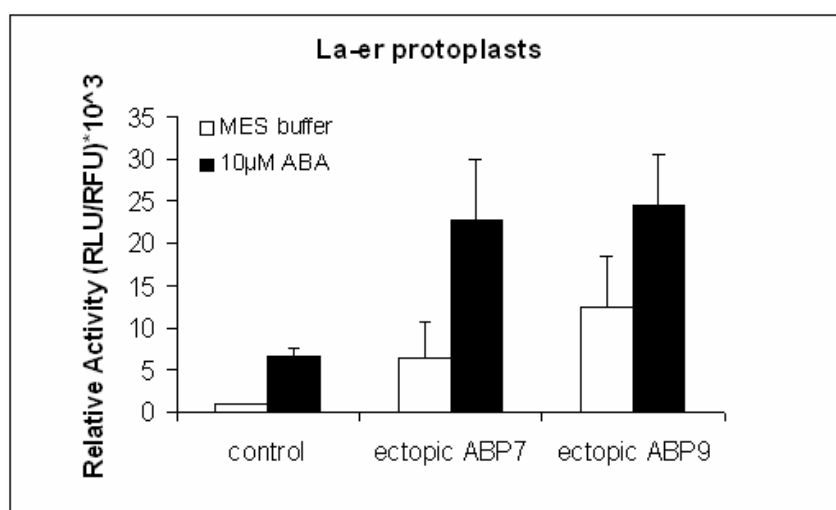
LUC (RLU/sec)*10^3	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	0.7	0.7	1.9	9.6	26.5	8.7
10 μM ABA	7.6	10.1	27.9	8.1	25.1	18.3
GUS (RFU/sec)	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	1.0	0.9	0.5	2.0	1.6	1.1
10 μM ABA	1.2	1.4	1.0	0.5	0.9	0.9

Figure 7. The effects of overexpression of ABP7 and ABP9 on ABA signalling in La-er wt protoplasts. LUC and GUS activity from each sample is shown in the table underneath the graph. In the La-er protoplasts system, overexpression of ABP7 resulted in an 8.5-fold signal induction in the absence of exogenous ABA and in a 3.4-fold signal induction in the presence of exogenous ABA. Overexpression of ABP9 resulted in a 16.5-fold ABA signal induction in the absence of exogenous ABA and in a 3.6-fold ABA signal induction in the presence of exogenous ABA.

LUC (RLU/sec)*10^2	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	0.3	0.1	25.6	32.5	36.8	48.7
25 μM ABA	0.8	1.8	114.6	175.2	135.7	112.4
GUS (RFU/sec)	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	1.8	0.6	3.0	4.1	4.2	4.1
25 μM ABA	0.7	0.9	3.1	5.9	5.2	5.9

Figure 8. ABA responses tested by lti65::LUC and overexpression of ABP7 and ABP9. Protoplasts were isolated from leaves of 4-week-old RLD seedlings. 5 μg effector plasmid DNA, 8 μg pSK lti65::LUC plasmid DNA, and 2.5 μg pSK 35S::GUS plasmid DNA were co-transfected in each sample. Data show that overexpression of ABP7 resulted in a 55-fold signal induction in the absence of exogenous ABA and in a 21-fold signal induction in the presence of exogenous ABA.   Overexpression of ABP9 resulted in a 69-fold ABA signal induction in the absence of exogenous ABA and in a 14-fold ABA signal induction in the presence of exogenous ABA.

To find out more information about the roles of ABP7 and ABP9 in ABA signalling, ectopic ABP7 and ABP9 constructs were tested in some mutant protoplasts as well. ABA2 is the dehydrogenase involved in the conversion of xanthoxin to ABA-aldehyde during ABA biosynthesis (Gonzalez-Guzman *et al.*, 2002). Arabidopsis mutant *aba2* is an ABA-deficient mutant with La-er background. Therefore, the plants only have low levels of endogenous ABA. Figure 9 shows that the ectopic expression of ABP7 and ABP9 could result in about 10-fold signal inductions (RLU/RFU) in the absence of exogenous ABA for both *aba2* mutant and La-er wild-type plants. With ABA treatment, the ectopic expression of ABP7 and ABP9 resulted in the assumed maximum response for both *aba2* and La-er. Furthermore, the ectopic-expression constructs of ABP7 and ABP9 have also been tested in protoplasts of ABA-insensitive mutant *abi1-1* and *abi2-1* (Figure 10 and Figure 11). The homologous protein ABI1 and ABI2 are two key negative regulators in ABA signalling. They encode type 2C protein phosphatases. Both ABP7 and ABP9 were identified to be interacting partners of ABI2, N-terminal truncated ABI1 (dNABI1, a.a. 123-434), and the point mutated form dN*abi1* (dNABI1^{Gly180Asp} with a functionally deficient catalytic domain of PP2C) in the yeast two-hybrid system by Dr. Yi Yang (Yang, 2003). They were further proven to interact with the point mutated form *abi2* (ABI2^{Gly168Asp} with a functionally deficient catalytic domain of PP2C). Unexpectedly, ectopic ABP7 and ABP9 could also induce the expression of Rab18::LUC, which is the ABA reporter in both *abi1-1* and *abi2-1* mutants. The DNA transfection to all the mutant protoplasts was the same like the DNA transfection described in Figure 6.
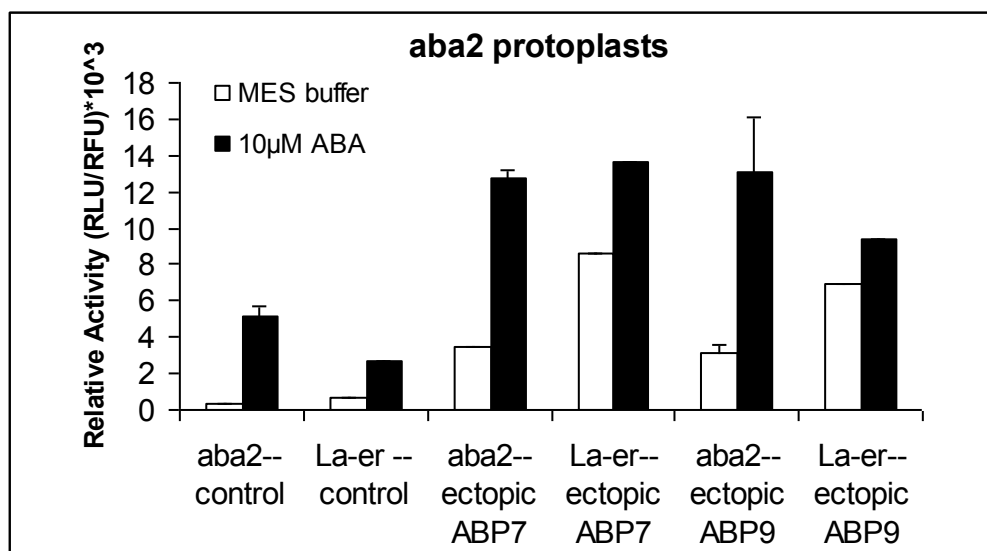
LUC in aba2 (RLU/sec)*10³	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	2.3	3.5	30.0	29.5	25.3	40.0
10 μM ABA	40.3	25.3	54.2	58.8	53.0	86.9
GUS in aba2 (RFU/sec)	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	7.6	10.0	8.8	8.5	9.1	11.6
10 μM ABA	7.3	5.3	4.4	4.5	4.8	5.7

Figure 9. ABA responses and overexpression of ABP7 and ABP9 in the transient system of the ABA-deficient mutant *aba2*. La-er wt protoplasts were used as comparison for *aba2*. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two independent transfections in *aba2* mutant, but presented as LUC activity divided by GUS activity (RLU/RFU) of a single transfection in La-er. In the *aba2* protoplasts system, overexpression of ABP7 resulted in a 10.4-fold signal induction in the absence of exogenous ABA and in a 2.5-fold signal induction in the presence of exogenous ABA. Overexpression of ABP9 resulted in a 9.5-fold ABA signal induction in the absence of exogenous ABA and in a 2.5-fold ABA signal induction in the presence of exogenous ABA.
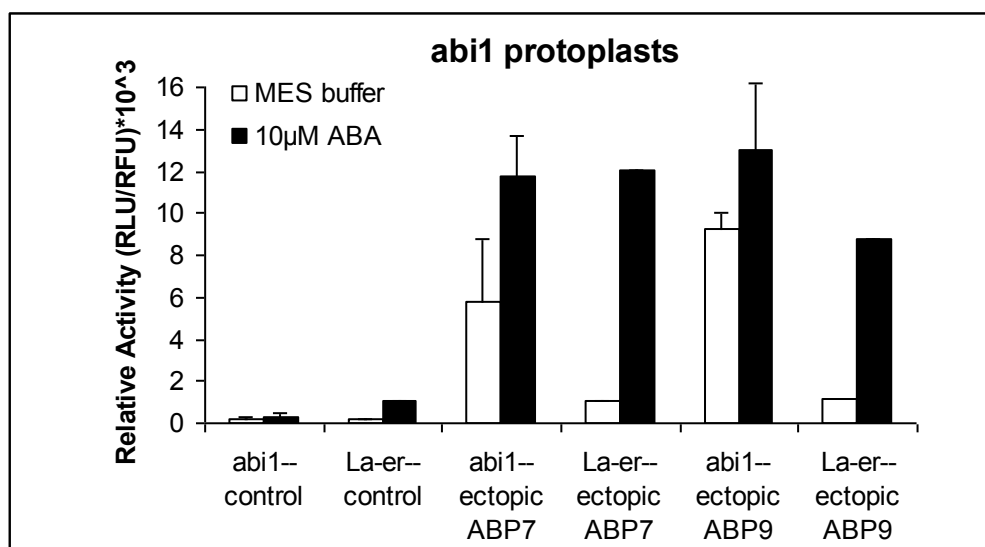
| LUC in abi1-1 (RLU/sec)*10^3	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	0.2	0.4	6.0	8.6	12.0	11.8
10 μM ABA	0.2	0.4	17.7	11.6	13.2	13.8
GUS in abi1-1 (RFU/sec)	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	1.3	1.4	1.6	1.1	1.4	1.2
10 μM ABA	1.0	0.9	1.7	0.9	1.2	0.9

Figure 10. ABA responses and overexpression of ABP7 and ABP9 in the transient system of the ABA-insensitive mutant *abi1-1*. La-er wt protoplasts were used as comparison for *abi1-1*. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two independent transfections in *abi1-1* mutant but presented as LUC activity divided by GUS activity (RLU/RFU) of a single transfection in La-er. In the *abi1-1* protoplasts system, overexpression of ABP7 resulted in a 27.9-fold signal induction in the absence of exogenous ABA and in a 36.1-fold signal induction in the presence of exogenous ABA. Overexpression of ABP9 resulted in a 44.7-fold ABA signal induction in the absence of exogenous ABA and in a 40.0-fold ABA signal induction in the presence of exogenous ABA.
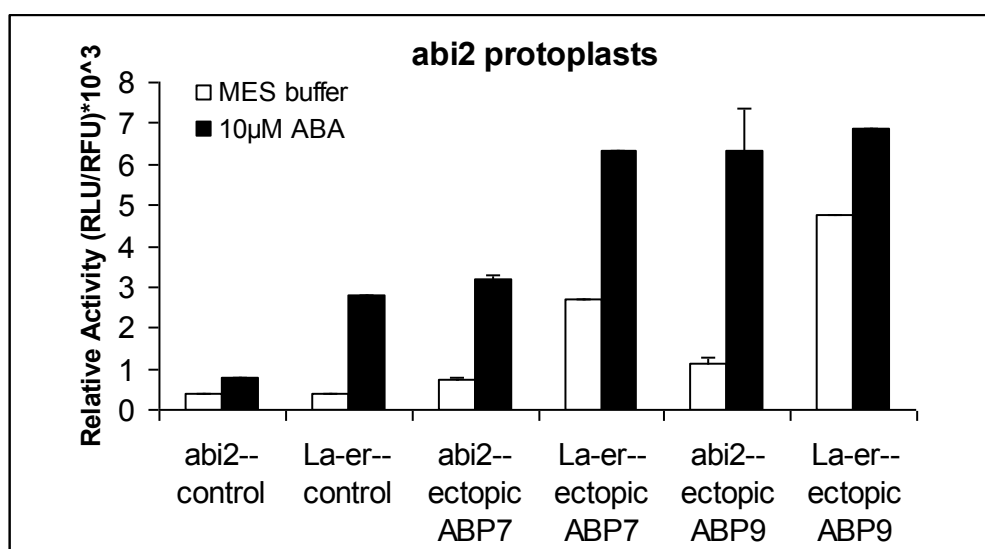
LUC in *abi2-1* (RLU/sec)*10^3	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	1.3	0.7	1.7	1.3	2.0	1.9
10 μM ABA	1.0	0.9	4.6	3.6	8.4	5.8
GUS in *abi2-1* (RFU/sec)	control-1	control-2	ectopic ABP7-1	ectopic ABP7-2	ectopic ABP9-1	ectopic ABP9-2
MES buffer	2.7	2.1	2.2	1.8	1.9	1.5
10 μM ABA	1.3	1.2	1.4	1.2	1.2	1.0

Figure 11. ABA responses and overexpression of ABP7 and ABP9 in the transient system of the ABA-insensitive mutant *abi2-1*. La-er wt protoplasts were used as comparison for *abi2-1*. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two independent transfections in *abi2-1* mutant but presented as LUC activity divided by GUS activity (RLU/RFU) of a single transfection in La-er. In the *abi2-1* protoplasts system, overexpression of ABP7 resulted in a 1.8-fold signal induction in the absence of exogenous ABA and in a 4.0-fold signal induction in the presence of exogenous ABA. Overexpression of ABP9 resulted in a 2.8-fold ABA signal induction in the absence of exogenous ABA and in a 7.9-fold ABA signal induction in the presence of exogenous ABA.

3.1.2 Effect of ABP7 and ABP9 overexpression on ABA responses in dependence of ABI1 and ABI2

The homologous protein ABI1 and ABI2 are two key negative regulators in ABA signalling. Both of them showed interactions with ABP7 and ABP9 in the yeast two-hybrid system (Yang, 2003). Thus, it is necessary to examine whether the positive regulating functions of ABP7 and ABP9 in ABA signalling would be influenced by ABI1 or ABI2 (Figure 12). Different amounts of pBI221 35S::ABI1 and pBI221 35S::ABI2 plasmids were co-transferred with a certain amount of pBI221 35S::ABP7 and pBI221 35S::ABP9 plasmid into RLD leaf cell protoplasts. Rab18::LUC was used as the ABA response reporter. The 35S::GUS construct was co-transfected to detect transformation efficiency. Six hours after transformation, the samples were treated with ABA to the final concentration of 25 μM. LUC (RLU/sec) and GUS (RFU/sec) activity were measured after 12 additional hours. Data were presented as the mean of RLU/RFU ± SD of two independent transfections. As shown in Figure 12, the ABP7- and ABP9-mediated stimulation of the ABA responses is antagonized by co-expression of ABI1 and ABI2. Compared with ABP7, ABP9 was more insensitive to the inhibition of ABI1 and ABI2.

Besides ABI1 and ABI2, the titration of pBI221 35S::*abi1* (ABI1^{Gly180Asp} with a functionally deficient catalytic domain of PP2C) in the protoplast system also impaired the ABA-signal induction effect of ABP9 (Figure 13). The protoplasts from the *abi1-1* mutant and wt La-er were transfected with 3 μg pBI221 35S::ABP9 plasmid DNA, the step-gradient amounts of pBI221 35S::*abi1* plasmid DNA, 10 μg pSK lti65::LUC, and 2 μg pSK 35S::GUS DNA. Six hours after transfection, all the samples were treated with ABA to a final concentration of 10 μM. Compared with wt La-er, ABP9 overexpression resulted in less induction of ABA signalling in the *abi1-1* protoplasts when titrated with the same amounts of 35S::*abi1* effectors. When 1μg or 3 μg of

35S::*abi1* effector was applied, the induction effect of ABP9 was completely impaired.
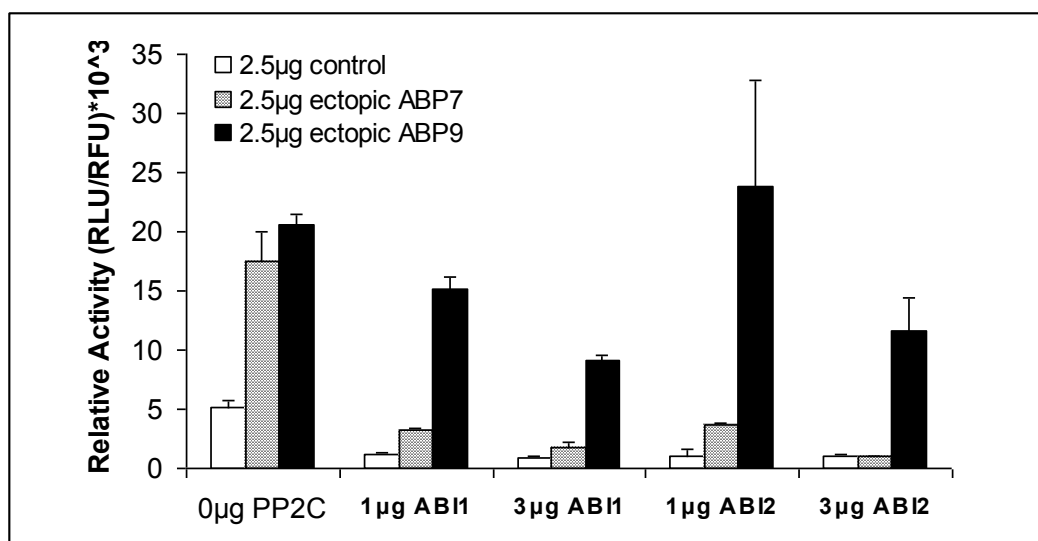
Figure 12. The ABP7- and ABP9-mediated stimulation of the ABA responses is antagonized by co-expression of ABI1 and ABI2. Protoplasts were isolated from leaves of 4-week-old RLD seedlings. 2.5 μg ectopic ABP7 or ectopic ABP9 effector plasmid DNA, 3 μg pSK Rab18::LUC plasmid DNA, 3 μg pSK 35S::GUS plasmid DNA, and the announced amounts of pBI221 35S::ABI1 and pBI221 35S::ABI2 plasmid DNA were co-transfected in each sample. GUS-deleted pBI221 constructs were used as an internal control for ectopic ABP7 and ectopic ABP9 effectors. All of the samples were treated with ABA to the final concentration of 25 μM 6 hours after transfection. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two independent transfections. When 1 μg pBI221 35S::ABI1 or pBI221 35S::ABI2 DNA was transfected, the signal induction from ABP7 was around 5 times reduced and that from ABP9 only got slightly influenced. When 3 μg pBI221 35S::ABI1 or 35S::ABI2 DNA was transfected, the signal induction from ABP7 was 10 times reduced and that from ABP9 was half reduced.
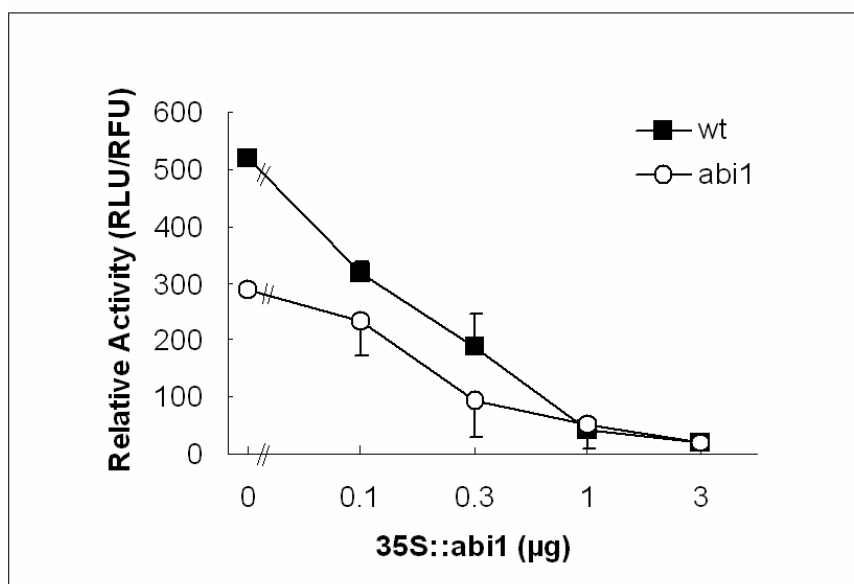
Figure 13. Titration of *abi1* to ectopic ABP9 in *abi1-1* and La-er wt protoplasts. 3 μg pBI221 35S::ABP9 plasmid DNA, the mentioned amounts of pBI221 35S::*abi1* plasmid DNA, 10 μg pSK lti65::LUC, and 2 μg pSK 35S::GUS DNA were co-transfected to *abi1-1* and La-er protoplasts. All the samples were treated with ABA to the final concentration of 10 μM 6 hours after transfection. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) ± SD of two independent transfections.

3.1.3 Knockdown of ABP7 and ABP9 expression by RNA interference and ABA responses

In addition to ABP7 and ABP9 overexpression constructs, the RNA interference construct was generated in pHannibal vector to knockdown the whole ABP7 and ABP9 gene family (see Appendix for details). This RNAi (RNA interference) construct was subsequently tested in the transient system (Figure 14). For this, 2.5 μg pHannibal (internal control) and RNAi plasmids were used as effectors in RLD protoplasts. The application of reporters, the

ABA treatment, and the signal measurement were the same as Result 3.1.1. The data in Figure 14 show that the RNAi effector resulted in a 0.6-fold reduction of ABA signalling in both the absence and the presence of exogenous ABA.
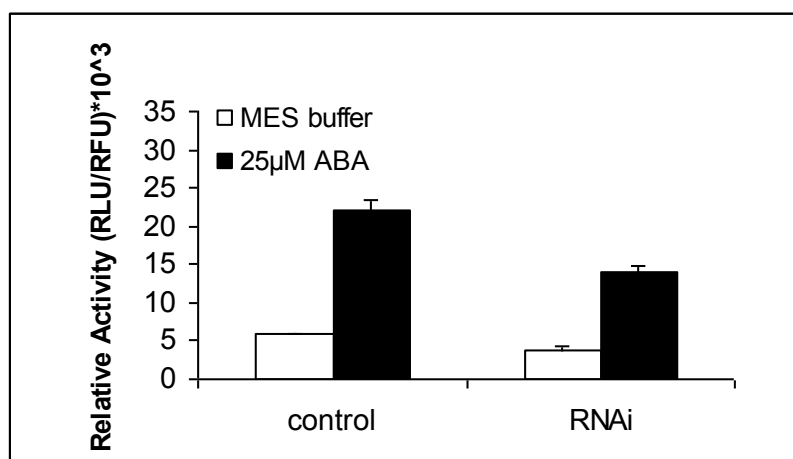
LUC (RLU/sec)*10³	control-1	control-2	RNAi-1	RNAi-2
MES buffer	91.2	81.0	53.3	49.6
25 μM ABA	252.5	203.0	153.4	178.9
GUS (RFU/sec)	control-1	control-2	RNAi-1	RNAi-2
MES buffer	15.1	13.4	16.2	12.1
25 μM ABA	11.0	9.5	11.6	12.3

Figure 14. RNAi (RNA interference) of ABP7 and ABP9's gene family and ABA signalling in RLD protoplasts. pHannibal vector was used as an internal control for RNAi effectors. ABA signalling presented as LUC activity divided by GUS activity (RLU/RFU) $\pm$ SD of two independent transfections in the graph.

3.2 Regulation of phytohormone responses in transgenic Arabidopsis by ABP7 and ABP9

Based on ABP7 and ABP9 overexpression vectors and RNAi knockdown vectors used in the transient system, corresponding binary vectors were constructed (RNAi construct was cloned into pART27 binary vector, while ectopic ABP7 and ABP9 constructs were cloned into pBI121 binary vector. See Appendix for details). The stable transgenic RLD Arabidopsis were generated afterwards. The T2 generation of the transgenic Arabidopsis with deregulated expression of ABP7 and ABP9 was tested for ABA responses. In addition, their phenotypes were checked in respect to exogenous phytohormones, such as ABA, auxin, cytokinin, and ethylene.

3.2.1 Physiological analysis of ABA responses in Arabidopsis with deregulated expression of ABP7 and ABP9

Root growth can be inhibited by ABA (Leung and Giraudat, 1998). Thus, root growth tests of seedlings on the MS medium with certain ABA concentrations can be used to detect the ABA sensitivity of plants. In the root growth tests, RNAi and ectopic ABP7 transgenic seedlings did not display any phenotypes compared with the controls (Figure 15). In contrast, all the ABP9 lines showed a clear reduction of root growth on the MS medium containing ABA (Figure 16).
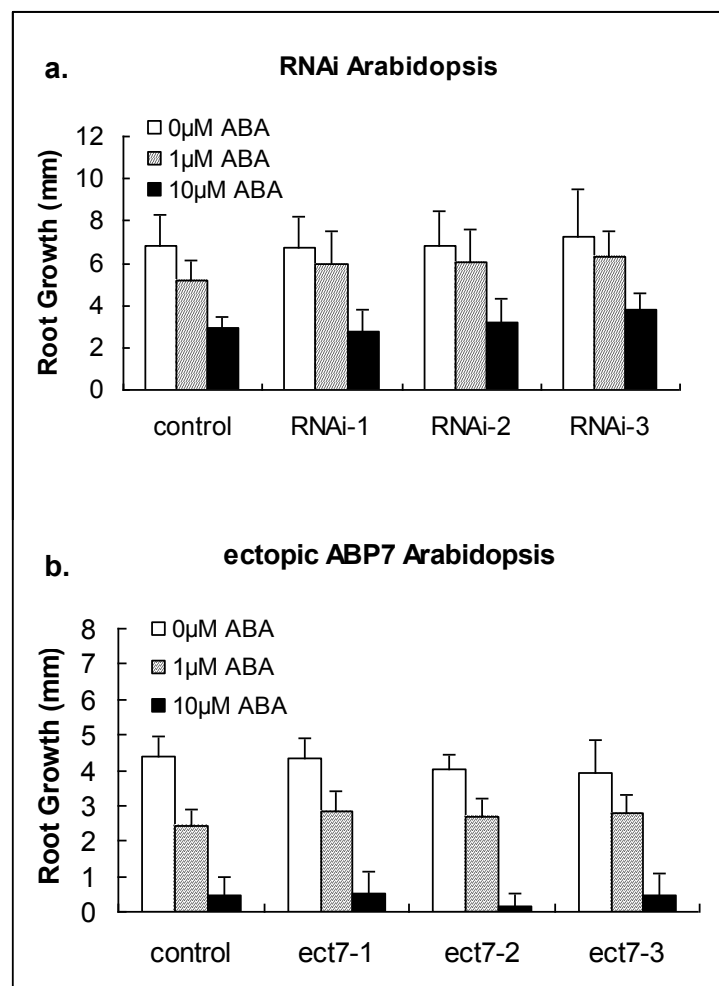
Figure 15. Root Growth Assay of RNAi transgenic Arabidopsis and ectopic ABP7 transgenic Arabidopsis. Four-day-old seedlings were transferred to the MS medium with ABA. Root growth measured within 48 hours (n＞16). One control line and three independent transgenic lines were chosen for each test. There was no clear difference of root growth between the control and the RNAi or ectopic ABP7 lines. **a.** The pART27 empty vector transgenic line was used as the internal control for the RNAi transgenic Arabidopsis. **b.** The pBI121 transgenic line was used as the internal control for the ectopic ABP7 (ect7) transgenic Arabidopsis.
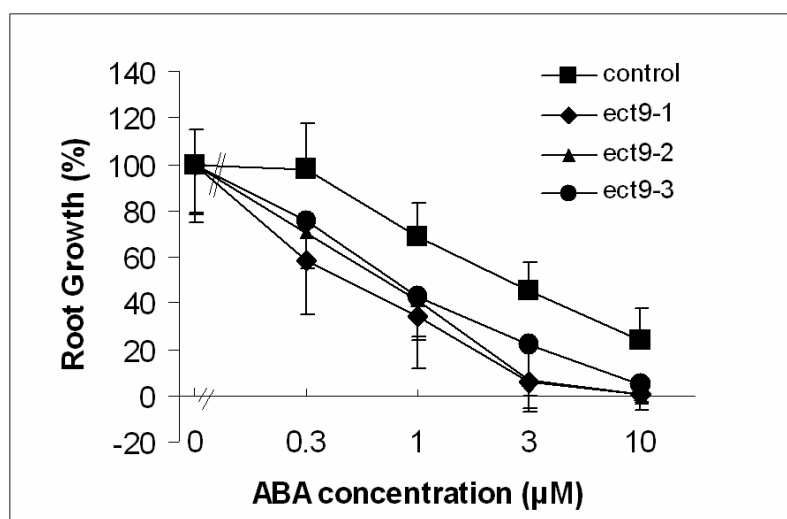
**Figure 16. Root Growth Assay of ABP9 transgenic lines.**
Four-day-old seedlings were transferred to the MS medium with
ABA. Root growth measured within 48 hours (n＞16). Compared
with the pBI121 control, all of the three independent ectopic ABP9
(ect9) transgenic lines showed the reduction of root growth in the
presence of exogenous ABA.

Plants that are hypersensitive or insensitive to ABA show phenotypes in seed
germination (Leung and Giraudat, 1998; Finkelstein *et al.*, 2002). Seed
germination of ABP7 and ABP9 deregulated transgenic plants was analyzed
(Figure 17 and Figure 18). The obtained results were consistent with the root
growth test. The ABP9 seeds were hypersensitive to ABA (Figure 18).
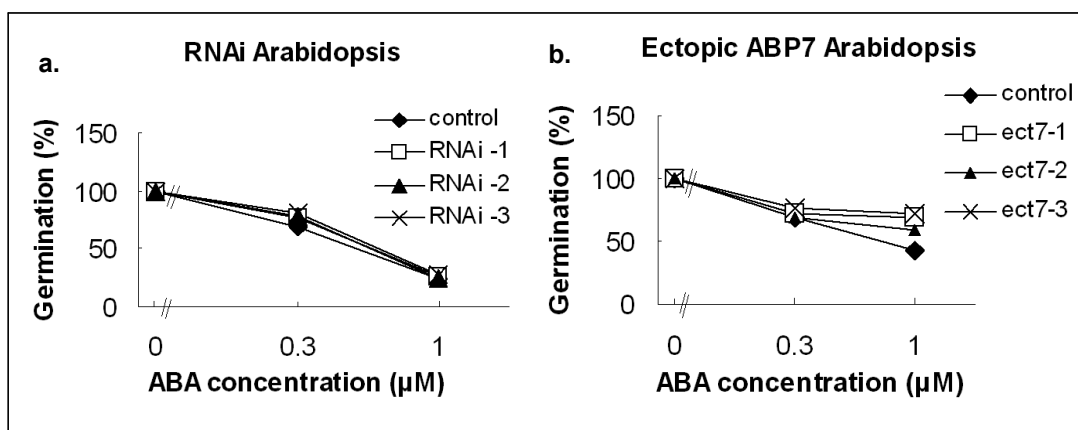
Figure 17. Germination assay of RNAi seeds and ectopic ABP7 seeds. All the seeds were stored at 4°C in the dark for 2 days to break dormancy. Subsequently, they were transferred into a culture room with 22°C and light. After 2 additional days, their germination was tested (n > 40). There was no difference of germination between the control line and the RNAi or ABP7 lines. **a.** One pART27 control line and three independent RNAi lines were randomly chosen for the test. **b.** One pBI121 control line and three independent ectopic ABP7 lines (ect7) were tested.

Plant transpiration is controlled by stomatal aperture. Stomatal aperture is reduced by ABA (Leung and Giraudat, 1998). The stomatal apertures of the transgenic seedlings were analyzed. Consistent with the result from the root growth test and the germination test, the ABP9 lines were hypersensitive to ABA in the stomatal aperture assay (Figure 19), while the RNAi and ABP7 lines were not (data not shown).
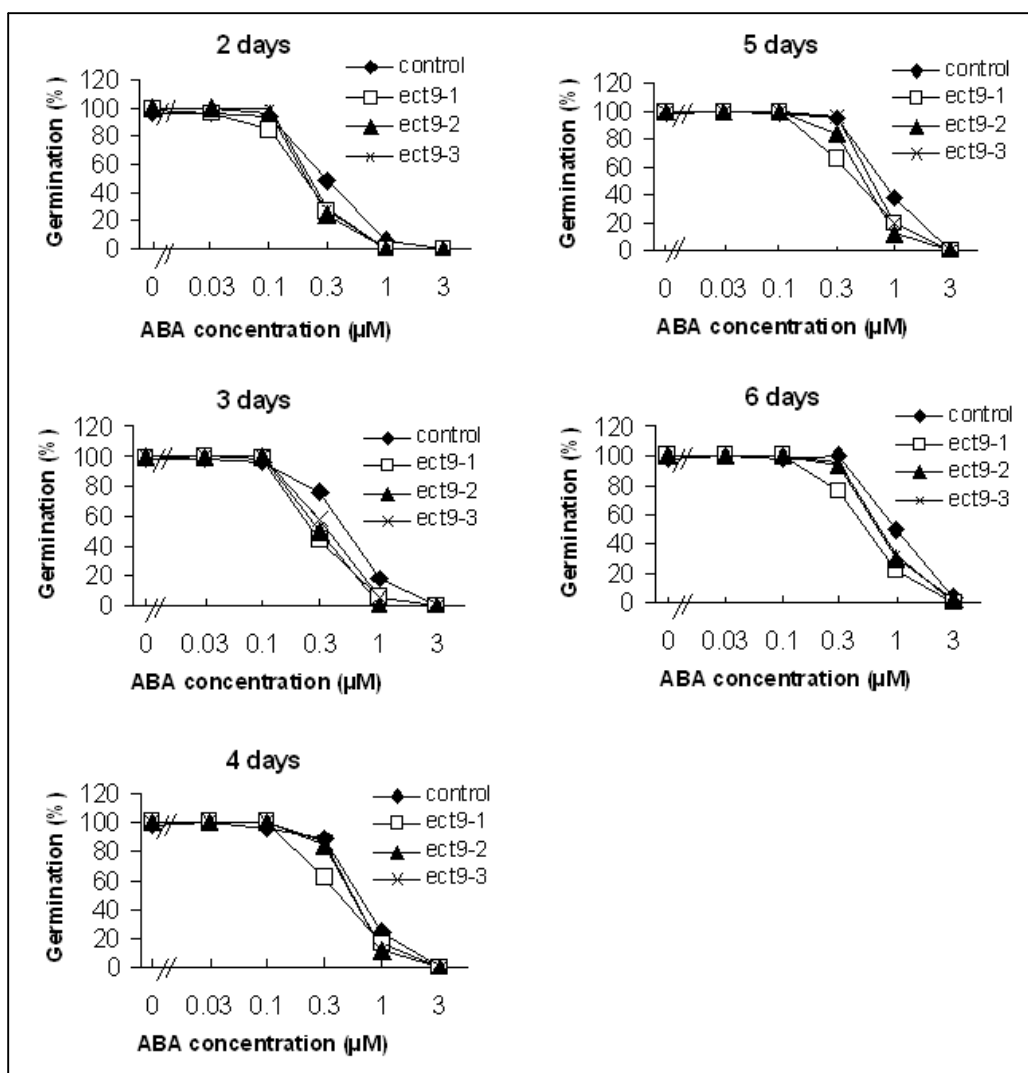
Figure 18. Germination assay of ABP9 seeds. Seeds from one pBI121 control and three independent ABP9 lines were tested (n＞50). All of the seeds were stored at 4°C in the dark for 2 days to break dormancy. Subsequently, they were transferred into a culture room with 22°C and light. Germination was tested after 2, 3, 4, 5, and 6 days. All of the ABP9 seeds displayed ABA hypersensitivity during the time course.
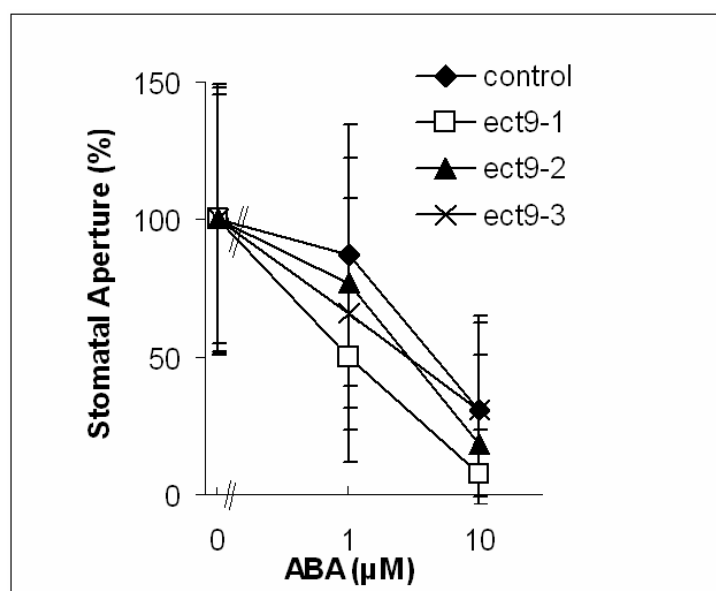
Figure 19. Stomatal aperture of ABP9 seedlings. 4-day-old seedlings were transferred to the MS medium containing ABA. The pictures from the lower surfaces of cotyledons were taken under a microscope after 24 hours (n>200). The stomata were measured by the ImageJ software. On the MS medium containing 1 μM ABA, three independent ectopic ABP9 (ect9) lines had 50%, 66%, and 77% stomatal aperture, while the control seedlings had 87%. On the MS medium containing 10μM ABA, three ABP9 lines had 7%, 18%, and 31% stomatal aperture, while the control had 31%.

All of the assays of root growth, germination, and stomatal aperture demonstrated that the ABP9 transgenic lines were hypersensitive to ABA, but the RNAi and ABP7 lines did not show altered sensitivity to ABA. However, in the transient expression system, both ABP9 and ABP7 overexpression resulted in a strong induction of ABA signalling, while the RNAi effector resulted in a clear reduction of ABA signalling. To discover the reason why the ABP7 and RNAi transgenic plants did not display altered sensitivity to ABA, their protoplasts were tested (Figure 20). The plasmids pSK Rab18::LUC and

pSK 35S::GUS as the reporters were co-transfected into the protoplasts isolated from the RNAi, ABP7, and ABP9 transgenic plants. No additional effectors were applied in these tests. The samples were treated with ABA to a final concentration of 25μM 6 hours after transformation. LUC (RLU/sec) and GUS (RFU/sec) activity was measured after 12 additional hours.
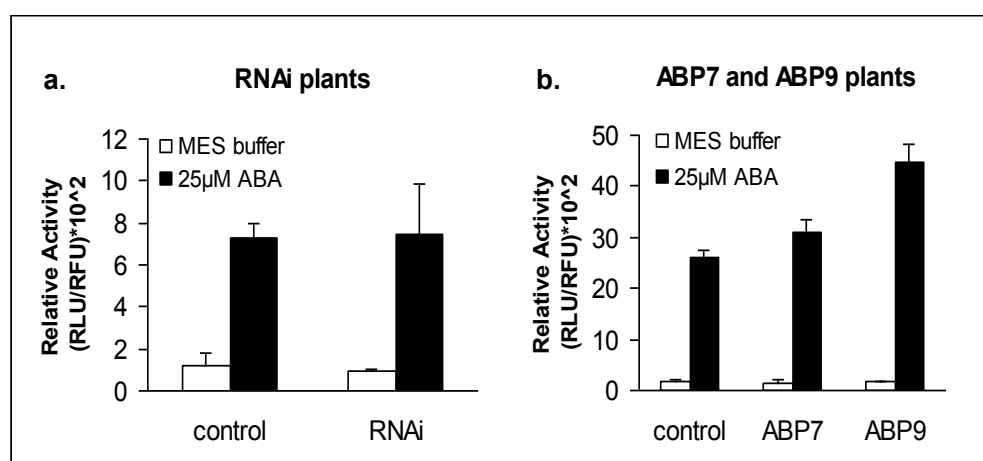
Figure 20. The ABA responses of the protoplasts from ABP7 and ABP9 de-regulated transgenic Arabidopsis. ABA signalling presented as the mean of LUC activity divided by GUS activity (RLU/RFU) ± SD of two independent transfections. GUS activity of each sample is comparable (data not shown). **a.** 2 μg pSK Rab18::LUC and 3 μg pSK 35S::GUS plasmid DNA was co-transfected to both pART27 control protoplasts and RNAi protoplasts. The RNAi protoplasts did not show any difference compared with the control. **b.** 3 μg pSK Rab18::LUC and 3 μg pSK 35S::GUS plasmid DNA was co-transfected to each sample. In the absence of exogenous ABA, the ABP7 and ABP9 protoplasts did not show any phenotype. In the presence of exogenous ABA, the ectopic ABP7 protoplasts only had a 1.2-fold induction of the ABA signal compared to the pBI121 control protoplasts, and ectopic ABP9 protoplasts had a 1.7-fold induction.

Figure 20 showed that the protoplasts of RNAi transgenic plants did not result in a reduction of ABA signalling. In addition, the protoplasts of ABP7 and ABP9 transgenic plants resulted in only a slight induction of ABA signalling in the presence of exogenous ABA. The protoplasts of the transgenic plants (Figure 20) and the protoplast transiently expressing the ABP7 or ABP9 de-regulated effectors (Figures 14 and 6) showed different results. In the transient expression system, the RNAi effector resulted in a 0.6-fold reduction of ABA signalling in both the absence and the presence of exogenous ABA. The ABP7 effector resulted in a 10-fold and a 2.5-fold induction in the absence and the presence of exogenous ABA, respectively. The ABP9 effector resulted in a 22-fold and a 3-fold induction, respectively. The difference between the result in Figure 20 and the results from the transient expression system implied that the expression levels of the RNAi and ABP7 constructs in the transgenic plants were too low to result in any phenotypes.

3.2.2 Phenotypes of transgenic lines in respect to exogenous phytohormones

3.2.2.1 ABA

Although RNAi (ABP7 and ABP9 knock-down expression) transgenic Arabidopsis did not show altered ABA sensitivity in the root growth, germination, and stomatal aperture tests, the RNAi seedlings displayed special phenotypes one to four weeks after germination and growing on the MS medium with 0.3 µM ABA (Figure 21). Further, 31-89% of the seedlings from all of the eight independent RNAi transgenic lines had very short roots. Some of these short-rooted seedlings had only a single large cotyledon and no primary leaves. Some possessed two normal cotyledons but also could not develop primary leaves. The rest of these short-rooted seedlings had normal shoots. However, the RNAi seedlings growing on the hormone-free MS medium were healthy. All of the control seedlings growing on the hormone-free

MS medium and the MS medium with 0.3 μM ABA looked healthy and normal.

The RNAi seedlings showed these phenotypes when they grew on the MS medium containing 0.3 μM ABA. But they had no phenotypes when they grew on the hormone-free MS medium. Therefore, all these phenotypes for the RNAi seedlings were ABA-induced. However, these ABA-induced phenotypes do not imply that the RNAi seedlings were hypersensitive to ABA. Because when the control seedlings grew on the MS medium containing higher concentrations of ABA, no similar phenotypes were found. The RNAi construct was designed to knock down the whole 14-member family of ABP7 and ABP9. When all the 14 family members and their interaction partners or down-stream components are taken into account, many signal pathways could be influenced in the RNAi seedlings. The development of the RNAi seedlings might be thereby impaired when exogenous ABA was applied.
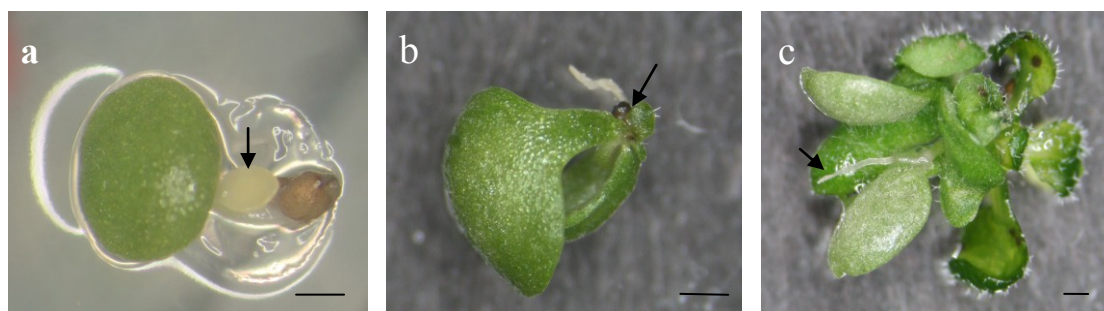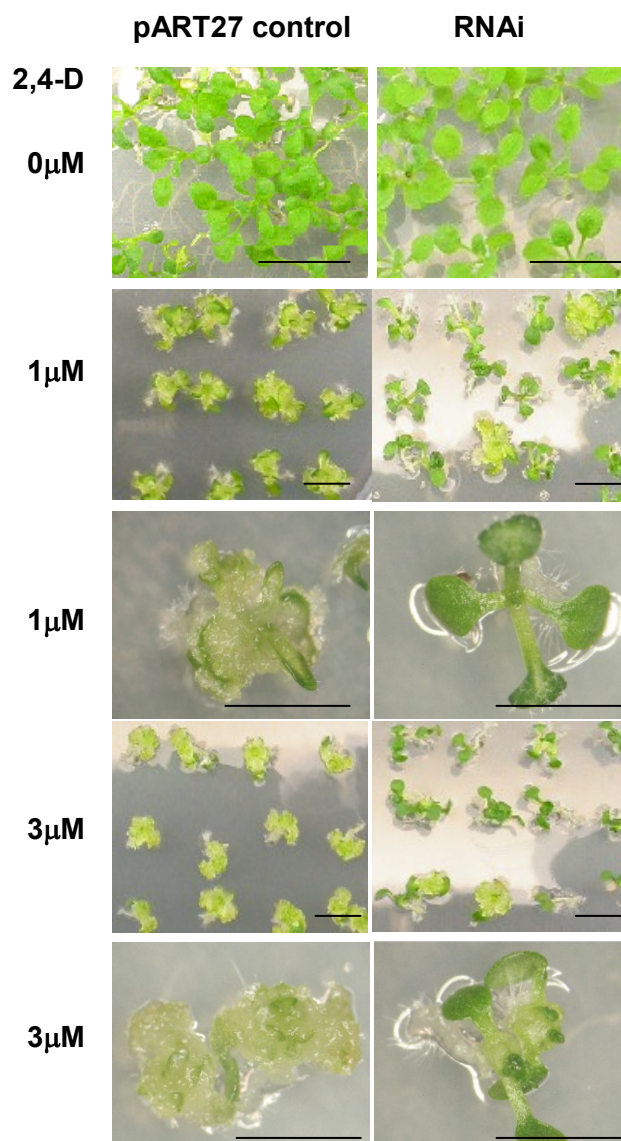
Figure 21. Phenotypes of the RNAi lines in respect to ABA. The RNAi seedlings grew on the MS medium containing 0.3μM ABA. **a.** The single-large-cotyledon phenotype. One cotyledon was green and in the correct size. The other cotyledon was extremely small and pale (shown by the arrow). The seedling was one-week-old.   **b.** No primary leaves. The primary leaves stopped developing and became dark brown (shown by the arrow). The seedling was four-week-old. **c.** The phenotype of the short root. The seedling had a normal shoot and a short root. The seedling was four-week-old. Bar=1 mm.

3.2.2.2 Auxin

Unexpectedly, the RNAi, ectopic ABP7, and ectopic ABP9 transgenic seedlings showed phenotypes in respect to auxin. High concentrations of auxin can induce calli formation (Sekiya *et al.*, 1977; Neibaur *et al.*, 2008). Thus, when both the pART27 control and the RNAi lines were directly grown on the MS medium with 1 μM or 3 μM 2,4-D, almost all the pART27 control lines formed calli after 3 weeks. However, only 11.5% of the RNAi lines from the MS medium with 1 μM 2,4-D and 20.6% of the RNAi lines from the MS medium with 3 μM 2,4-D formed calli (Figure 22). The percentages of the phenotypes likely resulted from the transgene segregation of the T2 generation of the RNAi lines.

The ratio of auxin to cytokinin is a well-known factor influencing calli formation (Sekiya *et al.*, 1977). Thus, the phenotype of the resistance of calli formation might imply that the RNAi lines were insensitive to auxin. An assay of auxin-inhibited root growth was set up to test this possibility (Figure 23). Auxin can induce ethylene synthesis. Therefore, the application of exogenous auxin in high concentrations can also inhibit root growth (Mulkey *et al.*, 1982). However, in the test (Figure 23), the RNAi lines did not behave like the auxin-resistant mutant *axr1-12* and they did not show any auxin insensitivity compared with the pART27 control. This result did not support the assumption that the RNAi lines possessed insensitivity to auxin.


Figure 22. Phenotypes of the RNAi lines in respect to auxin. The control and the RNAi lines grew on the MS medium with 0 μM, 1 μM, or 3 μM 2,4-D for three weeks. In the absence of exogenous 2,4-D, both the pART27 control and the RNAi lines looked normal. In the presence of 1 μM or 3 μM 2,4-D, most of the RNAi lines did not form calli like the pART27 control. Bar=1 cm.

83
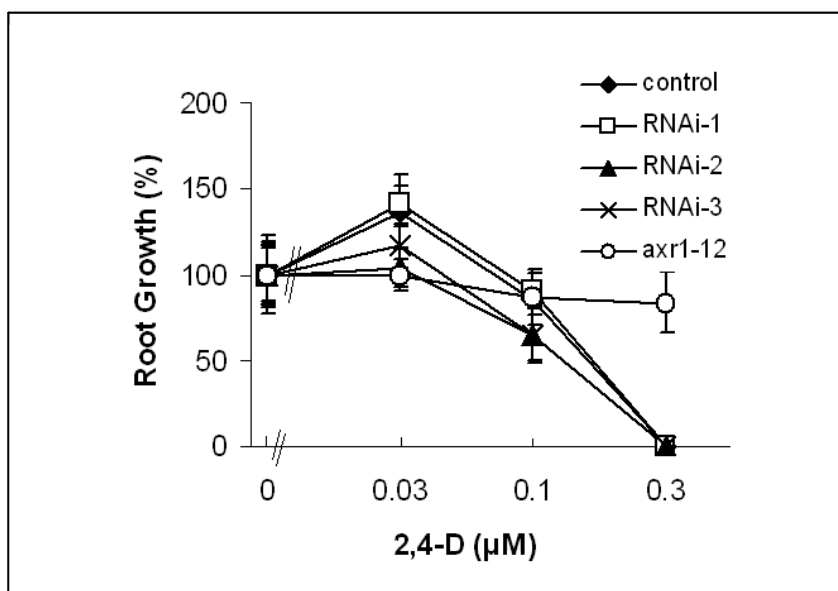
Figure 23. Root growth of RNAi lines in respect to 2,4-D.
4-day-old seedlings were transferred to the MS medium containing
various concentrations of 2,4-D. Root growth was measured after 3
days. The auxin-resistant mutant *axr1-12* was used as a positive
control. The data showed that 0.03 μM 2,4-D could stimulate root
growth, while 0.1 μM and 0.3 μM 2,4-D inhibited root growth. The
RNAi lines did not show 2,4-D insensitivity in this test.

Besides RNAi plants, the ectopic ABP7 Arabidopsis also displayed
phenotypes in respect to auxin. When the ABP7 lines germinated and grew on
the MS medium, some of them had vitreous shoots or even callus-like tissues,
especially on the petioles. When they grew on the MS medium with 0.3 μM
2,4-D, this phenotype became more clear (Figure 24). All of these vitreous
seedlings quickly dried out and died after being transferred to soil. The pBI121
transgenic control lines did not show this phenotype and grew healthily under
the same conditions.

pBl121 control   ectopic ABP7

MS

0.3μM 2,4-D
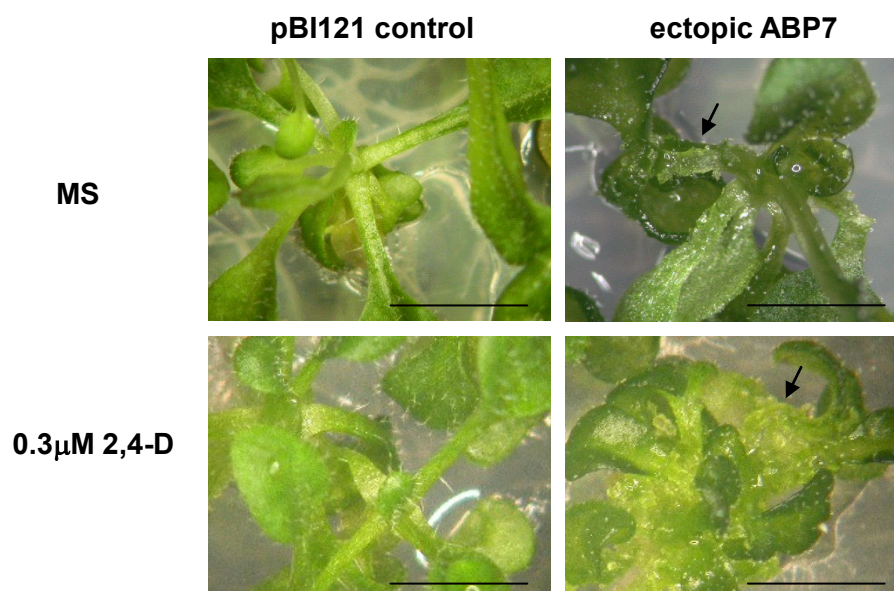
Figure 24. The vitreous and callus-like phenotypes of ABP7 lines. The photos were taken from 3-week-old ABP7 seedlings. The vitreous and callus-like phenotypes started from the petioles (shown by the arrows). Bar=5mm.

pBl121 control   ectopic ABP7   ectopic ABP7

2,4-D

1μM

3μM
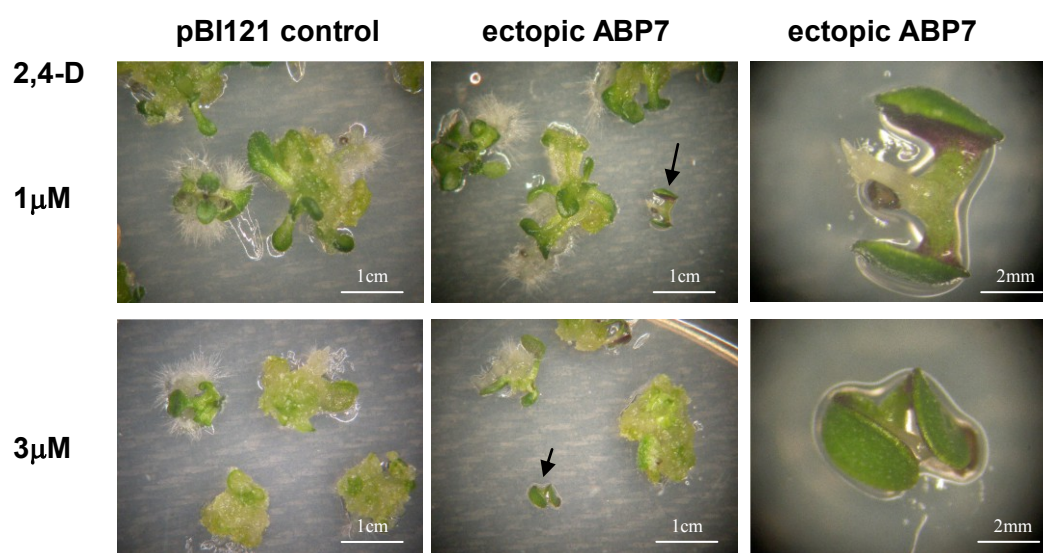
Figure 25. The tiny-seedling phenotype of ABP7 lines. The photos were taken from 3-week-old ABP7 seedlings. Some did not form calli. They were tiny seedlings. They had short hypocotyls and roots (highlighted by the arrows in the middle photos and magnified in the right photos).

When the control lines and the ABP7 lines grew on the MS medium with 1 μM or 3 μM 2,4-D, all the controls and most of the ABP7 seedlings formed calli in three weeks. However, besides calli, 9.5% of the ABP7 lines from the MS medium with 1 μM 2,4-D and 12.9% from the MS medium with 3 μM 2,4-D had the phenotype of tiny seedlings (Figure 25). These tiny seedlings had no primary leaves. They had extremely short hypocotyls and roots, instead of forming calli. When they were afterwards transferred to the hormone-free MS medium, they recovered and developed normal shorts and roots (data not shown).

The ectopic ABP9 lines also displayed very clear phenotypes when they grew on the MS medium with 1 μM and 3 μM 2,4-D (Figure 26). Ten days after germination, no control or ABP9 seedling formed calli. However, compared with the control lines, 65.5% of the ABP9 seedlings from the MS medium with 1 μM 2,4-D and 65.2% from the MS medium with 3 μM 2,4-D had extremely short hypocotyls. The rest looked like the control lines. In order to confirm that this phenotype resulted from ABP9 overexpression, genetic analysis by PCR was set up. Seven from the ABP9 seedlings that looked the same like control lines and seven from the ABP9 seedlings with short hypocotyls were randomly picked out for the test. The result showed that only the ABP9 seedlings with short hypocotyls contained the 35S::ABP9cDNA construct in their genome. Therefore, the transgene segregation of the ABP9 seedlings from the T2 generation caused the segregation of the phenotype of short hypocotyls. The ABP9 overexpression was the direct reason for this phenotype. The microscopy study showed that the ABP9 seedlings with short hypocotyls had normal mesophyll cells but very small hypocotyl cells. When the ABP9 seedlings continuously grew on the MS medium with 1 μM and 3 μM 2,4-D for another 10 days, all of them formed calli. ABP9 lines did not display the same

phenotype like ABP7 lines under the same conditions.

3.2.2.3 Cytokinin and ethylene

The RNAi and ectopic ABP7 transgenic plants were tested for cytokinin sensitivity. Cytokinin plays a major role in many different developmental and physiological processes in plants, such as cell division, regulation of root and shoot growth and branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Riou-Khamlichi *et al.*, 1999; O'Hare and Turnbul, 2004; Hare *et al.*, 2004). Leaf senescence of RNAi transgenic plants was analyzed to provide a first look at the cytokinin sensitivity of the RNAi lines. However, no difference was found between the RNAi and the pART27 control lines (data not shown). Cytokinin can also induce ethylene biosynthesis (Chae *et al.*, 2003). Thereby, the treatment of certain amounts of cytokinin can inhibit root growth. Root growth in respect to cytokinin was tested for the RNAi lines and ABP7 lines. The result also showed no difference between the control, RNAi, and ABP7 lines (Figure 27).

pBI121 control   **ectopic ABP9**

MS

1μM 2,4-D

1μM 2,4-D
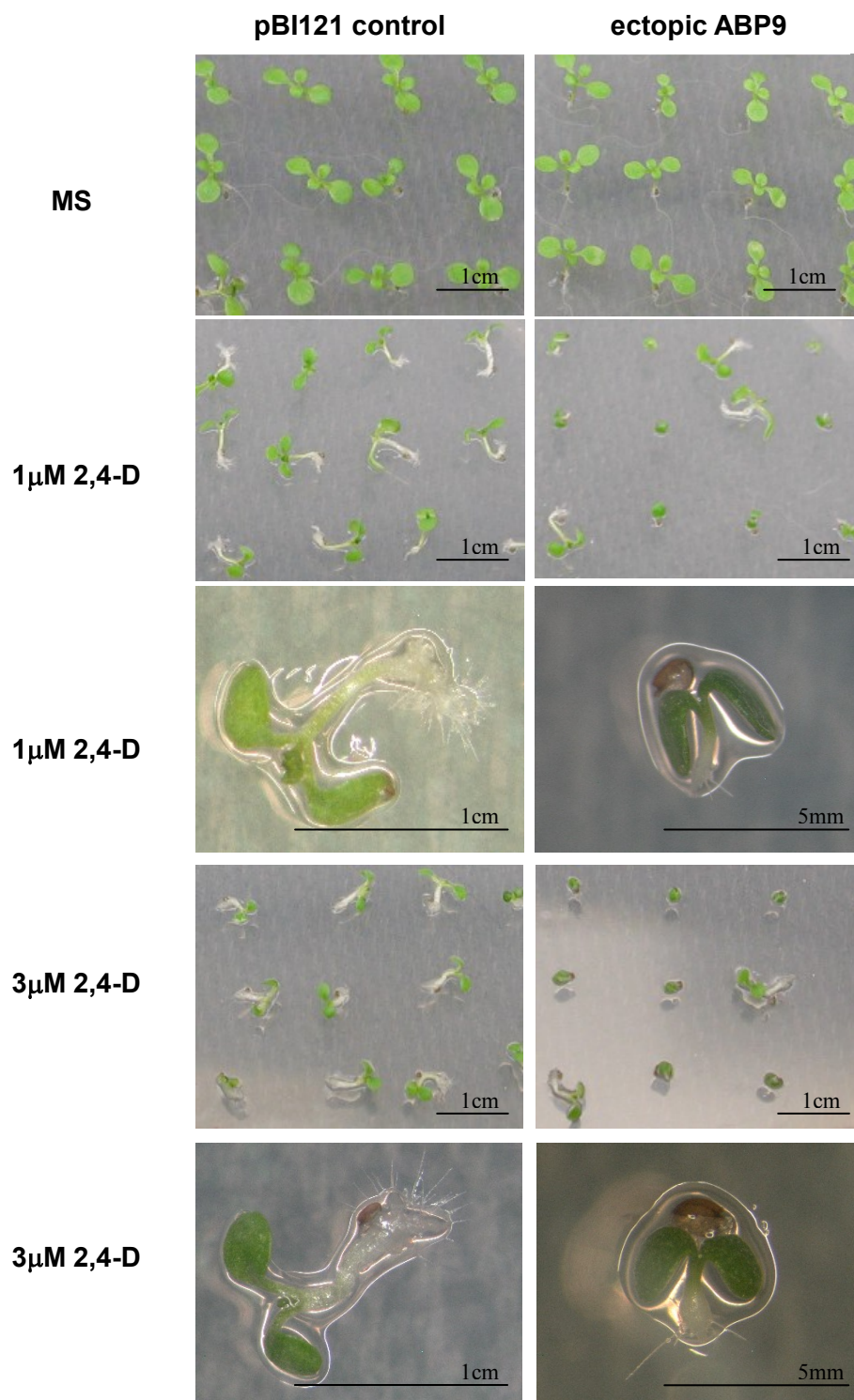
3μM 2,4-D

3μM 2,4-D

Figure 26. The phenotype of ABP9 seedlings in respect to auxin.
The photos were taken 10 days after germination.   65.5% of the ABP9
seedlings from the MS medium with 1 μM 2,4-D and 65.2% from the MS
medium with 3 μM 2,4-D showed the phenotype of short-hypocotyls.
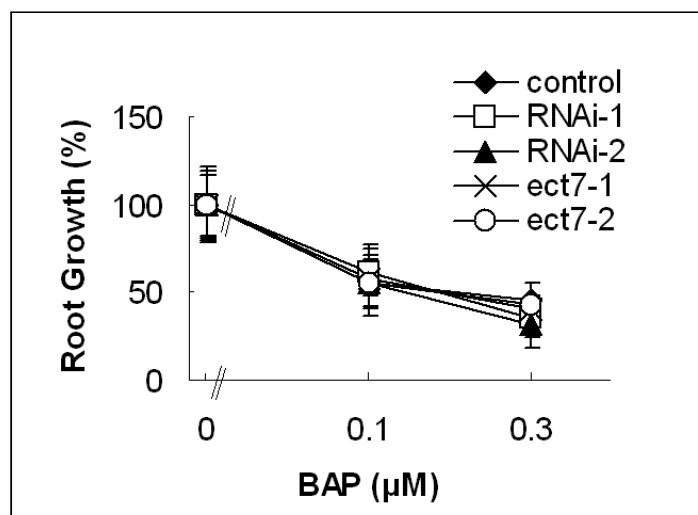
Figure 27. Root growth of RNAi and ABP7 lines in respect to cytokinin (BAP). 4-day-old seedlings were transferred to the MS medium with BAP. Root growth was measured in 2 additional days. The wt RLD Arabidopsis was used as the control. Two independent RNAi lines and two independent ABP7 lines were tested.

Ethylene is involved in many aspects of the plant life cycle, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Guo and Ecker, 2004). The treatment of ethylene or its metabolic precursor 1-aminocyclopropane-1-carboxylic acid (ACC) can result in the inhibition of root growth in seedlings. Root growth in the presence of ACC was analyzed for the RNAi and ABP7 lines. Altered sensitivity of ethylene was found in neither the RNAi lines nor the ABP7 lines (Figure 28).
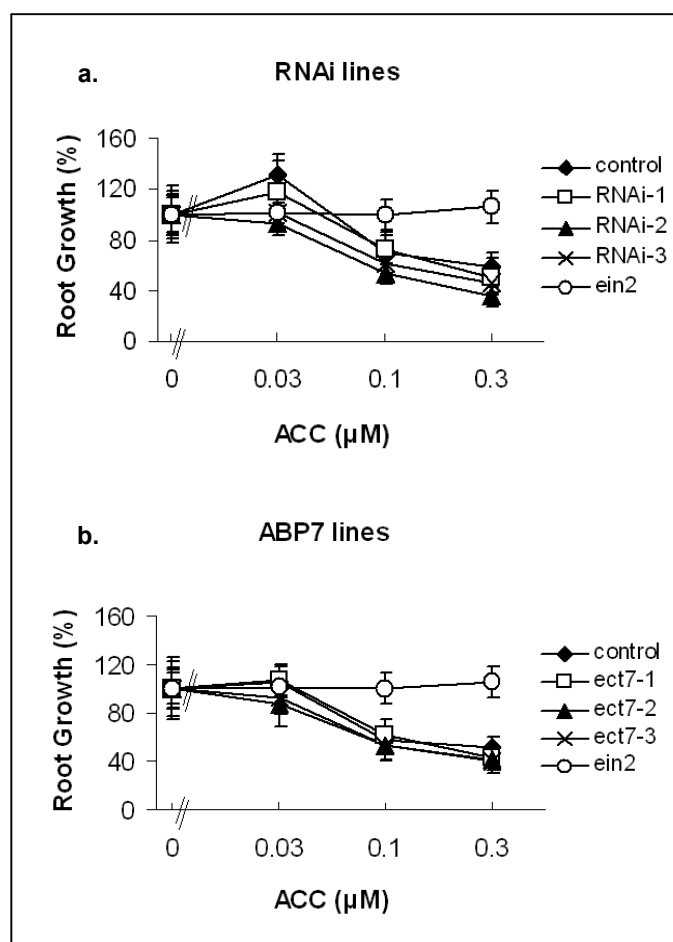
Figure 28. Root growth of RNAi and ABP7 lines in respect to ACC. 4-day-old seedlings were transferred to the MS medium with ACC. Root growth was measured after 48 hours. The ethylene-insensitive mutant *ein2* was used as a positive control in the test. **a.** The pART27 line was used as an internal control for the RNAi lines. **b.** The pBI121 line was used as an internal control for the ABP7 lines.

3.3 Expression studies of ABP9

3.3.1 Histochemical staining of endogenous promoter::genomic ABP9::
β-glucuronidase fusion transgenic plants

In order to study the expression pattern of ABP9 in Arabidopsis, stable
transgenic plants containing the construct of endogenous promoter::ABP9::
β-glucuronidase (GUS) were generated (see Appendix for cloning details).
Three independent T2 transgenic lines with the construct of ABP9
promoter::ABP9::GUS were analyzed by histochemical GUS staining. The
results showed that in seedlings the GUS staining was mainly concentrated in
root tips, root crowns, stipules, and stomata. It was also present in the
vascular system and mesophyll cells but never in hypocotyls. In flowers, GUS
staining was found only in the center of anthers (Figure 29).

The influence of ABA treatment on the expression of the construct
endogenous promoter::ABP9::GUS was investigated as well. Four-day-old
seedlings from these three independent T2 transgenic lines were transferred
to the hormone-free MS medium or the MS medium with 10 μM ABA.
Twenty-four hours after transformation, GUS staining was applied. The result
suggested that ABA treatment could not affect the expression pattern of
endogenous promoter::ABP9::GUS (pictures not shown).
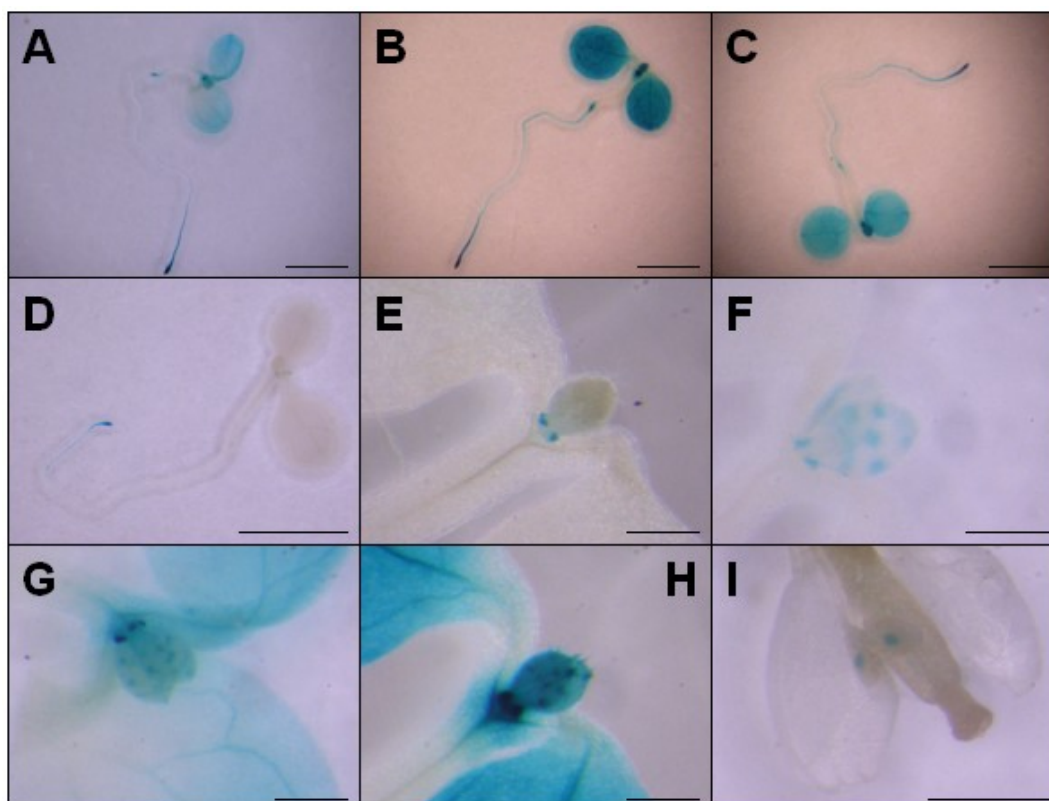
Figure 29. Histochemical localization of endogenous promoter::ABP9::GUS in Arabidopsis. Four-day-old seedlings and the flowers were stained by the GUS substrate. The reporter activity was found in different areas eg. root tips (A,B,C,D), root crowns (A, B), stipula (E, G, H), stomata (F, G, H), the vascular system and mesophyll cells (G, H), but never in hypocotyls. GUS activity was also found in the center of anthers (I). Bar=0.5 cm in A, B, C, D, and I. Bar=1 mm in E, F, G, and H.

3.3.2 Regulation of ABP9's expression

3.3.2.1 RT-PCR to test the possible regulations by ABA and auxin

ABP7 and ABP9 participate in ABA signalling. Meanwhile, ABP9 seems to be involved in the auxin signal pathway, according to the phenotype of ABP9 overexpression transgenic seedlings in respect to auxin (Figure 26). Therefore, it would be interesting to test whether ABP9's expression is regulated by ABA

and auxin. RT-PCR analysis was set up for the test. Five-day-old RLD wild-type seedlings were transferred to the hormone-free MS agarose medium, the MS agarose medium with 10 µM ABA, and the MS agarose medium with 10 µM 2,4-D. For each group, there were more than 30 seedlings. After 6 hours, total RNA was extracted from each group, and 2 µg RNA was used as the template to synthesize the first cDNA strands. The RT-PCR result showed that both ABP7 and ABP9 were down regulated by ABA at the transcriptional level. But auxin does not regulate the expression of ABP7 and ABP9 (Figure 30).
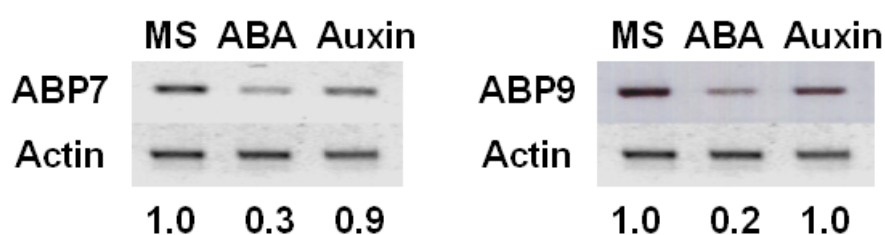

Figure 30. The expression of ABP7 and ABP9 in respect to ABA and auxin. The RT-PCR analysis was normalized by actin abundance. The induction factors indicated above were obtained according to the relative intensity of ABP7 and ABP9 bands compared with the actin control bands.

3.3.2.2 ABP9-GUS expression in response to Agrobacteria

ABP9 belongs to the PR10 pathogen-related protein family. Therefore, it might be involved in the defense of pathogen attacks. In addition, the transcription analysis by Genevestigator (Hruz *et al.*, 2008) showed that ABP9 expression was up regulated by *A.tumefaciens* treatment at the transcriptional level. Thus, it was necessary to check whether ABP9 expression would be regulated by

pathogen attacks at the protein level. Stable transgenic Arabidopsis seedlings containing the construct of endogenous promoter::ABP9::GUS were used for the test. Seeds (n>30) from three independent transgenic lines grew in the MS liquid medium for 9 days. Subsequently, the seedlings were infected by Agrobacterium GV3101 with a final concentration of 10^7/ml. After shaking overnight at 22°C, total protein from the seedlings was extracted and adjusted to 200 μg/ml. Then the GUS activity was measured. All three independent lines showed inductions of the GUS activity after the Agrobacterium GV3101 infection (Figure 31).
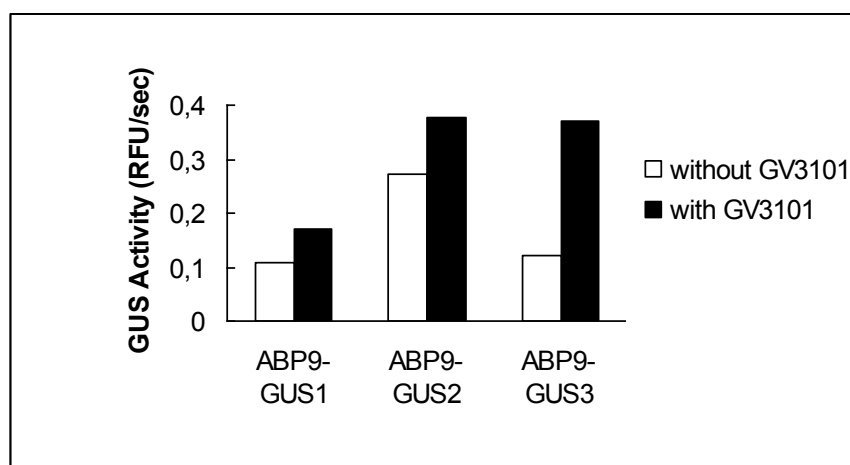
**Figure 31. ABP9 expression regulated by Agrobacterium GV3101.** Nine-day-old seedlings (n>30) from three independent lines were tested. Seedlings not infected by GV3101 were used as controls (open squares). Compared with the controls, all of the infected seedlings had induced GUS activity (solid squares).

3.4 Subcellular localization of ABP7 and ABP9 in Arabidopsis protoplasts

Besides the expression patter of ABP9, the subcellular localization of ABP9 was also studied. For this purpose, the construct of endogenous promoter::ABP9::EGFP was first tested in the protoplasts. However, the ABP9 promoter might be inactive in protoplasts or the endogenous expression level of ABP9 was too low. There was no visible EGFP signal in the protoplasts.

To resolve this problem, the constructs of 35S::ABP7 cDNA::EGFP and 35S::ABP9 cDNA::EGFP were cloned in the pSK vectors. These constructs were alternatively used to study the subcellular localization of ABP7 and ABP9 in protoplasts (see Appendix for cloning details). Leaves from 4-week-old RLD seedlings were used to isolate protoplasts. 15 μg pSK 35S::ABP7::EGFP or
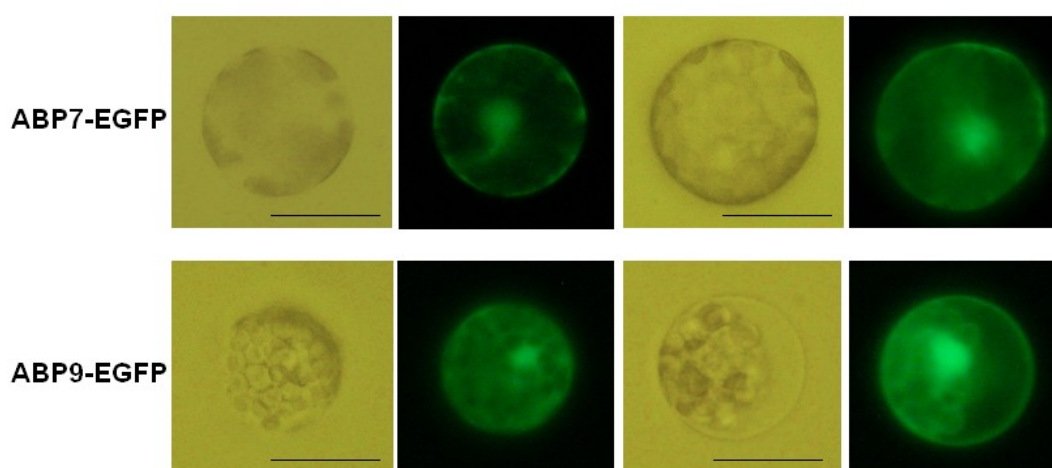
Figure 32. Subcellular localization of ABP7 and ABP9 in protoplasts. The constructs of 35S::ABP7::EGFP and 35S::ABP9::EGFP were expressed in the protoplasts. The ABP7-EGFP and ABP9-EGFP fusion protein concentrated in the nucleus. They appeared in the cytosol as well. Bar=20 μm.

pSK 35S::ABP9::EGFP DNA were co-transfected with 2 μg pSK 35S::GUS to each 100 μl protoplast sample. After shaking with 50 rpm at 22°C overnight in the dark, GUS activity was measured to check transformation efficiency. The fluorescent microscopy was applied to observe the protoplasts. The pictures showed that both ABP7 and ABP9 were mainly located in the nucleus, but they were also present in the cytosol (Figure 32).

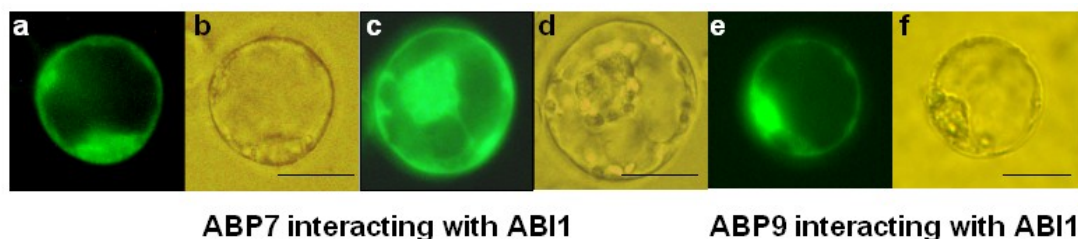### 3.5 Physical interaction between ABP7/9 and ABI1/*abi1*/ABI2/*abi2*

ABI1 and ABI2 are two major negative regulators of ABA signalling. ABP7 and ABP9 were shown to be interacting partners of N-terminal truncated ABI1 (dNABI1, a.a. 123-434), the point-mutated form dN*abi1* (dNABI1^{Gly180Asp} its PP2C activity is reduced more than ten times), full-length ABI2, and the point-mutated form *abi2* (ABI2^{Gly168Asp}) in yeast two-hybrid systems (Yang, 2003; Zhang, 2004). However, the interactions were never tested in plant cells. Bimolecular fluorescence complementation (BiFC) is one method to visualize protein-protein interactions in living plant cells. This approach relies on the formation of a fluorescent complex by two non-fluorescent fragments of the yellow fluorescent protein (YFP) brought together by association of interacting proteins fused to these fragments (Walter *et al*, 2004). The BiFC vectors used in this study were shown in Figure 33. Non-stop cDNA of ABP7, ABP9, ABI1, *abi1*, ABI2, and *abi2* were cloned into these pSPYNE-35S (YFP^N, a.a.1-155) and pSPYCE-35S (YFP^C, a.a. 156-239) vectors, respectively (see Appendix for cloning details). As a consequence, the non-fluorescent fragments YFP^N and YFP^C were fused to the N-termini of the tested proteins. If the tested proteins interacted with each other, YFP^N and YFP^C would be brought together and cause the YFP signal.

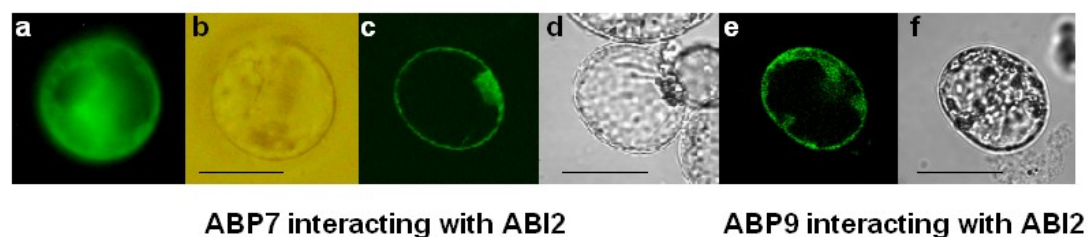The interactions between ABP proteins (ABP7 and ABP9) and ABI proteins

(ABI1, ABI2, *abi1,* and *abi2*) were tested in Arabidopsis protoplasts. The protoplasts were isolated from Col-0 cell culture, which was grown in the dark. 10 μg pSPYNE-35S plasmid DNA and 10 μg pSPYCE-35S plasmid DNA, which expressed the tested proteins, were co-transfected into 100 μl protoplasts. 2 μg pSK 35S::GUS plasmids were co-transfected into the protoplasts to check the transformation efficiency. After shaking with 50 rpm overnight at 22°C in the dark, the GUS activity was measured and the protoplasts were observed under the fluorescent microscope or the confocal microscope. The results showed that the interactions between ABI1 and ABP proteins mainly took place in the nucleus, while they also existed in the cytosol (Figure 34). This result was consistent with the subcellular localization of ABP7 and ABP9 as well as the subcellular localization of ABI1 (Figure 32; Moes *et al.*, 2008).
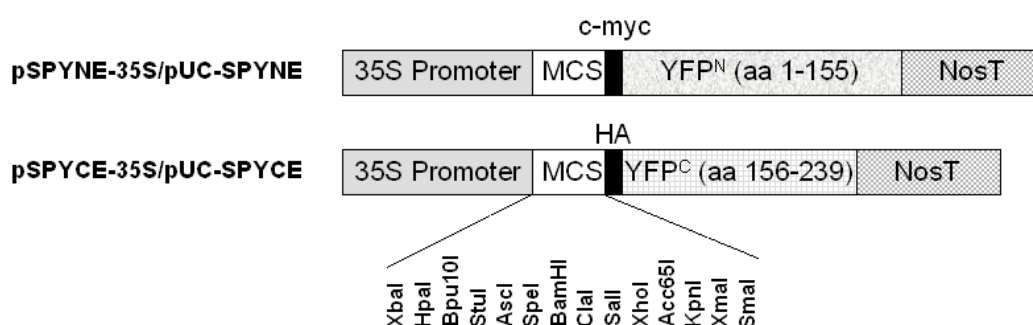
Figure 33. Schematic representation of pSPYNE-35S and pSPYCE-35S BiFC vectors. The non-stop cDNA of ABP7, ABP9, ABI1, abi1, ABI2, and abi2 were cloned into the multi-cloning sites (MCS) of these vectors. c-myc: c-myc affinity tag; HA: hemagglutinin affinity tag; 35S Promoter:35S promoter of the cauliflower mosaic virus; NosT: Nos terminator; YFP[N]:N-terminal fragment of YFP reaching from amino acid (aa) 1 to 155; YFP[C]: C-terminal fragment of YFP reaching from amino acid 156 to 239.

ABP7 interacting with ABI1   ABP9 interacting with ABI1

Figure 34. Bimolecular fluorescence complementation to visualize the interaction between ABP proteins and ABI1. The co-transfection of ABP7-YFP^C and ABI1-YFP^N fusion constructs (a,b), ABI1-YFP^C and ABP7-YFP^N fusion constructs (c,d), and ABI1-YFP^C and ABP9-YFP^N fusion constructs (e, f) caused the YFP signals in both the nucleus and the cytosol. However, the combination of ABP9-YFP^C and ABI1-YFP^N fusion constructs never resulted in any fluorescent signal. The co-transfection of YFP^C (original pSPYCE-35S vector) and YFP^N (original pSPYNE-35S vector) without fusion constructs was used as negative controls which never showed the YFP signal (pictures not shown). Bar=20 μm.

ABP7 interacting with ABI2   ABP9 interacting with ABI2

Figure 35. BiFC to visualize the interaction between ABP proteins and ABI2. The co-transfection of ABP7-YFP^C and ABI2-YFP^N fusion constructs resulted in the YFP signal in both the nucleus and the cytosol (a,b,c,d), while the co-transfection of ABP9-YFP^C and ABI2-YFP^N fusion constructs resulted in the YFP signal only in the cytosol (e,f). The combination of ABI2-YFP^C and ABP protein-YFP^N fusion constructs never showed any YFP signal. In addition, the negative control of the combination of YFP^C and YFP^N without fusion constructs did not show any YFP signal. The photos were taken under a fluorescent microscope (a, b) or a confocal microscope (c,d,e,f). Bar=20 μm.

In contrast to ABI1, ABI2 interacted with ABP proteins only in the cytosol (Figure 35). In addition, compared with the interaction between ABP7 and ABI2, the interaction between ABP9 and ABI2 always displayed much weaker signals although the transformation efficiencies were comparable to each other.

The previous study showed that *abi1* (ABI1^{Gly180Asp}) was localized in the nucleus (Moes *et al*., 2008). However, the BiFC-interacting tests between *abi1* and ABP proteins showed that their YFP signal was in the cytosol (Figure 36 a-f). The point-mutated form *abi2* (ABI2^{Gly168Asp}) was found to interact with

abi1 interacting with ABP7

abi1 interacting with ABP9   abi2 interacting with ABP7
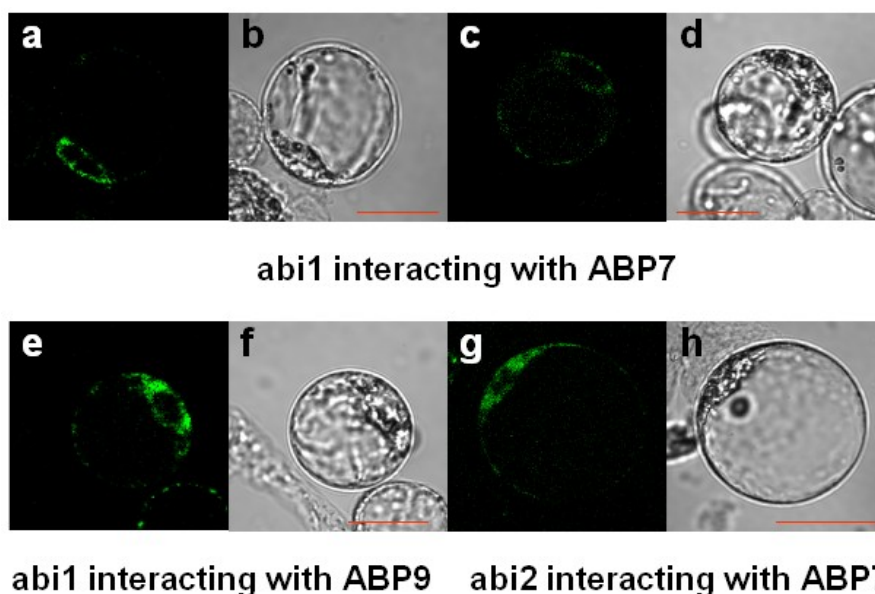
Figure 36. Interaction between *abi* proteins (*abi1, abi2*) and ABP proteins (ABP7, ABP9). The combination of *abi1*-YFP^{N} and ABP7-YFP^{C} (a,b), ABP7-YFP^{N} and *abi1*-YFP^{C} (c,d), ABP9-YFP^{N} and *abi1*-YFP^{C} (e,f), and *abi2*-YFP^{N} and ABP7-YFP^{C} (g,h) fusion constructs resulted in YFP signals in the cytosol of the protoplasts. All the other combinations between abi proteins and ABP proteins did not show any YFP fluorescence. The pictures were taken under a confocal microscope. Bar=20 μm.

ABP7 in the cytosol as well (Figure 36 g and h). Compared with ABI1 and ABI2, *abi1* and *abi2* interacted much more weakly with the ABP proteins in the protoplasts and the YFP signal was always slight. ABP9 was never found to interact with *abi2* by the BiFC method, although it was an interacting partner of *abi2* in the yeast two-hybrid system (Zhang, 2004).

3.6 Attempts to identify ligand(s) of ABP9

ABP7 and ABP9's family contains the domain Bet v 1-like. Bet v 1 is a 17-kDa protein abundantly present in the pollen of the White birch tree and is the primary cause of birth pollen allergy in humans (Mogensen *et al*, 2002). It belongs to the ubiquitous family of pathogenesis-related proteins (PR-10). Some researches showed that Bet v 1 can bind ligands such as fatty acids, flavonoids, and cytokinins (Mogensen *et al*, 2002). Its natural hypoallergenic isoform Bet v 1l interacts with brassinolide and 24-epicastasterone (Markovic-Housley *et al*, 2003). The three-dimensional structural prediction of ABP9 protein displays a cavity, which is similar to the cavities interacting with small ligands in Bet v 1 and Bet v 1l. Therefore, ABP9 might also have binding activity to ligands.

3.6.1 Expression and purification of histag proteins in *E.coli*

ABP7 and ABP9's cDNA were cloned into *QIAexpress* pQE 30 vectors for their protein expression in *E. coli*. After cloning, both were under the control of T5 promoter and were tagged by 6x histidine (6xHis) at the N-termini (see Appendix for details). The plasmids containing 6xHis-tagged ABP7 and 6xHis-tagged ABP9 were subsequently transfected into *E. coli* strain XL1 Blue, which produces enough *lac* repressor to block transcription efficiently. The addition of isopropyl-β-D-thiogalactoside (IPTG) can induce the expression of 6xHis-tagged proteins by binding to the *lac* repressor protein and inactivating it.

A time-course induction with the final concentration of 0.1 mM IPTG was conducted to search for the optimal induction time (Figure 37). The result
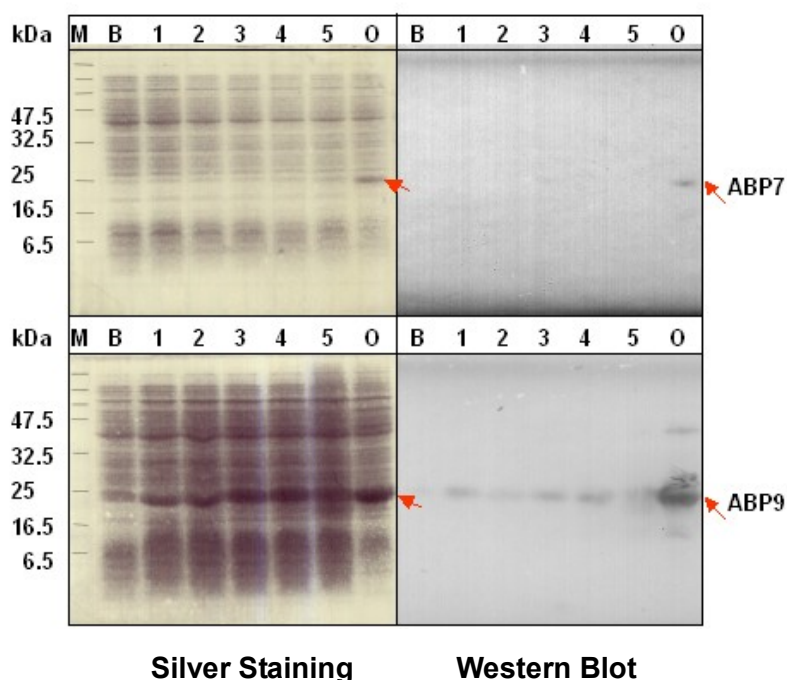
Figure 37. Time course study of IPTG induction to the expression of ABP7 and ABP9 in *E.coli.* IPTG was added to the *E.coli* cultures to a final concentration of 0.1 mM, when OD_{600} of the cultures reached 0.5-0.7. HRP-conjugated anti-Histag antibodies were used for detecting 6xHis-tagged proteins in the western blot after SDS-PAGE analysis. Silver staining and the western blot showed that overnight induction with IPTG resulted in the most expression of 6xHis-tagged fusion proteins in *E. coli*. The bands of 6xHis-tagged proteins are highlighted by arrows. M: protein marker; B: before IPTG induction; 1,2,3,4,5: 1,2,3,4,5 hour(s) of IPTG induction; O: overnight IPTG induction.

showed that the 6xHis-tagged ABP7 protein was 23.5 kDa and the 6xHis-tagged ABP9 protein was 23.3 kDa. Both were abundantly expressed in *E. coli* after overnight IPTG induction.

Ni-Nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography was used to purify 6xHis-tagged proteins under denaturing condition after overnight IPTG induction. The 6xhistidine can be bound to Ni-NTA resin with high affinity and can be eluted by low pH buffers or high concentrations of imidazole as a
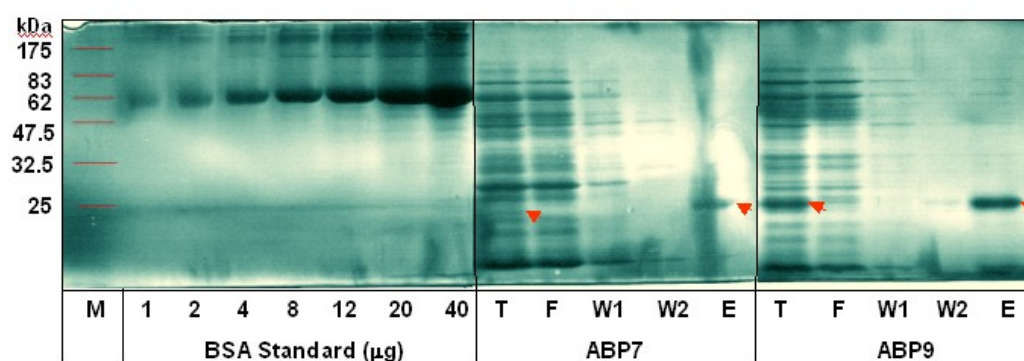
Figure 38. Purificaiton and quantification of 6xHistagged ABP7 and ABP9. Denatured *E. coli* lysates (pH 8.0) were loaded into Ni-NTA affinity columns after overnight IPTG induction. The columns were subsequently washed by washing buffers with pH 6.3 and pH 5.9 to remove the unspecific binding componants. The 6xHis-tagged proteins were finally eluted by elution buffer with pH 4.5. According to the BSA standard from SDS-PAGE, the 10 μl ABP7 eluate sample (obtained from 0.25 ml *E. coli* culture) contained about 1 μg protein and the 10 μl ABP9 eluate sample (obtained from 0.25 ml *E. coli* culture) contained about 7 μg protein. The 6xHis-tagged proteins are highlighted by red arrows. M: protein marker; 1-40: 1-40 μg BSA; T: denatured *E.coli* lysates loading into the column (total proteins); F: flowthrough; W1: washing fraction 1 (washed with pH 6.3 buffer); W2: washing fraction 2 (washed with pH 5.9 buffer); E: eluate (eluted with pH 4.5 buffer).

competitor (see Materials and Methods for details). The purification results and quantification results are shown in Figure 38. The expression levels of 6xHis-tagged proteins were calculated based on the BSA (bovine serum albumin) standard. The result showed that 1 ml *E. coli* culture produced 4 μg ABP7 protein or 28 μg ABP9 protein after overnight induction with 0.1 mM IPTG.

3.6.2 Analysis of anti-ABP7 and anti-ABP9 antibodies

Around 600 µg 6xHistagged ABP7 and 600 µg 6xHistagged ABP9 proteins were finally purified from E. coli to inject into rabbits for polyclonal antibodies. A 65-day FAST polyclonal antibody protocol was applied by the Eurogentec company to obtain the anti-ABP7 and anti-ABP9 sera. The rabbits were injected with 150 µg 6xHistagged protein on the 1st, 14th, 28th, and 50th day. The bleed was taken on the 1st and 38th day and finally harvested on the 65th day. The sera taken on the 1st day (before injection) and the sera taken on the 38th day (after two injections) were analysed by ELISA (Figure 39). The result suggested that there was a cross-reaction between the ABP7 protein and the anti-ABP9 serum. In addition, there was also a cross-reaction between the ABP9 protein and the anti-ABP7 serum. After 5,000-fold or 10,000-fold dilution, both the anti-ABP7 and anti-ABP9 sera could still produce clear signals.
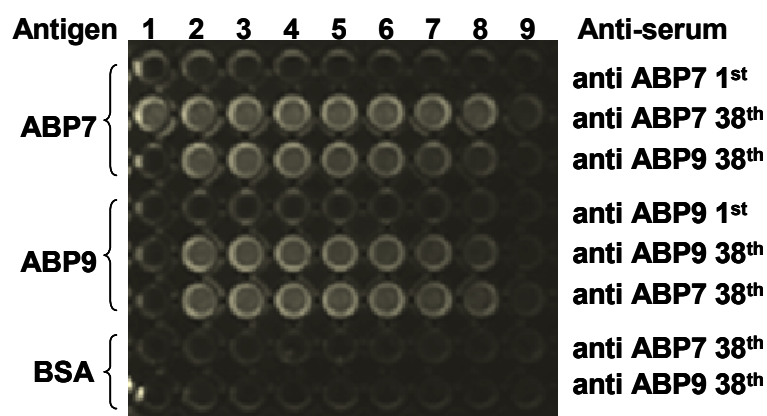
Figure 39. The analysis of the anti-ABP7 and anti-ABP9 sera by ELISA. 100 ng antigen was applied in each well. BSA was used as a negative antigen control. Anti-sera taken from the 1st (negative control) and 38th day were used as the first antibodies. HRP conjugated goat-anti-rabbit IgG was used as the second antibodies. 1: anti-sera without dilution; 2: 100-fold diluted anti-sera; 3: 200-fold diluted anti-sera; 4: 500-fold diluted anti-sera; 5: 1,000-fold diluted anti-sera; 6: 2,000-fold diluted anti-sera; 7: 5,000-fold diluted anti-sera; 8: 10,000-fold diluted anti-sera; 9: 100,000-fold diluted anti-sera.

A blot analysis was set up to test the anti-ABP7 and anti-ABP9 sera taken on the 38th day (after two injections). The result showed that the 5,000-fold diluted anti-ABP7 serum could detect a minimum of 1 ng ABP7 protein in the blot, while the 5,000-fold diluted anti-ABP9 serum could detect a minimum of 10 ng ABP9 protein (Figure 40).
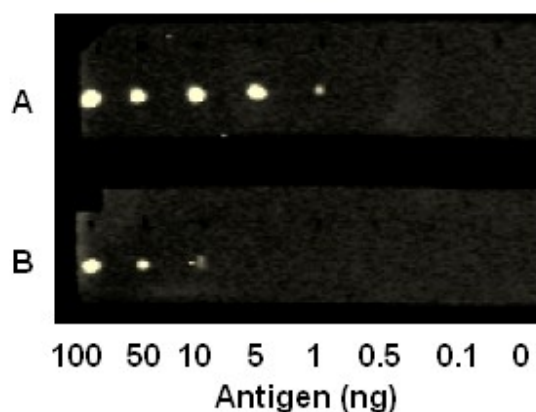
Figure 40. Blot analysis of the anti-ABP7 and anti-ABP9 antibodies. The indicated amounts of antigens (ABP7 or ABP9) were loaded on the blot. The 5,000-fold diluted anti-ABP7 or anti-ABP9 serum from the 38th day was used as the first antibody. The HRP conjugated goat-anti-rabbit IgG was used as the second antibody. A: The ABP7 protein was the antigen and the anti-ABP7 serum was the first antibody. B: The ABP9 protein was the antigen and the anti-ABP9 serum was the first antibody.

3.6.3 Binding test between ABP9 and phytohormones

ABP9 is likely to interact with ligand(s) due to its structural similarity to Bet v 1, Bet v 1l, and to a cytokinin-specific binding protein CSBP (Mogensen *et al*, 2002; Markovic-Housley *et al*, 2003; Fujimoto *et al*, 1998). In addition, the previous results showed that ABP9 overexpression caused dramatic induction of the ABA signal in the transient expression system and ABA hypersensitivity for transgenic plants. Thereby, ligands such as cytokinin and ABA are promising candidates for interaction with ABP9.

During Ni-NTA affinity chromatography purification, 6xHistagged ABP9 was

denatured by 6 M urea. A gradient washing step from 6 M urea to 0 M urea was applied to refold the denatured 6xHistagged ABP9. After refolding, the native 6xHistagged ABP9 was stored in the ABP9 storage buffer (pH 7.4) containing 150 mM NaCl, 20% glycerol, 10 mM Tris·Cl, 2 mM β-ME, and 0.02% NaN_3. Subsequently, the refolded 6xHistagged ABP9 was tested for interaction with ^{14}C-ABA and ^{14}C-zeatin.

3.6.3.1 ABP9 and ^{14}C-ABA

Equilibrium dialysis equipment was used for the binding tests. As shown in Figure 33A, two chambers were separated by a semipermeable membrane that only allows small molecules to diffuse through it. Big molecules like BSA and 6xHistagged ABP9 can not pass through it. An ABA diffusion test was conducted first. For this, 500 μl 0.2 μM ^{14}C-ABA (4.4 x 10^3 cpm) in the ABP9 storage buffer was added into one chamber, and 500μl ABP9 storage buffer without ABA was added into the other chamber (Figure 41A). To check the distribution of radioactivity, a 50 μl sample from each chamber was taken at different time points. Figure 41B showed that ^{14}C-ABA was evenly distributed in these two chambers within about 24 hours.

The binding test between ABP9 and ABA was set up in these chambers. 3 μM refolded 6xHis-tagged ABP9, 0.1 μM ^{14}C-ABA, and 1 μM azide in the ABP9 storage buffer was added in one chamber, while 3 μM BSA, 0.1 μM ^{14}C-ABA, and 1 μM azide in the same buffer was added in the other chamber. If ABP9 protein binds ABA under this buffer condition, the ^{14}C-ABA from the BSA chamber should diffuse towards the ABP9 chamber. However, the sample from the ABP9 chamber did not have more radioactivity than the sample from the BSA chamber after 24 hours (Figure 41C). Nevertheless, the study with isothermal titration calorimetry (ITC) afterwards showed that ABP9 could bind ABA with a high apparent dissociation constant. Therefore, the binding test

between ^{14}C-ABA and ABP9 was not sensitive enough to detect the weak interaction.

A   **B**   **C**
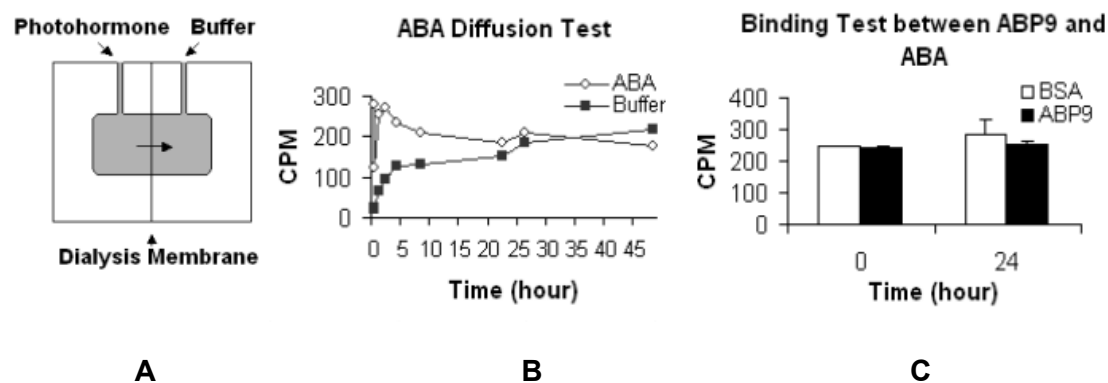
Figure 41. ABA diffusion and binding assay. A: The equipment consists of two chambers separated by a dialysis membrane. The dialysis membrane allows small molecules like ABA and zeatin to pass through it, but proteins not. **B:** The ABA diffusion test showed that 0.2 μM ^{14}C-ABA needed about 24 hours to evenly distribute in the chambers. **C.** 3 μM native ABP9 protein was added in one chamber, while 3 μM BSA was added in the other chamber to balance the protein. 0.1 μM ^{14}C-ABA was added in both the chambers. After 24 hours, the sample from the ABP9 chamber did not show more radioactivity than that from the BSA chamber.

3.6.3.2 ABP9 and ^{14}C-zeatin

The same instrument shown in Figure 33A was used for the diffusion test of ^{14}C-zeatin and its binding test with ABP9. 0.1 μM ^{14}C-zeatin and 1 μM azide in ABP9 storage buffer was put in one chamber, while 1 μM azide in the same buffer was put in the other chamber. A 50 μl sample was taken from each chamber at different time points to check ^{14}C-zeatin diffusion. The data showed that ^{14}C-zeatin was equally distributed in these two chambers after 24

hours (Figure 42A).

A   **B**
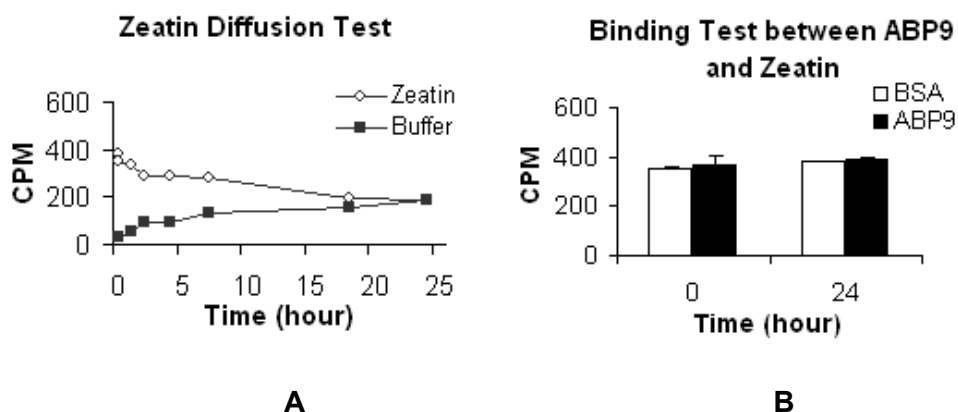
Figure 42. Zeatin diffusion and binding assay. A: Zeatin diffusion test showed that 24 hours were sufficient for ^{14}C-zeatin to distribute in the chambers equally. **B.** 3μM native ABP9 protein was added in one chamber, while 3 μM BSA was added in the other chamber to balance the protein. 0.1 μM ^{14}C-zeatin was added in both chambers. After 24 hours, the samples from these chambers still had equal radioactivity.

The binding test between ABP9 and ^{14}C-zeatin was set up similarly to the binding test between ABP9 and ^{14}C-ABA. 3 μM refolded 6xHis-tagged ABP9 was added in one chamber and 3 μM BSA was added into the other chamber to balance protein. 0.1 μM ^{14}C-zeatin and 1 μM azide in ABP9 storage buffer were added in both chambers. After 24 hours, no changes of radioactivity were found (Figure 42B).

3.6.4 Protein phosphatase 2C activity tests of ABI1 and ABI2 in the presence of ABP7 and ABP9

ABI1 and ABI2 are two negative key regulators in ABA signalling. Both belong to the type 2C serine/threonine protein phosphatases (PP2C) subfamily. Their enzymatic activities are Mg^{2+} or Mn^{2+} dependent and pH sensitive (Leube *et al*, 1998; Meinhard *et al*, 2002). ABP7 and ABP9 have been demonstrated to be the interacting partners of ABI1 and ABI2 in the yeast two-hybrid system and Arabidopsis protoplasts. Thus, it was interesting to test whether their interactions would influence the PP2C activity of ABI proteins.

The effect on the PP2C activity of ABI2 was first studied in the presence of ABP9 and ABA (Figure 43). The result showed that ABP9 could efficiently inhibit the phosphatase activity of ABI2 in an *S*-ABA-dependent manner. When ABP9 was denatured or *S*-ABA was replaced by *R*-ABA and *trans*-ABA, the inhibition was abrogated.

The subsequent study demonstrated that the PP2C activity of ABI1 was also inhibited by ABP9 in an *S*-ABA-dependent manner. In addition, the half-maximal inhibition of ABI1 and ABI2 occurred at approximately 60 nM and 70 nM, respectively (Figure 44).
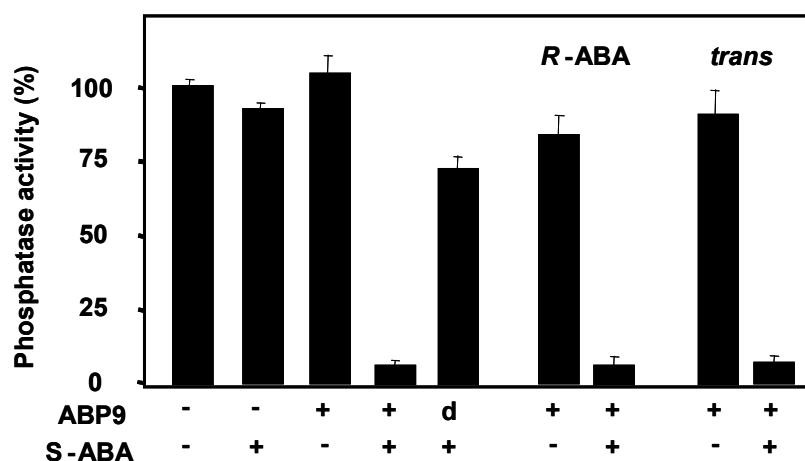
Figure 43. Inhibition of ABI2 by ABP9 and *S*-ABA. In vitro analysis conducted with ABI2, ABP9, and heat-denatured ABP9 (d, 95 °C, 10 min) in the presence or absence of *S*-ABA (1 µM) and/or *R*-ABA and *trans*-ABA (10 µM). The assay was performed with umbelliferylphosphate. Activity of ABI2 without ABP9 and phytohormone was set to 1.
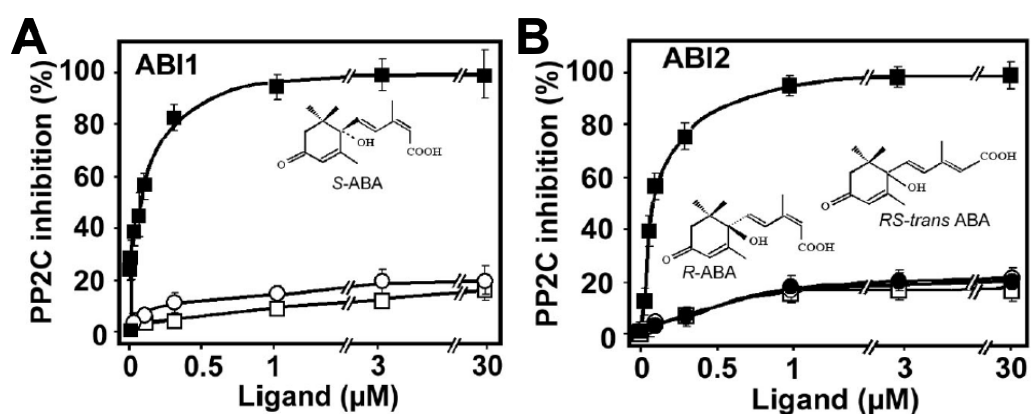
Figure 44. Inhibition of ABI1 and ABI2 by *S*-ABA (filled squares), *R*-ABA (open squares), and *trans-R,S*-ABA (open circles) in the presence of ABP9. The effect of *S*-ABA on ABI2 activity is marked by filled circles in the absence of ABP9 in (B).

4. <u>Discussion</u>

This work characterized the homologous proteins ABP7 and ABP9 as positive regulators, interacting with and antagonizing the key negative regulators clade-A PP2Cs ABI1 and ABI2 in ABA signalling in Arabidopsis. ABP9 has been shown to mediate ABA responses to a large extent, including gene expression, stomatal closure, seed germination, and inhibition of vegetative growth. Further study has demonstrated that ABP7 (finally named as RCAR3, <u>R</u>egulatory <u>C</u>omponents of <u>A</u>BA <u>R</u>eceptor 3) and ABP9 (RCAR1) are able to physically interact with ABA. Both ABP7 and ABP9 can block the phosphatase activities of ABI1 and ABI2 in an ABA-dependent manner (Szostkiewicz *et al.,* 2010; Figures 46 and 47; Ma *et al.*, 2009). Thus, ABP7 and ABP9 essentially function as ABA receptors. They first bind to ABA and clade-A PP2Cs, then transmit the ABA signal by controlling protein phosphatase activities of the PP2Cs.

Recent studies (Fujii *et al.,* 2009; Umezawa *et al.,* 2009) have shown that clade-A PP2Cs can inactivate their downstream SnRK2 kinases through physical interaction and dephosphorylation. The inactive SnRK2s are therefore not capable of phosphorylating the basic leucine zipper (bZIP) ABF/AREB/ABI5 transcription factors. In the presence of ABA, the ABA receptors disrupt the interaction between the PP2Cs and SnRK2s, thus preventing PP2C-mediated inactivation of SnRK2s. This results in phosphorylation of bZIP transcription factors by SnRK2s and the subsequent ABA-induced gene expression. Two independent studies have demonstrated that another two members of ABP9's family, PYRABACTIN RESISTANCE 1 (PYR1, product of At4g17870) and PYR1-Like 5 (PYL5; product of At5g05440), function as ABA receptors. They physically interact with ABA and inhibit phosphatase activities of clade-A PP2Cs (Park *et al.,* 2009; Santiago *et*

al., 2009b). Several structural studies have confirmed the identities of PYR1, PYL1 (At5g46790), and PYL2 (At2g26040) in ABP9's family as ABA receptors (Figure 45). The receptors such as PYL1 (Figure 46) enclose ABA in their ligand-binding pockets and change their conformations to interact with PP2Cs in a similar manner (Nishimura *et al.,* 2009; Santiago *et al.,* 2009a; Miyazono *et al.,* 2009; Melcher *et al.,* 2009). Thirteen members in ABP9's family interacted with clade-A PP2Cs in the yeast two-hybrid system and antagonized ABI1 to regulate the ABA-dependent induction of RD29B::LUC expression in the transient expression system, while the last member PYL13 (At4g18620) was not tested (Ma *et al.,* 2009; Park *et al.,* 2009; Fujii *et al.,* 2009). Taken together, it is highly likely that all of the members in ABP9's family function as ABA receptors, redundantly regulating ABA responses through a network composed of diverse PP2Cs, SnRK2s and ABFs.
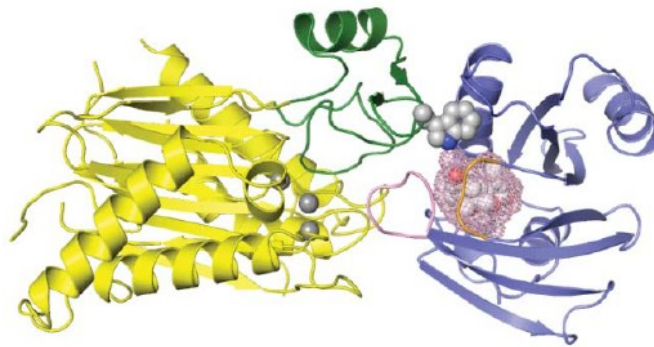
Figure 45. Structure of the PYL2-ABA-HAB1 co-receptor complex (Melcher *et al.,* 2009). HAB1 is shown with its catalytic domain in yellow (Mg^{2+} ions as balls) and the ABA-interacting domain in green. PYL2 is shown in blue and ABA as a ball model with its surrounding ligand binding pocket as mesh.
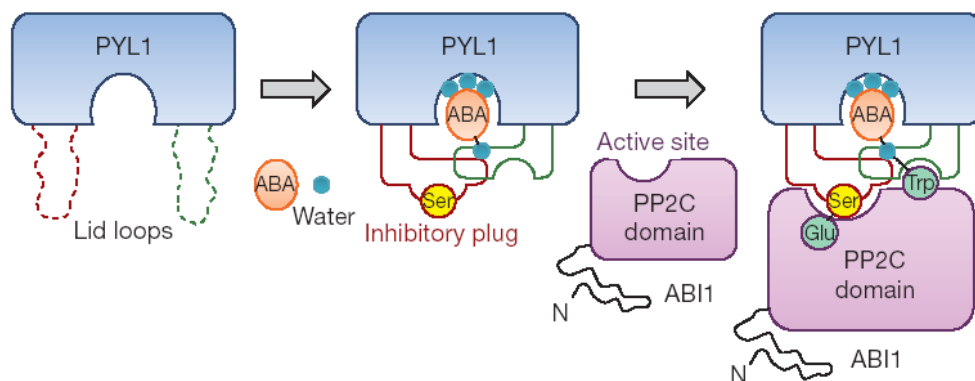
Figure 46. A model of (+)-ABA-induced ABI1 regulation by PYL1 (Miyazono *et al.,* 2009). When a (+)-ABA molecule enters into an ABA-binding site of PLY1, the conformations of lid loops change from the open state to the closed state. The closed loops of PLY1 act as a scaffold to interact with the PP2C domain of ABI1. Thus, PLY1-(+)-ABA activates the SnRK2-dependent phosphorylation pathways by inhibiting the phosphatase activity of ABI1.

4.1 Roles of ABP7 and ABP9 in ABA signal pathway and in phytohormones crosstalk

ABP7 and ABP9, as homologous proteins, function similarly in mediating ABA responses to a large extent. However, whether all the ABA-related responses are regulated via ABP7 and ABP9 is still unclear. Although ABP7 and ABP9 are acting as ABA co-receptors, their functions are not completely understood. ABP9 might have more functions than an ABA receptor, according to the clear phenotype from ABP9 overexpression transgenic seedlings in respect to 2,4-D (Data represented in Results 3.2.2.2).

- *Regulation of ABA-induced gene expression by ABP7 and ABP9*

The results obtained from the transient expression system in this work showed that overexpression of ABP7 and ABP9 dramatically induced ABA-dependent Rab18::LUC or Lti65::LUC expression, in the absence and presence of exogenous ABA, in both wild-type (wt) and diverse mutants (the ABA-deficient mutant *aba2*, ABA-insensitive mutants *abi1-1* and *abi2-1*). These results indicate that ABP7 and ABP9 are upstream to the ABF/ABRE/ABI5 bZIP transcription factors. Recently, members of ABP9's family, clade-A PP2Cs, SnRK2s and ABF/AREB/ABI5 bZIP transcription factors have been demonstrated as the only core components necessary to complete the ABA regulation of gene expression (Fujii *et al.,* 2009). However, besides the pathway, there are probably other signal pathways to regulate ABA-dependent gene expression. It is interesting to consider whether ABP9's family regulates other types of transcription factors, such as AtHB6 (Himmelbach *et al*., 2002), MYB/MYC (Abe *et al.*, 1997), and ABI4 (Niu *et al.*, 2002). The homeodomain protein AtHB6 is a target of ABI1. The phosphatase domain of ABI1 and the N-terminal domain of AtHB6 containing the DNA binding site are critical for their interaction, although whether AtHB6 is dephosphorylated by ABI1 is not known (Himmelbach *et al.,* 2002). As ABP9's homologues PYL1 and PLY2 have been shown to interact with the phosphatase domains of PP2Cs (Miyazono *et al.,* 2009; Melcher *et al.,* 2009), ABP9's family is theoretically capable of preventing the interaction between AtHB6 and ABI1 through competitively interacting with the phosphatase domain of ABI1. MYB/MYC and ABI4 transcription factors have never been shown to be regulated by PP2Cs in the ABA signal pathway. But they seem to act downstream of the Mg-chelatase H subunit (CHLH), which is a chloroplast protein in Arabidopsis and has been suggested as a candidate for an ABA receptor (Shen *et al.,* 2006; Shang *et al.,* 2010). However, the receptor nature of its homologue

XanF in barley is questionable (Muller and Hansson, 2009). The relations between ABP9's family and MYB/MYC, ABI4, or CHLH are still unclear.

In this work, overexpression of ABP7 and ABP9 pronouncedly induced Rab18::LUC expression in the absence of exogenous ABA in *aba2* protoplasts (data presented in Results 3.1.1, Figure 9), indicating that overexpression of ABP7 and ABP9 activated ABA signalling in the ABA-deficient mutant. The ectopic expression of ABP7 and ABP9 rescued the inefficient inhibition of PP2Cs resulting from a low ABA level.

In the ABA-insensitive mutant *abi1-1* and *abi2-1*, the PP2Cs are point-mutated in their phosphatase domains, leading to markedly decreased PP2C activities and impaired interactions with ABP7 or ABP9 (Leung *et al*., 1997; Leung and Giraudat, 1998; Zhang 2004; Results 3.5, Figure 36). In these mutants, overexpression of ABP7 and ABP9 also dramatically induced the ABA signal in both absence and presence of exogenous ABA (data presented in Results 3.1.1, Figures 10 and 11), implying that ABP7 and ABP9 may antagonize abi1-1 and abi2-1 to regulate ABA signalling. However, Umezawa and his colleagues have recently shown that the mutant protein abi1-1 did not respond to ABP9's homologue PYR1 and constitutively deactivated SnRK2.6 when its phosphatase activity was tested in vitro (Umezawa *et al.,* 2009).This result indicates that PYR1 cannot antagonize abi1-1 as it does ABI1 to repress ABA responses. Thus, when ABP9 and ABP7 were ectopically expressed in the *abi1-1* and *abi2-1* mutants, they might not fully inhibit the phosphatase activities of the mutant proteins abi1 and abi2. However, the abundant ABP proteins might fully inhibit other wt homologous PP2Cs such as HAB1 and AHG3 to induce expression of the reporter genes.

The reason why *abi1-1* and *abi2-1* mutants display dominant ABA-insensitive phenotypes has been debated for years. The previous popular explanation

was that abi1 and abi2 might bind to a putative substrate and prevent activity of the wild-type protein (Merlot and Giraudat, 1997; Allen *et al*., 1999). However, ABP9's homologues have recently been suggested to inhibit PP2Cs through physically interacting with their phosphatase domains (Miyazono *et al.,* 2009; Santiago *et al*., 2009a; Melcher *et al.,* 2009). But the abi1-1 and abi2-1 mutant proteins do not interact, or interact only weakly, with ABP proteins (Result 3.5, Figure 36). Therefore, abi proteins can escape the regulation through ABP9's family and disrupt ABA signalling through their residual phosphatase activities. A new model was proposed to explain why *abi1-1* is a dominant ABA-insensitive mutant (Umezawa *et al.,* 2009). In this model, the abi1-1 mutant protein does not interact with ABA-bound ABP/RCAR/PYR and constitutively dephosphorylates SnRK2 (Figure 47). The protein kinases SnRK2s can therefore not phosphorylate or activate ABF/AREB/ABI5 bZIP transcription factors to induce gene expression. This model can also explain why both wt PP2Cs and abi1-1 mutant protein repressed ABA responses in a dose-dependent manner in the ABP9-overexpressed protoplasts (Results 3.1.2, Figures 12 and 13). When ABI1 and ABI2 were too abundant to be efficiently blocked by overexpressed ABP9, the unblocked PP2Cs dephosphorylated their downstream SnRK2s and consequently inhibited ABA responses. When abi1-1 protein was too abundant, its residual phosphatase activity complemented the insufficient active PP2Cs in the ABP9-overexpressed protoplasts and consequently also inhibited ABA responses. Another support for the model in Figure 47 is the mutant *abi1-1R2* (Gosti *et al.*, 1999; Park *et al.*, 2009). This mutant contains *abi1-1* mutation and a suppressor mutation. It displays completely abolished PP2C activity and ABA hypersensitive phenotype.
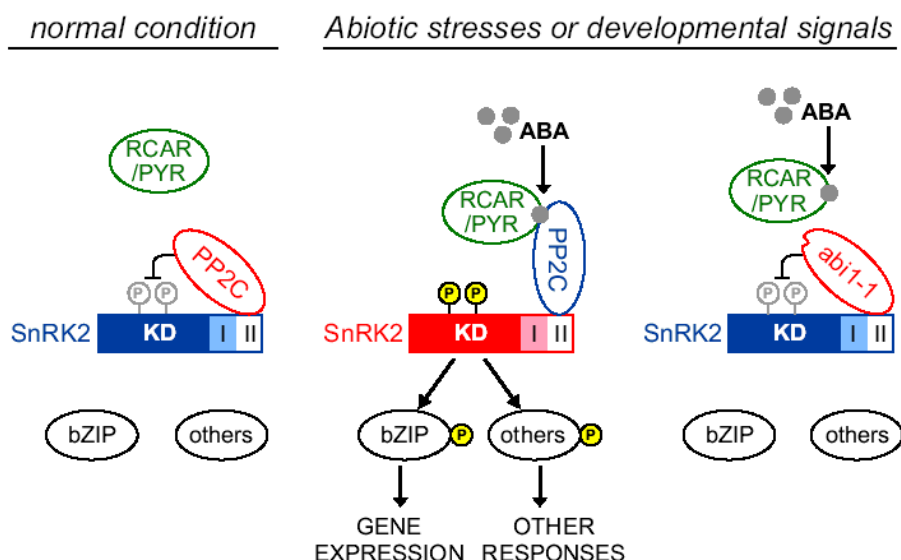
Figure 47. A proposed model of the ABA signalling pathway in Arabidopsis (Umezawa et al., 2009). Left, in the absence of ABA, PP2C dephosphorylates and inactivates SnRK2. Middle, in the presence of ABA, RCAR/PYR/ABP interacts with PP2C, and SnRK2 is released from negative regulation and converted to an active form. Right, even in the presence of ABA, the *abi1–1* mutant of PP2C constitutively dephosphorylates SnRK2, consequently conferring dominant ABA insensitivity.

● *Regulation of other ABA responses by ABP7 and ABP9*

The transgenic Arabidopsis with ABP9 overexpression displayed a clear ABA hypersensitive phenotype in root growth, seed germination, and stomatal closure tests, indicating that ABP9 regulates ABA responses in diverse aspects. In contrast, ABP7 overexpression transgenic Arabidopsis did not show phenotypes in any of these three ABA sensitivity tests. It seems that ABP7 only regulated ABA-dependent gene expression, while it did not regulate other ABA responses. However, the test on protoplasts isolated from ABP7 overexpression plants showed that the expression level of ABP7 was too low

117

to result in clearly altered ABA sensitivity in transgenic Arabidopsis (Results 3.2.1, Figure 20b). This is the main reason why ABP7 overexpression transgenic plants did not show phenotypes in the assays of ABA sensitivity in this work. Actually, ABP7 regulates ABA-mediated seed germination, root growth, and drought response (Saavedra *et al.,* 2010). Thus, both ABP9 and ABP7, as ABA receptors, regulate ABA responses in many aspects. The RNAi knockdown transgenic plants also did not show phenotypes in ABA sensitivity tests, indicating that the members in ABP9's family function redundantly in ABA signalling.

- *ABP7 and ABP9's involvement in phytohormones crosstalk*

In addition to ABA, the ABP7- and ABP9-deregulated transgenic plants displayed phenotypes in respect to auxin (Results 3.2.2.2, Figures 22, 24, 25, and 26). Treatment of exogenous auxin can induce calli formation and 2,4-D is the most effective auxin variety for calli induction (Sekiya *et al.*, 1977; Neibaur et al., 2008). When the T2 RNAi knockdown plants grew on the MS medium containing 1 µM and 3 µM 2,4-D, 88.5% and 79.4% of the seedlings respectively displayed resistance to forming calli after three weeks, while no control seedling had this resistance. The auxin-to-cytokinin concentration ratio is an important factor regulating postembryonic de novo organogenesis (Skoog and Miller, 1957). Auxin triggers organogenesis, whereas cytokinin modulates it via regulating the auxin efflux (Pernisova *et al.,* 2009). Therefore, this phenotype indicated that the RNAi knockdown plants might be either insensitive to 2,4-D or hypersensitive to the endogenous cytokinin.

Figure 27 (Results 3.2.2.3 and a leaf senescence test (data not shown) have afterwards demonstrated that the RNAi plants were not hypersensitive to cytokinin. Moreover, when protoplasts were co-transferred with the RNAi knockdown effector and a cytokinin-dependent reporter pARR6::LUC, the

protoplasts also did not display altered sensitivity to kinetin (Ma *et al.,* 2009). Taken together, the RNAi transgenic plants are likely insensitive to auxin. Consistent with this assumption, ABP7 (Results 3.2.2.2, Figures 24 and 25) and ABP9 (Figure 26) overexpression plants also displayed phenotypes in respect to 2,4-D. The only contradictory evidence appears in Figure 23 (Results 3.2.2.2), which showed that the RNAi knockdown seedlings did not have phenotypes in respect to 2,4-D in the root growth test. High concentrations of auxin promote ethylene biosynthesis and consequently inhibit root growth (Kim *et al.,* 1992). In addition, auxin mediates multiple aspects of plant development, such as apical and basal axia formation, root patterning, tropisms, and organogenesis (Kim *et al.,* 1992; Friml *et al*., 2003; Blilou *et al.,* 2005; Friml *et al*., 2002; Pernisova *et al*., 2009). The signal pathways of auxin-promoted ethylene biosynthesis and auxin-triggered organogenesis do not seem to largely overlap with each other (Tsuchisaka and Theologis, 2004; Pernisova *et al.,* 2009). Although the RNAi knockdown seedlings did not show altered auxin sensitivity in auxin-promoted ethylene biosynthesis, they can still be insensitive to auxin during calli formation.

.

Receptors of other phytohormones have never been reported to be involved in phytohormones crosstalk. Nevertheless, AXR2/IAA7, which interacts with the auxin receptor TIR1 and encodes a member of AUX/IAA protein family, has been demonstrated to play a role in ABA signalling (Gray *et al.,* 2001; Nagpal *et al.,* 2000; Wilson *et al.,* 1990). The interaction between AUX/IAA and auxin-bound TIR1 can result in ubiquitination and subsequent degradation of the transcription repressors AUX/IAA (Tan *et al.,* 2007). The *axr2/iaa7* mutant showed insensitivity to both auxin and ABA (Nagpal *et al.,* 2000; Wilson *et al.,* 1990). In contrast to AXR2/IAA7, ABI1 and ABI2 have been suggested to affect auxin responses (Rock & Sun, 2005). The *abi1-1* and *abi2-1* mutants had reduced expression of an ABA- and auxin-inducible reporter ProDc3::GUS in response to 2,4-D. The crosstalk between auxin signalling and ABA

signalling is far from fully understood. Recent studies have suggested that auxin and ABA converge on the DELLA proteins (Figure 48). DELLA proteins are negative regulators in gibberellin responses. The interaction between DELLA proteins and the gibberellin receptor GID1 results in their ubiquitination and subsequent degradation. Auxin promotes gibberellin-induced destabilization of some of the DELLA proteins, while ABA stabilizes RGA (a specific DELLA protein) and inhibits its degradation (Santner and Estelle, 2009). According to this model, auxin and ABA should always antagonize each other. However, phenotypes of some mutants indicate that the mechanism of the crosstalk between auxin and ABA is more complicated. In addition to *axr2/iaa7*, the mutant *indole-3-butyric acid response-5* (*ibr5*) also has decreased sensitivity to auxin and ABA (Monroe-Augustus *et al.,* 2003). On the other hand, the mutant *sensitive to abscisic acid and auxin* (*sax1*) is hypersensitive to both of the phytohormones (Ephritikhine *et al.,* 1999). In this work, the ABP9 overexpression plants also displayed hypersensitivity to both ABA (Results 3.2.1, Figures 16, 18, and 19) and auxin (Results 3.2.2.2, Figure 26).

The ABP7 and ABP9 overexpression plants showed completely different phenotypes in respect to auxin. Some of the ABP7 overexpression transgenic plants appeared vitreous or even had callus-like tissues on petioles when they grew on the MS medium or MS medium containing 0.3 µM 2,4-D, while the control plants started to form calli when they grew on the MS medium containing a higher level of 2,4-D.(Figures 24 and 25). The ABP9 overexpression plants displayed phenotypes of short hypocotyls, while they did not show altered sensitivity to calli formation (Figure 26). This indicated that ABP7 and ABP9 affect auxin responses in different aspects, although they regulate ABA signalling in a redundant manner. ABP7 and ABP9, as ABA receptors, are involved in the crosstalk between ABA and auxin.
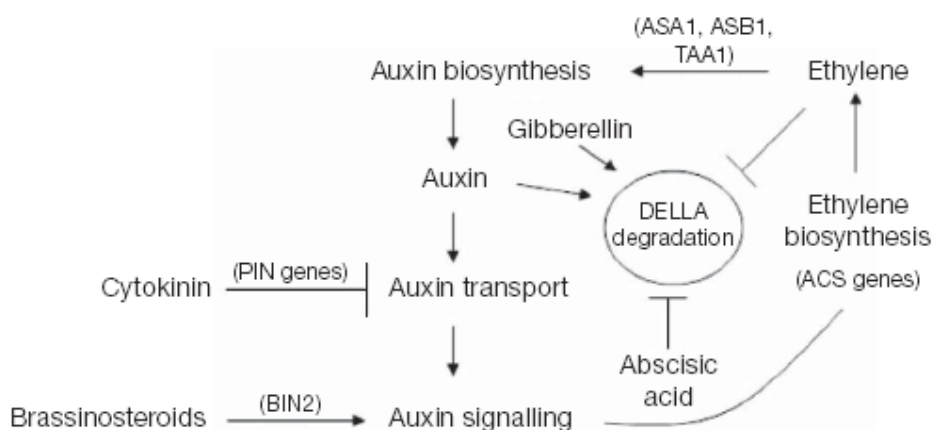
Figure 48. A model of phytohormones' crosstalk (Santner and Estelle, 2009). Auxin, ABA, ethylene, and gibberellin all converge on DELLA proteins.

4.2 The ABP7/ABP9's family and their signal pathways

The ABP7 and ABP9's family in Arabidopsis consists of fourteen homologous members. The major functional properties and regulatory properties of the members in this family are shown in Table 2 (Ma *et al*., 2009; Park *et al*., 2009; Szostkiewicz *et al.,* 2010; Miyazono *et al.,* 2009; Santiago *et al.,* 2009b; Melcher *et al.,* 2009; Hruz *et al*., 2008). Up to now, all of the tested members positively interacted with (+)-ABA, blocked PP2Cs phosphatase activity in an ABA-dependent manner, and interacted with PP2Cs in the yeast two-hybrid system. Thus, the whole family is highly likely to function redundantly as ABA receptors.

Table 2. Properties of the Members in ABP7 and ABP9's Family.

Gene	Other Names	Bind to (+)-ABA	Block PP2Cs, with (+)-ABA	Interact with PP2Cs In Y2H			Expression Regulation
				ABI1; ABI2	HAB 1	AHG 3	
At1g01360	ABP9/ RCAR1/ PYL9	Yes	Block ABI1/2	Yes	Yes		Upregulated by Agrobacteria
At4g01026	PCAR2/ PYL7				Yes		Upregulated by Agrobacteria
At5g53160	ABP7/ RCAR3/ PYL8	Yes	Block ABI1/2 HAB1	Yes	Yes		Upregulated by Agrobacteria and high CO_2
At4g27920	RCAR4/ PYL10				Yes		
At5g45860	RCAR5/ PYL11				Yes		
At5g45870	RCAR6/ PYL12				Yes	Yes	
At4g18620	RCAR7/R YL13						
At5g05440	RCAR8/ PYL5	Yes	Block ABI1/2 HAB1		Yes		Upregulated by high CO_2
At2g40330	RCAR9/ PYL6				Yes		Downregulated by ABA; Upregulated by high CO_2
At2g38310	RCAR10/ RYL4		Block ABI1/2 HAB1		Yes	Yes	Downregulated by ABA; upregulated by high CO_2
At4g17870	RCAR11/ PYR1	Yes	Block ABI1/2 HAB1	Yes	Yes	Yes	Upregulated by high CO_2
At5g46790	RCAR12/ PYL1	Yes	Block ABI1/2		Yes	Yes	
At1g73000	RCAR13/ PYL3				Yes	Yes	
At2g26040	RCAR14/ PYL2	Yes			Yes	Yes	

Y2H: The Yeast Two-hybrid System; The empty fields: unknown answers.

In the nucleus, these ABA-bound receptors physically interact with the phosphatase domain of clade-A PP2Cs (ABI1, ABI2, HAB1, HAB2, AHG1, and AHG3) and thereby inhibit their phosphatase activity. The downstream SnRK2 protein kinases (SnRK2.2, SnRK2.3, and SnRK2.6) are consequently released from PP2Cs and phosphorylate the ABF/AREB/ABI5 bZIP transcription factors to induce gene expression (Figure 49; Fujii *et al.,* 2009; Umezawa *et al.,* 2009; Kim, 2006). In addition to calcium-independent protein kinase SnRK2s, several calcium-dependent protein kinases have been suggested to interact with and phosphorylate ABF/AREB/ABI5 transcription factors as well. CPK4 and CPK11 were showed to phosphorylate ABF1 and ABF4 in vitro (Zhu *et al.,* 2007). CPK32 phosphorylated ABF4 in vitro and interacted with ABF1, ABF2, and ABF3 (Choi *et al.,* 2005). But it is not known whether CPK4, CPK11, and CPK32 can interact with and be inhibited by PP2Cs.

In the cytosol of guard cells, the ABA-bound ABP/RCAR/PYR/PYL receptors interact and inhibit the phosphatase activity of some clade-A PP2Cs (ABI1, ABI2, and AHG3/PP2CA). The SnRK2.6/OST1 protein kinase is thereby released and phosphorylates the guard cell anion efflux channel SLAC1 (SLOW ANION CHANNEL-ASSOCIATED 1) and a guard cell K^+ influx channel KAT1 (K+ATPase 1). SLAC1 and KAT1 are activated and deactivated, respectively, resulting in turgor decrease and subsequent stomatal closure (Figure 50; Lee *et al.,* 2009; Geiger *et al.,* 2009; Sato *et al.,* 2009). In contrast to ABA signalling to induce gene expression, some PP2Cs (HAB1 and HAB2) and some SnRK2 protein kinases (SnRK2.2 and SnRK2.3) do not seem to play roles in this pathway to induce stomatal closure (Sato *et al*., 2009; Fujii *et al.,* 2007). However, some calcium-dependent protein kinases (CPK21 and CPK23) have been shown to regulate stomatal closure (Geiger *et al.,* 2010). ABI1 physically interacted with CPK23 and could suppress the protein kinase activity of CPK21 and CPK23, which phosphorylated and activated SLAC1 in
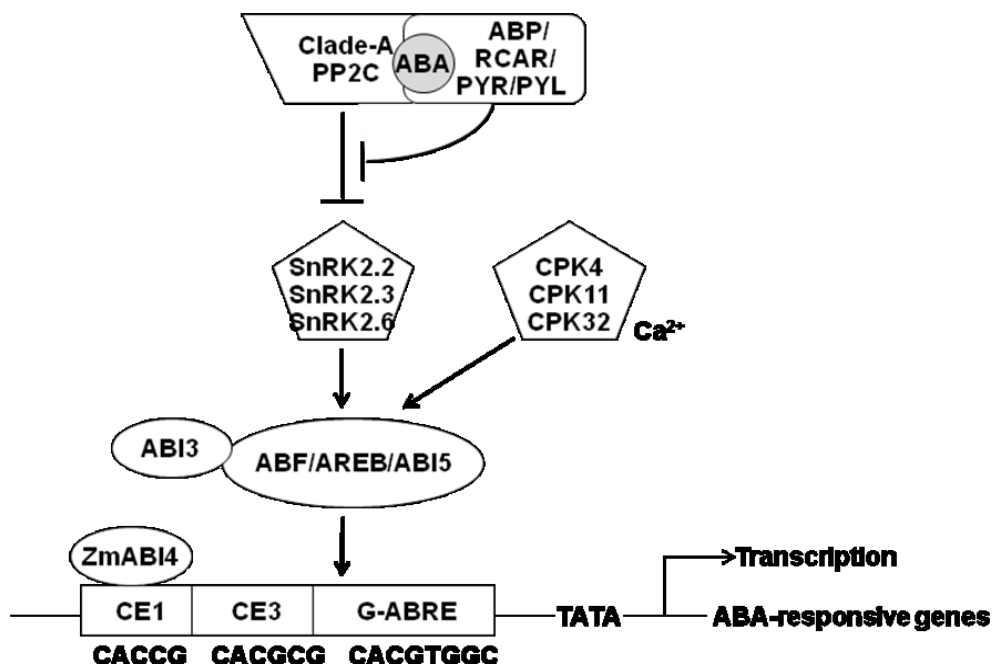
Figure 49. Regulation of ABA-responsive genes by ABP/RCAR/PYR/ PYL receptors in the nucleus. Physical interactions between ABA-bound receptors and PP2Cs prevent the dephosphorylation of SnRK2s by PP2Cs. The activated SnRK2s (SnRK2.2, SnRK2.3, and SnRK2.6) consequently phosphorylate ABF/AREB/ABI5 transcription factors. ABI5 acts in combination with ABI3. The AP2-type transcription factor *maize* ABI4 is a transcriptional repressor and binds a coupling element CE1. The calcium-dependent protein kinases (CPK4, CPK11, and CPK32) phosphorylate ABF/AREB/ABI5 transcription factors in line with SnRK2s. Protein kinases are indicated by pentagons. Transcription factors are indicated by ovals. *cis*-Regulatory elements are boxed and their core sequences are presented below each one. CE: coupling element; G-ABRE: G-box ABA-responsive element.
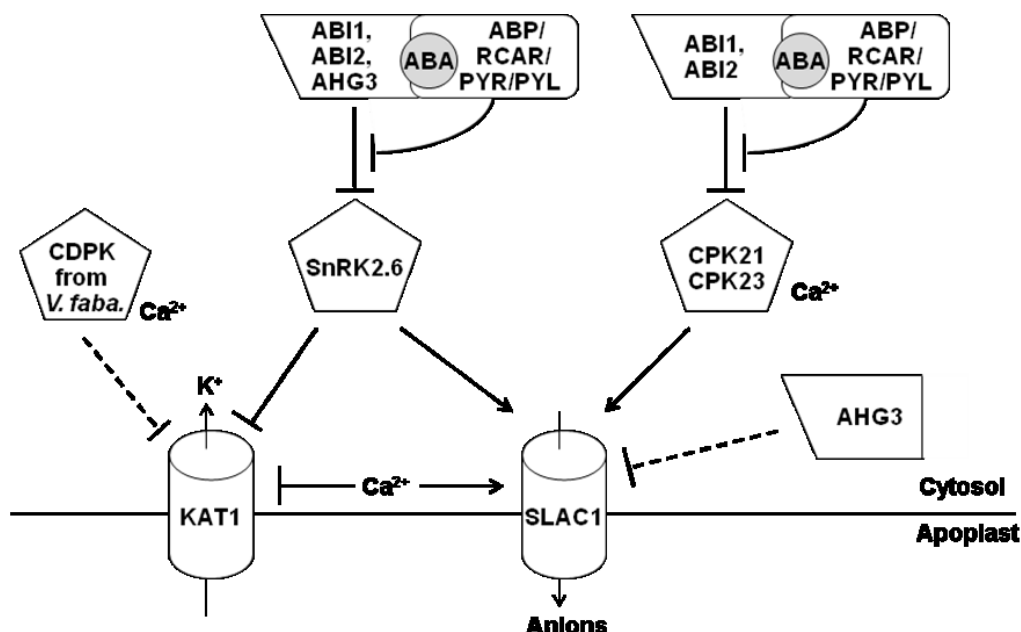
Figure 50. Regulation of SLAC1 and KAT1 by ABP/RCAR/PYR/PYL

receptors in guard cells. The interactions between PP2Cs and the

ABA-bound receptors inhibit the inactivation of SnRK2.6, CPK21, and

CPK23. The calcium-independent protein kinase SnRK2.6 and

calcium-dependent protein kinases CPK21 and CPK23 are consequently

capable of activating the anion channel SLAC1. SnRK2.6 inhibits a K^+ efflux

channel KAT1. A calcium-dependent protein kinase (CDPK) from *V. faba*

phospholats KAT1 and probably inhibits KAT1 in line with the inhibition by

SnRK2.6. The PP2C AHG3 physically interacts with SLAC1 and may be able

to directly regulate the activity of SLAC1. PP2Cs are indicated by irregular

quadrilaterals. Calcium-dependent and calcium-independent protein kinases

are indicated by pentagons. Ion channels are indicated by columns.

line with the activation by OST1. ABI2 was also shown to inhibit the

CPK23-mediated activity of SLAC1, but HAB1 and HAB2 did not. Of note,

CPK6 interacted with SLAC1 while CPK3 did not, although both have been

demonstrated to regulate guard cell S-type anion channels and stomatal

125

closure (Mori *et al.,* 2006). This indicated the existence of other pathways through CPKs to regulate stomatal closure. A calcium-dependent protein kinase expressed in guard cells of *Vicia faba* phosphorylated the K^+ influx channel KAT1, implying a possible inhibition of KAT1 by CPKs in Arabidopsis (Li *et al.,* 1998). Lee and his colleagues have demonstrated physical interaction between SLAC1 and AHG3/PP2CA, suggesting the possibility of direct inhibition of SLAC1 by PP2Cs (Lee *et al.,* 2009).

The main interacting partners of PP2Cs include SnRK2s and several CPKs, as well as several CIPKs. CIPKs are CBL (calcineurin B-like protein)-interacting protein kinases. The diverse combinations of CBL-CIPK complexes play important roles in the stress tolerance of plants. Some CBLs and CIPKs have been demonstrated as positive and negative regulators in ABA responses, such as CBL4, CBL1, CBL9, CIPK15, and CIPK3 (Guo *et al.,* 2002; Pandey *et al.,* 2008). CBL1 and CBL9 interact with CIPK23. The complexes of CBL1-CIPK23 and CBL9-CIPK23 activate an inward-rectifying potassium channel AKT1 by phosphorylation (Lee *et al.,* 2007). AKT1 functions in K^+ nutrition in roots. It can physically interact with and be inactivated by a putative PP2C AIP1. AIP1 also interacts with CIPK23. Interestingly, another potassium channel AKT2 that participates in K^+ loading in the phloem interacts with and is dephosphorylated and inhibited by PP2CA/AHG3 (Cherel *et al.,* 2002). These results indicate a possible regulation of K^+ homeostasis by ABP/RCAR/PYR/PYL receptors through PP2Cs (Figure 51a). CIPK24 is an interacting partner of ABI2. CIPK24 acts in combination with root-specific CBL4 to mediate the Na^+/H^+ antiporter SOS1 (salt overly sensitive 1). The activated SOS1 can extrude Na^+ into the extracellular space to enhance salt detoxification of plants (Guo *et al.,* 2001; Weinl and Kudla, 2009). CIPK24 can also act in combination with shoot-specific CBL10 to mediate sodium detoxification. But they seem to activate an unknown channel on the tonoplast to transport Na^+ into the vacuolar compartment (Kim *et al.,* 2007; Weinl and

a.
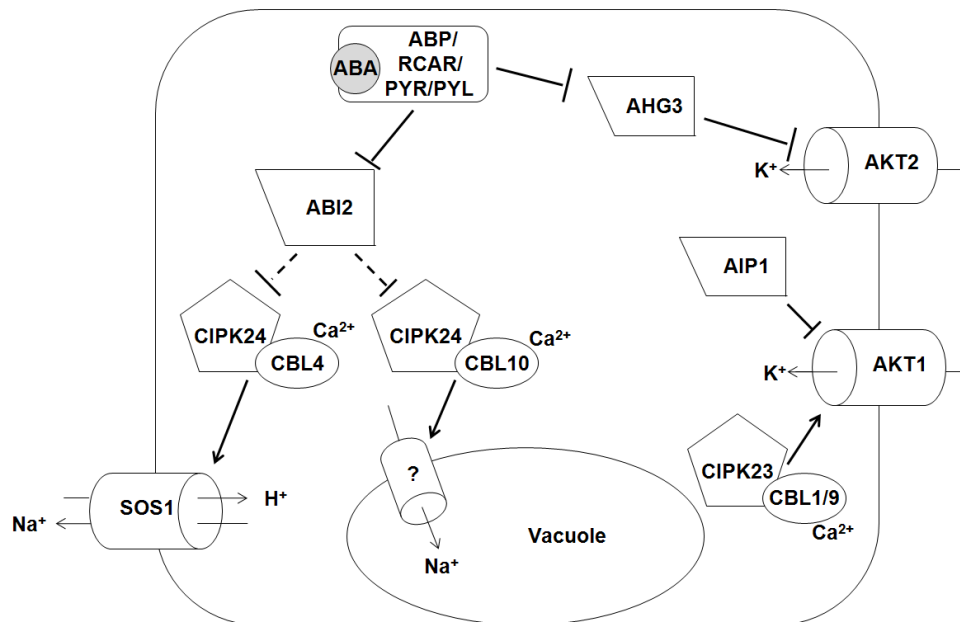
b.
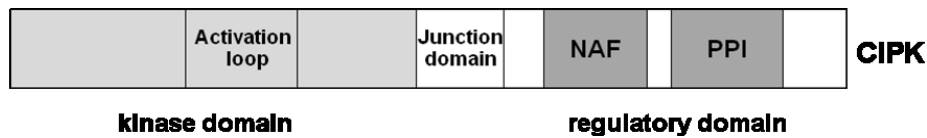
Figure 51. Regulation pathways via ABPs/RCARs, PP2Cs, and CIPK-CBL complexes. a. PP2Cs are indicated by irregular quadrilaterals. CIPKs are indicated by pentagons. CBLs are indicated by small ovals. Ion channels are indicated by columns. **b.** The overall structure of CIPKs (Weinl and Kudla, 2009). The structure of CIPKs comprises an N-terminal kinase domain and a regulatory C-terminal domain that are separated by a junction domain. The regulatory C-terminal domain contains two conserved interaction domains: the NAF domain, which is responsible for the CBL–CIPK interaction, and the adjacent protein–phosphatase interaction (PPI) domain mediating interaction with 2C-type protein phosphatase (PP2C)-type phosphatases.

Kudla, 2009). These results imply a regulation of salt tolerance by ABP/RCAR/PYR/PYL receptors through ABI2 and CIPK24 (Figure 51a). PP2Cs interact with the PPI domain of CIPKs (figure 51b), while CBLs bind to the NAF, as well as partly binding to the PPI domain of CIPKs (Kudla *et al.,* 2010). Therefore, the ABI2 interaction with CIPK24 can probably lead to competitive replacement of CBL4 and CBL10, resulting in inhibition of CIPK24.

The ABP/RCAR/PYR/PYL proteins regulate gene expression, stomatal closure, K^+ homeostasis, and probably also regulate salt tolerance. Although several other proteins also have been reported as candidates for ABA receptors, only ABP/RCAR/PYR/PYL proteins provide a connection between ABA perception and the downward signal transduction through PP2Cs. These ABP/RCAR/PYR/PYL proteins function redundantly in ABA signalling. But they have diverse expression patterns and expression regulations (Table 2), indicating their different roles and importance during different developmental stages or under different stresses. They control the activity of PP2Cs, which can act in combination with SnRK2s, CPKs, or CIPK-CBL complexes to switch on/off many responses through dephosphorylation/phosphorylaion. The ABP/RCAR/PYR/PYL proteins, as cytosolic ABA receptors, mediate ABA responses as well as many other responses.

5. <u>Appendix</u>

5.1 Plasmids for the expression of 6xHis-tagged proteins in *E. coli*

5.1.1 The map of the plasmid to generate 6xHistagged proteins

In this study, the *QIAexpress* pQE30 vector was used to generate all kinds of DNA constructs to express 6xHis-tagged proteins in *E. coli*. After cloning, the proteins are under the control of T5 promoter and are tagged by 6x histidine (6xHis) at the N-termini. The map of the *QIAexpress* pQE30 vector is shown below (Figure 52).
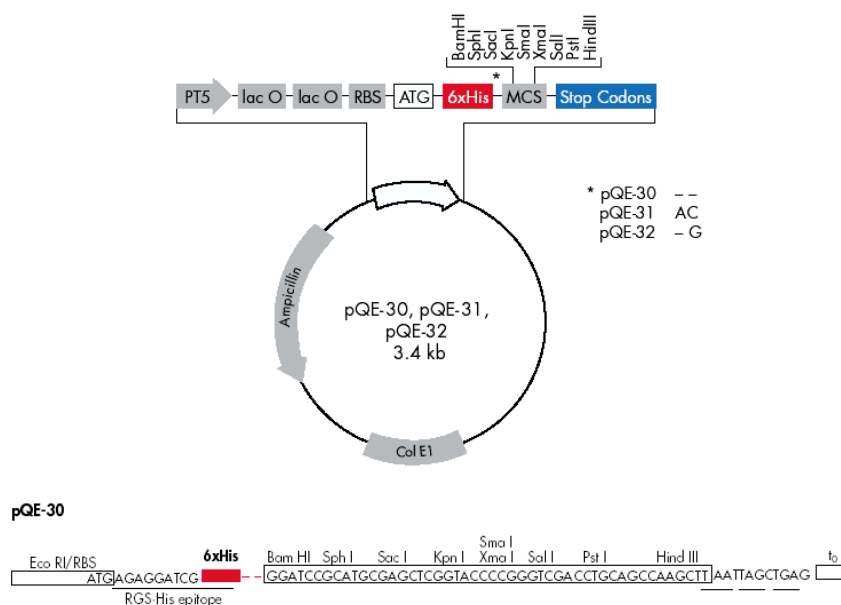
Figure 52. The plasmid map and the multiple-cloning-sites (MCS) sequence of the *QIAexpress* pQE 30 vector (from The QIAexpressionist Handbook).

5.1.2 Plasmid for the expression of 6xHis-tagged ABP7 fusion protein

ABP7 (At5g53160.2) cDNA was amplified with the primer pair 5'-ATTCTGGATCCGCATGCATGGAAGCTAACGGG-3' and 5'-TGGGAGCTCCTTTAGACTCTCGATTCTGTC-3'. The PCR fragment was subsequently cloned into the *QIAexpress* pQE30 vector via BamHI and SacI sites, yielding the plasmid pQE30-ABP7 to express 6xHis-tagged ABP7 fusion protein in *E. coli*.

5.1.3 Plasmid for the expression of 6xHis-tagged ABP9 fusion protein

ABP9 (At1g01360) cDNA was amplified with the primer pair 5'-TAATCTAGCTAGCGTCGACATATGATGGACGGCGTTGAAGGCGGC-3' and 5'-TGGGAGCTCGTCGACTGATTATGTAGTTCACTG-3'. The PCR fragment was subsequently cloned into the *QIAexpress* pQE30 vector via Sal1 site and the orientation of ABP9 cDNA in the pQE30 vector was checked after cloning. This yielded the plasmid pQE30-ABP9 to express 6xHis-tagged ABP9 fusion protein in *E. coli*.

5.1.4 Plasmid for the expression of 6xHis-tagged △N-ABP9 and △C-ABP9 fusion proteins

The cDNA of N-terminus truncated ABP9 (delta N-ABP9 or ∆N-ABP9, a.a. 70-187, without N-terminal domain) and C-terminus truncated ABP9 (delta C-ABP9 or ∆C-ABP9, a.a. 1-137, without C-terminal domain) were amplified with the primer pairs 5'-ATCGGAGCTCACAGTAATCGGTGATCC-3' and 5'-CCCGAAGCTTTCACTGAGTAATGTCCTGAG-3', 5'-TAACTGAGCTCATGATGGACGGCGTTG-3' and 5'-TAAGAAAGCTTTCCTGCTCTTCCCTCG-3', respectively. The PCR fragments were subsequently cloned into the *QIAexpress* pQE30 vector via SacI and HindIII sites, yielding pQE30 △N-ABP9 and pQE30 △C-ABP9 constructs to express 6xHis-tagged truncated ABP9 fusion proteins in *E. coli*.

5.2 Plasmids for generation of the transgenic plants

5.2.1 Plasmids for generation of ABP7 and ABP9 deregulated transgenic Arabidopsis

5.2.1.1 Plasmid for generation of ABP7 overexpression transgenic Arabidopsis

In this study, the binary vector pBI121 (Figure 53) was used to create ABP7 and ABP9 overexpression constructs for transgenic plants. To create pBI121-ABP7 plasmid (Figure 53), the GUS gene in pBI121 was replaced by ABP7 cDNA and it was achieved by the following steps:

I.   The pBI121 vector was first digested with SmaI and SacI to remove GUS gene, and subsequently blunted with Klenow fragment.

II. The ABP7 cDNA was obtained by digesting pQE30-ABP7 plasmid (see Appendix *5.1.2*) with SphI and SacI.

III. The obtained ABP7 cDNA was blunted with Klenow fragment and subsequently ligated to the blunt-ends pBI121 (without GUS).

Before Cloning
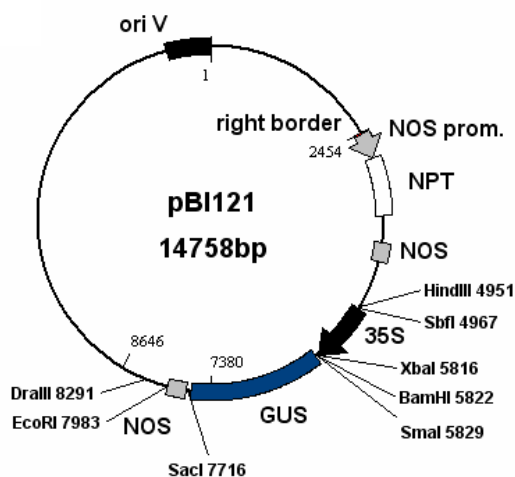
gi|19569229|gb|AF485783.1| Binary vector pBI121

After Cloning
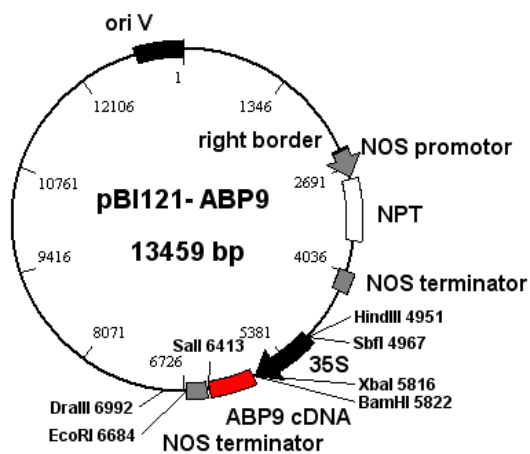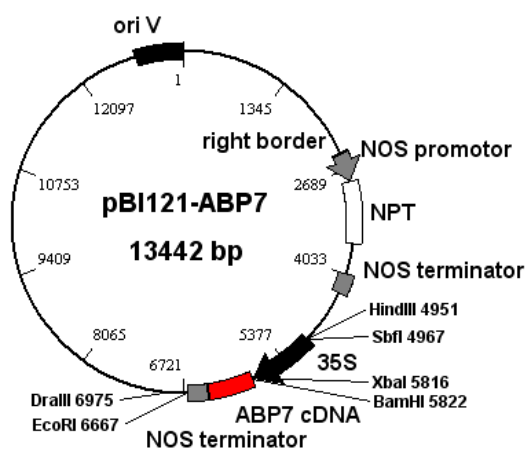
Figure 53. The plasmid maps of pBI121, pBI121-ABP7, and pBI121-ABP9.

IV. Check the orientation of ABP7 cDNA in the pBI121 ABP7 plasmid, by using HindIII digestion.

5.2.1.2 Plasmid for generation of ABP9 overexpression transgenic Arabidopsis

The binary vector pBI121 ABP9 was created as the ABP9 overexpression construct pBI121-ABP9 (Figure 53) for transgenic Arabidopsis, and this was achieved by the following steps:

V. The pBI121 vector was first digested with SmaI and SacI to remove GUS gene, and subsequently blunted with Klenow fragment.

VI. The ABP9 cDNA was obtained by digesting pQE30-ABP9 plasmid (see Appendix *5.1.3*) with Sal1.

VII. The obtained ABP9 cDNA was blunted with Klenow fragment and subsequently ligated to the blunt-ends pBI121 (without GUS).

VIII. Check the orientation of ABP9 cDNA in the pBI121 ABP9 plasmid, by using HindIII digestion and XhoI digestion.

5.2.1.3 Plasmids for generation of RNAi knockdown expression transgenic Arabidopsis

The sense sequence of the ABP9 cDNA conservative fragment (bp 100-371 from ABP9 cDNA) was amplified with the primer pair 5'-CCGGAATTCTGTACCTCTGCTCTTGTC-3' and 5'-GCCGGTACCGACGAGTAATTCTTAAGTCTG-3'. The antisense sequence of the conservative fragment from ABP9 cDNA was amplified with the primer pair 5'-GAAGGATCCTGTACCTCTGCTCTTGTC-3' and 5'-CCATCGATGACGAGTAATTCTTAAGTCTGTG-3'. The two obtained PCR fragments were subsequently cloned into the pHannibal expression vector via EcoRI and KpnI sites (sense orientation) or BamHI and ClaI sites (antisense orientation), yielding pHannibal RNAi construct (Figure 54). The RNAi cassette

was finally cut out of the pHannibal RNAi plasmid with NotI and cloned into pART27 (Gleave, 1992) binary vector via NotI site, yielding pART27 RNAi binary vector to transfer into Arabidopsis.

5.2.2 Plasmids for generation of transgenic Arabidopsis expressing endogenous promoter::genomic ABP9:: β-Glucuronidase (GUS)

The 3.4 kb fragment consisting of ABP9 endogenous promoter, 5'UTR, and non-stop genomic DNA was amplified from Arabidopsis total genomic DNA with the primer pair 5'-CCCAACCGCGGTAAGAGTTGTGTGTGTGTTAATG-3' and 5'-TATCCGGATCCCTGAGTAATGTCCTGAGAAGC-3'. The PCR fragment was subsequently cloned into pSKAscIGUSTer expression vector via SacII and BamHI sites, yielding pSKAscIABP9GUSTer construct (Figure 55). The expression cassette comprising ABP9 endogenous promoter::ABP9 non-stop genomic DNA::GUS::NOS terminator was then cut out of pSKAscIABP9GUSTer plasmid with AscI and subsequently cloned into the binary vector pBIAscIBAR (Figure 56) via AscI site, yielding pBIAscIBAR ABP9GUS plasmid to transfer into Arabidopsis.
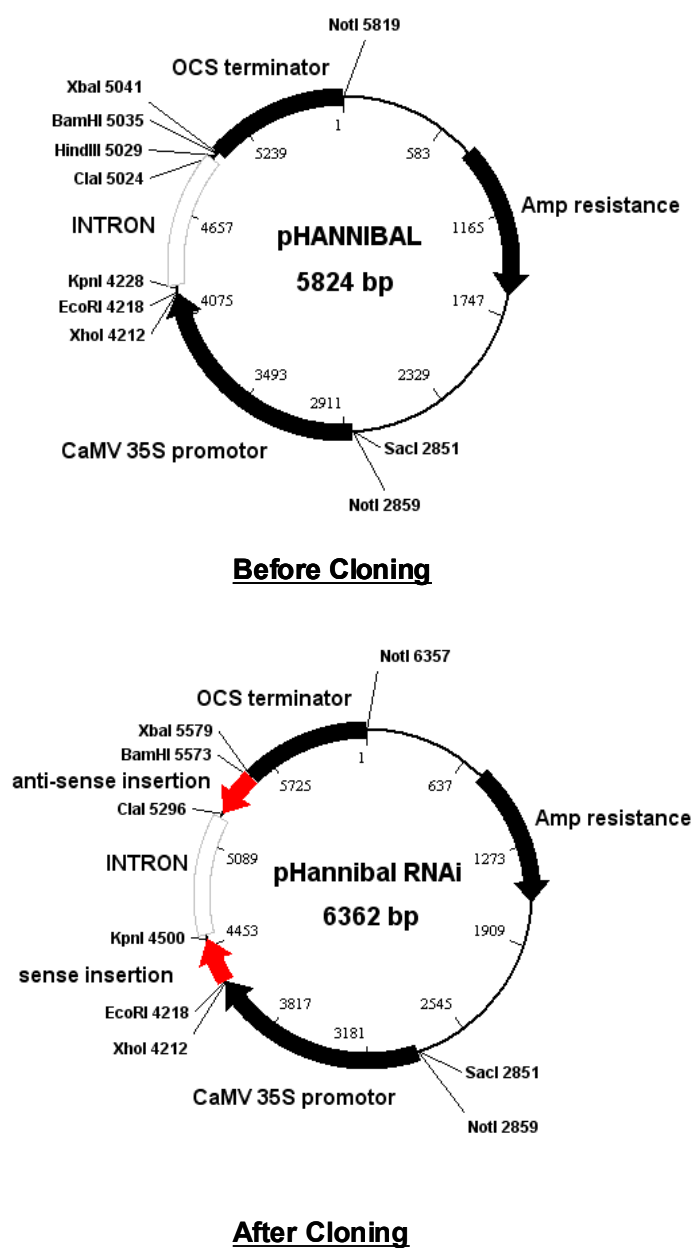
Figure 54. The plasmid maps of pHannibal and pHannibal RNAi expression vectors. The sense and antisense sequences of the ABP9 cDNA conservative fragment are highlighted with red arrows.
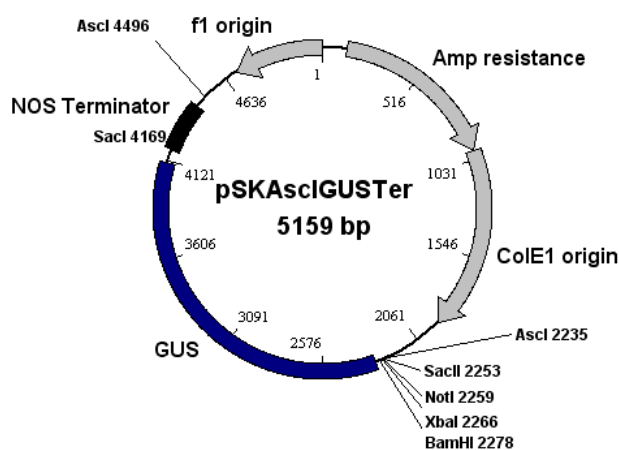
Before Cloning
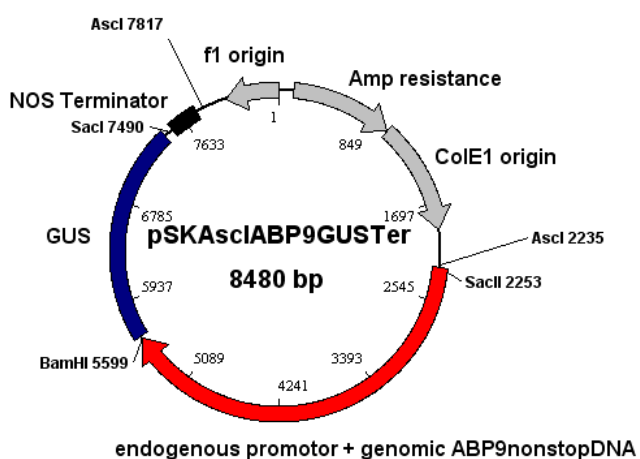
After Cloning

Figure 55. The plasmid maps of pSKAsclGUSTer and pSKAsclABP9GUSTer. The ABP9 endogenous promoter::genomic non-stop DNA is highlighted with the red arrow.
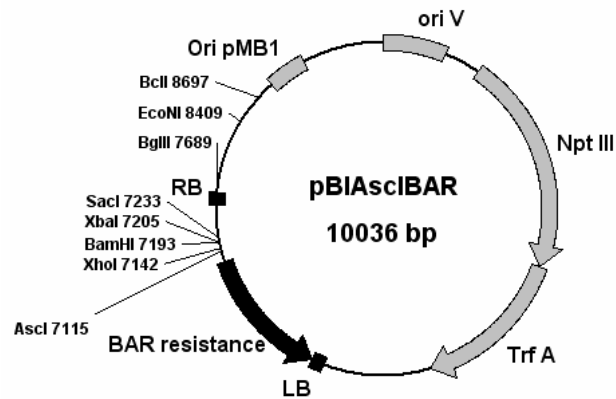
Figure 56. The plamid map of binary vector pBIAscIBAR.

5.3 Plasmids for the transient expression system

The plasmids pSK Rab18::LUC, pSK lti65::LUC, pSK 35S::GUS, pBI221 35S::ABI1, and pBI221 35S::*abi1* were cloned as described in the paper by Moes *et al*. (Moes *et al.*, 2008). The plasmid pBI221 35S::ABI2 was cloned as described in the paper by Ma *et al*. (Ma *et al.*, 2009). The plasmids pHannibal and pHannibal RNAi were the same as described in Appendix 5.2.1.3 (Figure54).

The expression vector pBI221 (Figure 57) was used to generate the over-expression constructs pBI221 ABP7 and pBI221 ABP9, for the transient system. For this purpose, the GUS-NOS terminator cassette in pBI221 was replaced via BamHI and EcoRI sites, by the ABP7cDNA-NOS terminator cassette from binary vector pBI121 ABP7 (Figure 53) or the ABP9cDNA-NOS terminator cassette from binary vector pBI121 ABP9 (Figure 53), respectively.
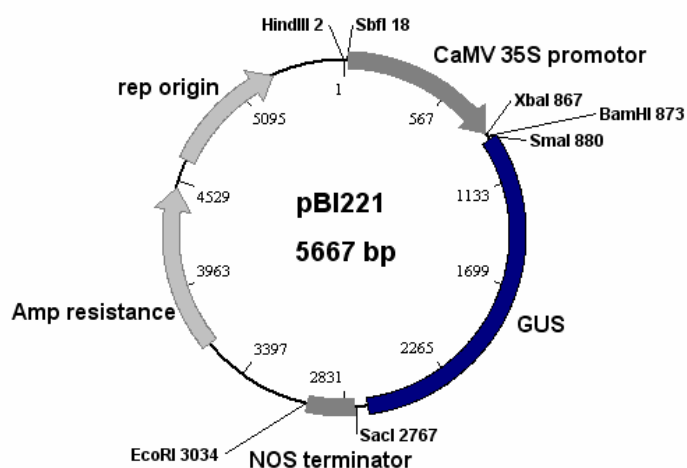
Figure 57. The map of the expression vector pBI221.

5.4 Plasmids for the study of subcellular localization of ABP7 and ABP9

The plasmids pSK 35S::ABP7::EGFP and pSK 35S::ABP9::EGFP were created to study the subcellular localization of ABP7 and ABP9 in Arabidopsis protoplasts. For this purpose, the non-stop cDNA of ABP7 and ABP9 were first amplified with the primer pairs 5'-TCTCGTCGACATGGAAGCTAACGGGATTG-3' and 5'-ATACCCGGGAGACTCTCGATTCTGTCG-3', 5'-TAATCTAGCTAGCGTCGACATATGATGGACGGCGTTGAAGGCGGC-3' and 5'-ATACCCGGGACTGAGTAATGTCCTGAG-3', respectively. The PCR fragments were subsequently linked to the N-terminus of EGFP in pSK 35S::EGFP vector (Moes *et al.*, 2008) via SalI and SmaI sites, yielding pSK 35S::ABP7::EGFP or pSK 35S::ABP9::EGFP.

5.5 Plasmids for the study by bimolecular fluorescence complementation

The expression vectors pSPYNE-35S (YFP^N, a.a. 1-155) and pSPYCE-35S (YFPC, a.a. 156-239) are used to generate all of the split-YFP constructs in this study (Figure 58; Walter *et al.*, 2004)).
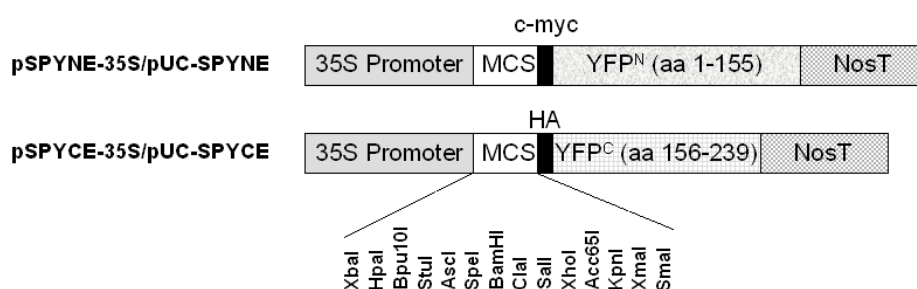
Figure 58. Schematic representation of pSPYNE-35S and pSPYCE-35S BiFC vectors (Walter *et al.*, 2004). c-myc: c-myc affinity tag; HA: hemagglutinin affinity tag; 35S Promoter:35S promoter of the cauliflower mosaic virus; NosT: Nos terminator; YFP^N:N-terminal fragment of YFP reaching from amino acid (aa) 1 to 155; YFP^C: C-terminal fragment of YFP reaching from amino acid 156 to 239.

For the ABP7 and ABP9 split-YFP constructs, the non-stop cDNA of ABP7 and ABP9 were amplified with the primer pairs 5'-ATCTTGGATCCATGGAAGCT AACGGG-3' and 5'-AATACCCGGGGACTCTCGATTCTGTCG-3', 5'-TAA GGATCCATGATGGACGGCGTTG-3' and 5'-TAACCCGGGCTGAGTAATGTC CTGAG-3', respectively. The PCR fragments were subsequently cloned into both pSPYNE-35S and pSPYCE-35S vectors via BamHI and SmaI sites, yielding pSPYNE 35S::ABP7::YFP^N, pSPYCE 35S::ABP7::YFP^C, pSPYNE 35S::ABP9::YFP^N, and pSPYCE 35S::ABP9::YFP^C constructs.

The ABI1 and *abi1* split-YFP constructs were cloned as described in the dissertation of Dr. Moes (Moes, 2006). For the ABI2 and *abi2* split-YFP constructs, the non-stop cDNA of ABI2 and *abi2* were amplified from their corresponding templates with the primer pair 5'-TTACTAGTATGGACGAA GTTTCTCC-3' and 5'-TTGTCGACTCTATTCAAGGATTTGCTC-3'. The PCR fragments of ABI2 and *abi2* were subsequently cloned into both pSPYNE-35S and pSPYCE-35S vectors via SalI and SpeI sites, yielding pSPYNE 35S::ABI2::YFP^N, pSPYCE 35S::ABI2::YFP^C, pSPYNE 35S::*abi2*::YFP^N, and pSPYCE 35S::*abi2*::YFP^C constructs.

6. <u>References</u>

Abe, H., K. Yamaguchi-Shinozaki, T. Urao, T. Iwasaki, D. Hosokawa and K. Shinozaki (1997). "Role of arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression." <u>Plant Cell</u> 9(10): 1859-68.

Adie, B. A., J. Perez-Perez, M. M. Perez-Perez, M. Godoy, J. J. Sanchez-Serrano, E. A. Schmelz and R. Solano (2007). "ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis." <u>Plant Cell</u> 19(5): 1665-81.

Aleksandra Wasilewska, F. V., Caroline Sirichandra, Yulia Redko, Fabien Jammes, Christiane Valon, Nicolas Frei dit Frey and Jeffrey Leung (2008). "An Update on Abscisic Acid Signaling in Plants and More ..." <u>Molecular Plant</u> 1(2): 198-217.

Allen, G. J., K. Kuchitsu, S. P. Chu, Y. Murata and J. I. Schroeder (1999). "Arabidopsis abi1-1 and abi2-1 phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells." <u>Plant Cell</u> 11(9): 1785-98.

Audran, C., C. Borel, A. Frey, B. Sotta, C. Meyer, T. Simonneau and A. Marion-Poll (1998). "Expression studies of the zeaxanthin epoxidase gene in nicotiana plumbaginifolia." <u>Plant Physiol</u> 118(3): 1021-8.

Baker, D. B. and P. M. Ray (1965). "Relation between Effects of Auxin on Cell Wall Synthesis and Cell Elongation." <u>Plant Physiol</u> 40(2): 360-8.

Bensmihen, S., S. Rippa, G. Lambert, D. Jublot, V. Pautot, F. Granier, J. Giraudat and F. Parcy (2002). "The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis." <u>Plant Cell</u> 14(6): 1391-403.

Biesiadka, J., G. Bujacz, M. M. Sikorski and M. Jaskolski (2002). "Crystal structures of two homologous pathogenesis-related proteins from yellow lupine." J Mol Biol 319(5): 1223-34.

Blilou, I., J. Xu, M. Wildwater, V. Willemsen, I. Paponov, J. Friml, R. Heidstra, M. Aida, K. Palme and B. Scheres (2005). "The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots." Nature 433(7021): 39-44.

Blum, H., H. Beier and H. J. Gross (1987). "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels." Electrophoresis 8(2): 93-99.

Boudsocq, M., H. Barbier-Brygoo and C. Lauriere (2004). "Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana." J Biol Chem 279(40): 41758-66.

Bright, J., R. Desikan, J. T. Hancock, I. S. Weir and S. J. Neill (2006). "ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis." Plant J 45(1): 113-22.

Bufe, A., C. Information, M. D. Spangfort, H. Kahlert, M. Schlaak and W.-M. Becker (1995). "The major birch pollen allergen, Bet v 1, shows ribonuclease activity." Planta 199(3): 413-415.

Campanoni, P. and P. Nick (2005). "Auxin-dependent cell division and cell elongation. 1-Naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid activate different pathways." Plant Physiol 137(3): 939-48.

Chae, H. S., F. Faure and J. J. Kieber (2003). "The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in Arabidopsis by increasing the stability of ACS protein." Plant Cell 15(2): 545-59.

Cheng, W. H., A. Endo, L. Zhou, J. Penney, H. C. Chen, A. Arroyo, P. Leon, E. Nambara, T. Asami, M. Seo, T. Koshiba and J. Sheen (2002). "A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions." Plant Cell 14(11): 2723-43.

Cherel, I., E. Michard, N. Platet, K. Mouline, C. Alcon, H. Sentenac and J. B. Thibaud (2002). "Physical and functional interaction of the Arabidopsis K(+) channel AKT2 and phosphatase AtPP2CA." Plant Cell 14(5): 1133-46.

Choi, H. I., H. J. Park, J. H. Park, S. Kim, M. Y. Im, H. H. Seo, Y. W. Kim, I. Hwang and S. Y. Kim (2005). "Arabidopsis calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid-responsive gene expression, and modulates its activity." Plant Physiol 139(4): 1750-61.

Christensen, A. B. C., Baik HO; Nasby, Michael; Gregersen, Per L.; Brandt, Jakob; Madriz-Ordenana, Kenneth; Collinge, David B.; Thordal-Christensen, Hans (2002). "The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins." Molecular Plant Pathology, 3(3): 135-144.

Christmann, A., D. Moes, A. Himmelbach, Y. Yang, Y. Tang and E. Grill (2006). "Integration of abscisic acid signalling into plant responses." Plant Biol (Stuttg) 8(3): 314-25.

Christmann, A. and E. Grill (2009). "Are GTGs ABA's biggest fans?" Cell 136(1): 21-3.

Clough, S. J. (2004). "Floral Dip: Agrobacterium-Mediated Germ Line Transformation." Transgenic Plants: Methods and Protocols 286: 91-101.

143

Constabel, C. P., C. Bertrand and N. Brisson (1993). "Transgenic potato plants overexpressing the pathogenesis-related STH-2 gene show unaltered susceptibility to Phytophthora infestans and potato virus X." <u>Plant Mol Biol</u> 22(5): 775-82.

Corpet, F. (1988). "Multiple sequence alignment with hierarchical clustering." <u>Nucleic Acids Res</u> 16(22): 10881-90.

de Torres-Zabala, M., W. Truman, M. H. Bennett, G. Lafforgue, J. W. Mansfield, P. Rodriguez Egea, L. Bogre and M. Grant (2007). "Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease." <u>Embo J</u> 26(5): 1434-43.

Ebner, C., K. Hoffmann-Sommergruber and H. Breiteneder (2001). "Plant food allergens homologous to pathogenesis-related proteins." <u>Allergy</u> 56 Suppl 67: 43-4.

Edreva, A. (2005). "PATHOGENESIS-RELATED PROTEINS: RESEARCH PROGRESS IN THE LAST 15 YEARS." <u>GEN. APPL. PLANT PHYSIOLOGY,</u> 31: 105-124.

Ephritikhine, G., M. Fellner, C. Vannini, D. Lapous and H. Barbier-Brygoo (1999). "The sax1 dwarf mutant of Arabidopsis thaliana shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid." <u>Plant J</u> 18(3): 303-14.

Fedoroff, N. V. (2002). "Cross-talk in abscisic acid signaling." <u>Sci STKE</u> 2002(140): RE10.

Felsenstein, J. (2004). "PHYLIP (Phylogeny Inference Package), version 3.6.

Distributed by the author. Department of Genome Sciences, University of Washington, Seattle."

Finkelstein, R. R., S. S. Gampala and C. D. Rock (2002). "Abscisic acid signaling in seeds and seedlings." Plant Cell 14 Suppl: S15-45.

Flores, T., A. Alape-Giron, M. Flores-Diaz and H. E. Flores (2002). "Ocatin. A novel tuber storage protein from the andean tuber crop oca with antibacterial and antifungal activities." Plant Physiol 128(4): 1291-302.

Friml, J., J. Wisniewska, E. Benkova, K. Mendgen and K. Palme (2002). "Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis." Nature 415(6873): 806-9.

Friml, J., A. Vieten, M. Sauer, D. Weijers, H. Schwarz, T. Hamann, R. Offringa and G. Jurgens (2003). "Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis." Nature 426(6963): 147-53.

Fujii, H., P. E. Verslues and J. K. Zhu (2007). "Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis." Plant Cell 19(2): 485-94.

Fujii, H., V. Chinnusamy, A. Rodrigues, S. Rubio, R. Antoni, S. Y. Park, S. R. Cutler, J. Sheen, P. L. Rodriguez and J. K. Zhu (2009). "In vitro reconstitution of an abscisic acid signalling pathway." Nature 462(7273): 660-4.

Fujimoto, Y., R. Nagata, H. Fukasawa, K. Yano, M. Azuma, A. Iida, S. Sugimoto, K. Shudo and Y. Hashimoto (1998). "Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (Vigna radiata)." Eur J Biochem 258(2): 794-802.

Gao, Y., Q. Zeng, J. Guo, J. Cheng, B. E. Ellis and J. G. Chen (2007). "Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in Arabidopsis." Plant J 52(6): 1001-13.

Geiger, D., S. Scherzer, P. Mumm, A. Stange, I. Marten, H. Bauer, P. Ache, S. Matschi, A. Liese, K. A. Al-Rasheid, T. Romeis and R. Hedrich (2009). "Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair." Proc Natl Acad Sci U S A 106(50): 21425-30.

Geiger, D., S. Scherzer, P. Mumm, I. Marten, P. Ache, S. Matschi, A. Liese, C. Wellmann, K. A. Al-Rasheid, E. Grill, T. Romeis and R. Hedrich (2010). "Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca2+ affinities." Proc Natl Acad Sci U S A 107(17): 8023-8.

George, E. F., M. A. Hall and G.-J. De Klerk (2008 Copyright). "The Anatomy and Morphology of Tissue Cultured Plants." Plant Propagation by Tissue Culture 1(Chapter 13): 465-477.

Gleave, A. P. (1992). "A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome." Plant Mol Biol 20(6): 1203-7.

Gonzalez-Guzman, M., N. Apostolova, J. M. Belles, J. M. Barrero, P. Piqueras, M. R. Ponce, J. L. Micol, R. Serrano and P. L. Rodriguez (2002). "The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde." Plant Cell 14(8): 1833-46.

Gosti, F., N. Beaudoin, C. Serizet, A. A. Webb, N. Vartanian and J. Giraudat (1999). "ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling." Plant Cell 11(10): 1897-910.

Gray, W. M., A. Ostin, G. Sandberg, C. P. Romano and M. Estelle (1998). "High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis." <u>Proc Natl Acad Sci U S A</u> 95(12): 7197-202.

Gray, W. M., S. Kepinski, D. Rouse, O. Leyser and M. Estelle (2001). "Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins." <u>Nature</u> 414(6861): 271-6.

Guo, Y., U. Halfter, M. Ishitani and J. K. Zhu (2001). "Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance." <u>Plant Cell</u> 13(6): 1383-400.

Guo, Y., L. Xiong, C. P. Song, D. Gong, U. Halfter and J. K. Zhu (2002). "A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis." <u>Dev Cell</u> 3(2): 233-44.

Guo, H. and J. R. Ecker (2004). "The ethylene signaling pathway: new insights." <u>Curr Opin Plant Biol</u> 7(1): 40-9.

Guo, J., Q. Zeng, M. Emami, B. E. Ellis and J. G. Chen (2008). "The GCR2 gene family is not required for ABA control of seed germination and early seedling development in Arabidopsis." <u>PLoS ONE</u> 3(8): 2982.

Guse, A. H. (2008). "Back from the dormant stage: second messenger cyclic ADP-ribose essential for Toxoplasma gondii pathogenicity." <u>Sci Signal</u> 1(17): 18.

Hall, T. A. (1999). "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT." <u>Nucleic Acids Symposium Series</u> 41: 95-98.

HANSEN, H. and K. GROSSMANN (2000). "Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition." Plant physiology 124: 1437-1448.

Hare, P. D., W. A. Cress and J. van Staden (2004). "The involvement of cytokinins in plant responses to environmental stress." Plant growth regulation 23: 79-103.

Himmelbach, A., M. Iten and E. Grill (1998). "Signalling of abscisic acid to regulate plant growth." Philos Trans R Soc Lond B Biol Sci 353(1374): 1439-44.

Himmelbach, A., T. Hoffmann, M. Leube, B. Hohener and E. Grill (2002). "Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis." Embo J 21(12): 3029-38.

Himmelbach, A., Y. Yang and E. Grill (2003). "Relay and control of abscisic acid signaling." Curr Opin Plant Biol 6(5): 470-9.

Holm, R. E. and J. L. Key (1969). "Hormonal regulation of cell elongation in the hypocotyl of rootless soybean: an evaluation of the role of DNA synthesis." Plant Physiol 44(9): 1295-302.

Hoth, S., M. Morgante, J. P. Sanchez, M. K. Hanafey, S. V. Tingey and N. H. Chua (2002). "Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant." J Cell Sci 115(Pt 24): 4891-900.

Hruz, T., O. Laule, G. Szabo, F. Wessendorp, S. Bleuler, L. Oertle, P. Widmayer and W. a. Z. Gruissem, P. (2008). "Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes." Advances in Bioinformatics 420747.

Hugouvieux, V., J. M. Kwak and J. I. Schroeder (2001). "An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in Arabidopsis." Cell 106(4): 477-87.

Iten, M. (1992). "Identifizierung und Charakterisierung von Abscisinsaeure-insensitiven Mutanten von Arabidopsis thaliana." Diplomarbeit, ETH Zürich, Abteilung XA.

Iuchi, S., M. Kobayashi, T. Taji, M. Naramoto, M. Seki, T. Kato, S. Tabata, Y. Kakubari, K. Yamaguchi-Shinozaki and K. Shinozaki (2001). "Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis." Plant J 27(4): 325-33.

Jensen, P. J., R. P. Hangarter and M. Estelle (1998). "Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis." Plant Physiol 116(2): 455-62.

Jiang, K. and L. J. Feldman (2003). "Root Meristem Establishment and Maintenance: The Role of Auxin." Journal of Plant Growth Regulation 21(4): 432-440.

Kaliff, M., J. Staal, M. Myrenas and C. Dixelius (2007). "ABA is required for Leptosphaeria maculans resistance via ABI1- and ABI4-dependent signaling." Mol Plant Microbe Interact 20(4): 335-45.

Kazuo, N. and Y.-S. Kazuko (2005). "Molecular studies on stress-responsive gene expression in Arabidopsis and improvement of stress tolerance in crop plants by regulon biotechnology." JARQ. Japan Agricultural Research Quarterly.

Kim, W. T., A. Silverstone, W. K. Yip, J. G. Dong and S. F. Yang (1992).

"Induction of 1-Aminocyclopropane-1-Carboxylate Synthase mRNA by Auxin in Mung Bean Hypocotyls and Cultured Apple Shoots." Plant Physiol 98(2): 465-471.

Kim, S. Y. (2006). "The role of ABF family bZIP class transcription factors in stress response." Physiologia Plantarum 126: 519-527.

Kim, B. G., R. Waadt, Y. H. Cheong, G. K. Pandey, J. R. Dominguez-Solis, S. Schultke, S. C. Lee, J. Kudla and S. Luan (2007). "The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis." Plant J 52(3): 473-84.

Koistinen, K. M. and P. A. M. G. Viivi H. Hassinen, Satu J. Lehesranta, Sirpa I. Keinänen,   Harri I. Kokko, Elina J. Oksanen, Arja I. Tervahauta, Seppo Auriola, Sirpa O. Kärenlampi, (2002). "Birch PR-10c is induced by factors causing oxidative stress but appears not to confer tolerance to these agents." New Phytologist 155(3): 381-391.

Koistinen, K. M., P. Soininen, T. A. Venalainen, J. Hayrinen, R. Laatikainen, M. Perakyla, A. I. Tervahauta and S. O. Karenlampi (2005). "Birch PR-10c interacts with several biologically important ligands." Phytochemistry 66(21): 2524-33.

Koncz, C. and J. Schell (1986). "The promoter of T-L DNA gene 5 controls the tissue-secific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector." Molecular & general genetics 204: 383-396.

Koornneef, M., L. Bentsink and H. Hilhorst (2002). "Seed dormancy and germination." Curr Opin Plant Biol 5(1): 33-6.

Kudla, J., O. Batistic and K. Hashimoto (2010). "Calcium signals: the lead currency of plant information processing." Plant Cell 22(3): 541-63.

Kuhn, J. M., A. Boisson-Dernier, M. B. Dizon, M. H. Maktabi and J. I. Schroeder (2006). "The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in Arabidopsis, and effects of abh1 on AtPP2CA mRNA." Plant Physiol 140(1): 127-39.

Kwak, J. M., I. C. Mori, Z. M. Pei, N. Leonhardt, M. A. Torres, J. L. Dangl, R. E. Bloom, S. Bodde, J. D. Jones and J. I. Schroeder (2003). "NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis." Embo J 22(11): 2623-33.

Lang, V. and E. T. Palva (1992). "The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process of Arabidopsis thaliana (L.) Heynh." Plant Mol Biol 20(5): 951-62.

Leckie, C. P., M. R. McAinsh, G. J. Allen, D. Sanders and A. M. Hetherington (1998). "Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose." Proc Natl Acad Sci U S A 95(26): 15837-42.

Lee, H.-S. a. M., B.V. (1997). "Endogenous biosynthetic precursors of (+)-abscisic acid. V. Inhibition by tungstate and its removal by cinchonine shows that xanthoxal is oxidised by a molybdo-aldehyde oxidase." Aust. J. Plant Physiol. 24(6): 727-732.

Lee, S. C., W. Z. Lan, B. G. Kim, L. Li, Y. H. Cheong, G. K. Pandey, G. Lu, B. B. Buchanan and S. Luan (2007). "A protein phosphorylation/dephosphorylation network regulates a plant potassium channel." Proc Natl Acad Sci U S A 104(40): 15959-64.

Lee, S. C., W. Lan, B. B. Buchanan and S. Luan (2009). "A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling

in plant guard cells." Proc Natl Acad Sci U S A 106(50): 21419-24.

Leube, M. P., E. Grill and N. Amrhein (1998). "ABI1 of Arabidopsis is a protein serine/threonine phosphatase highly regulated by the proton and magnesium ion concentration." FEBS Lett 424(1-2): 100-4.

Leung, J., S. Merlot and J. Giraudat (1997). "The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction." Plant Cell 9(5): 759-71.

Leung, J. and J. Giraudat (1998). "Abscisic Acid Signal Transduction." Annu Rev Plant Physiol Plant Mol Biol 49: 199-222.

Li, J., Y. R. Lee and S. M. Assmann (1998). "Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel." Plant Physiol 116(2): 785-95.

Linsmaier, E. M. and F. Skoog (1965). "Organic growth factor requirements of tobacco tissue cultures." Physiol. Plant. 18: 100-127.

Liscombe, D. K., B. P. MacLeod, N. Loukanina, O. I. Nandi and P. J. Facchini (2005). "Evidence for the monophyletic evolution of benzylisoquinoline alkaloid biosynthesis in angiosperms." Phytochemistry 66(20): 2501-20.

Liu, J. J. and A. K. M. Ekramoddoullah (2006). "The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses." Physiological and Molecular Plant Pathology 68: 3-13.

Liu, X., B. Huang, J. Lin, J. Fei, Z. Chen, Y. Pang, X. Sun and K. Tang (2006). "A

novel pathogenesis-related protein (SsPR10) from Solanum surattense with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible." <u>J Plant Physiol</u> 163(5): 546-56.

Ma, Y., I. Szostkiewicz, A. Korte, D. Moes, Y. Yang, A. Christmann and E. Grill (2009). "Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors." <u>Science</u> 324(5930): 1064-8.

MacRobbie, E. A. C. (1995). "ABA-induced ion efflux in stomatal guard cells: multiple actions of ABA inside and outside the cell." <u>The Plant Journal</u> 7(4): 565-576.

Markovic-Housley, Z., M. Degano, D. Lamba, E. von Roepenack-Lahaye, S. Clemens, M. Susani, F. Ferreira, O. Scheiner and H. Breiteneder (2003). "Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier." <u>J Mol Biol</u> 325(1): 123-33.

Mathur, J., and Koncz, C. (1998). "Establishment and maintenance of cell suspension cultures." <u>Methods in Molecular Biology; Arabidopsis protocols</u>: 27-30.

Mauch-Mani, B. and F. Mauch (2005). "The role of abscisic acid in plant-pathogen interactions." <u>Curr Opin Plant Biol</u> 8(4): 409-14.

Meinhard, M. and E. Grill (2001). "Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis." <u>FEBS Lett</u> 508(3): 443-6.

Meinhard, M., P. L. Rodriguez and E. Grill (2002). "The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling." <u>Planta</u> 214(5): 775-82.

Meinke, D. W. (1992). "A Homoeotic Mutant of Arabidopsis thaliana with Leafy Cotyledons." Science 258(5088): 1647-1650.

Meinke, D. W., L. H. Franzmann, T. C. Nickle and E. C. Yeung (1994). "Leafy Cotyledon Mutants of Arabidopsis." Plant Cell 6(8): 1049-1064.

Melcher, K., L. M. Ng, X. E. Zhou, F. F. Soon, Y. Xu, K. M. Suino-Powell, S. Y. Park, J. J. Weiner, H. Fujii, V. Chinnusamy, A. Kovach, J. Li, Y. Wang, F. C. Peterson, D. R. Jensen, E. L. Yong, B. F. Volkman, S. R. Cutler, J. K. Zhu and H. E. Xu (2009). "A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors." Nature 462(7273): 602-8.

Melotto, M., W. Underwood, J. Koczan, K. Nomura and S. Y. He (2006). "Plant stomata function in innate immunity against bacterial invasion." Cell 126(5): 969-80.

Merlot, S. and J. Ciraudat (1997). "Genetic Analysis of Abscisic Acid Signal Transduction." Plant Physiol 114: 751-757.

Milborrow, B. V. and R. C. Noddle (1970). "Conversion of 5-(1,2-epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-cis-2-trans-4-dienoi c acid into abscisic acid in plants." Biochem J 119(4): 727-34.

Mishra, G., W. Zhang, F. Deng, J. Zhao and X. Wang (2006). "A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis." Science 312(5771): 264-6.

Miyazono, K., T. Miyakawa, Y. Sawano, K. Kubota, H. J. Kang, A. Asano, Y. Miyauchi, M. Takahashi, Y. Zhi, Y. Fujita, T. Yoshida, K. S. Kodaira, K. Yamaguchi-Shinozaki and M. Tanokura (2009). "Structural basis of abscisic acid

signalling." Nature 462(7273): 609-14.

Mizoguchi, T., A. Kikuchi, T. Kato, H. Kamada and K. Shinozaki (2005). "Isolation of Arabidopsis mutants that have altered sensitivity to staurosporine and ABA." Plant Biotechnology 22: 303-310.

Moes, D. (2006). "Signaltransduktion des Phytohormons Abscisinsaeure: Rolle der nukleaeren Lokalisation der Proteinphosphatase ABI1." Technical University, Munich.

Moes, D., A. Himmelbach, A. Korte, G. Haberer and E. Grill (2008). "Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards ABA responses in Arabidopsis." Plant J 54(5): 806-19.

Mogensen, J. E., R. Wimmer, J. N. Larsen, M. D. Spangfort and D. E. Otzen (2002). "The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands." J Biol Chem 277(26): 23684-92.

Moiseyev, G. P., L. I. Fedoreyeva, Y. N. Zhuravlev, E. Yasnetskaya, P. A. Jekel and J. J. Beintema (1997). "Primary structures of two ribonucleases from ginseng calluses. New members of the PR-10 family of intracellular pathogenesis-related plant proteins." FEBS Lett 407(2): 207-10.

Monroe-Augustus, M., B. K. Zolman and B. Bartel (2003). "IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis." Plant Cell 15(12): 2979-91.

Mori, I. C., Y. Murata, Y. Yang, S. Munemasa, Y. F. Wang, S. Andreoli, H. Tiriac, J. M. Alonso, J. F. Harper, J. R. Ecker, J. M. Kwak and J. I. Schroeder (2006). "CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca(2+)-permeable channels and stomatal closure." PLoS Biol 4(10): e327.

Mulkey, T. J., K. M. Kuzmanoff and M. L. Evans (1982). "Promotion of growth and shift in the auxin dose/response relationship in maize roots treated with the ethylene biosynthesis inhibitors aminoethoxyvinylglycine and cobalt." Plant Science Letters 25: 43-48.

Muller, A. H. and M. Hansson (2009). "The barley magnesium chelatase 150-kd subunit is not an abscisic acid receptor." Plant Physiol 150(1): 157-66.

Murashiqe, T. and F. Skoog (1962). "A revised medium for rapid growth and bioassays with tobacco tissue cultures." Physiologia Plantarum 15(3): 473-497.

Mustilli, A. C., S. Merlot, A. Vavasseur, F. Fenzi and J. Giraudat (2002). "Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production." Plant Cell 14(12): 3089-99.

Nagpal, P., L. M. Walker, J. C. Young, A. Sonawala, C. Timpte, M. Estelle and J. W. Reed (2000). "AXR2 encodes a member of the Aux/IAA protein family." Plant Physiol 123(2): 563-74.

Nakamura, S., T. J. Lynch and R. R. Finkelstein (2001). "Physical interactions between ABA response loci of Arabidopsis." Plant J 26(6): 627-35.

Nakashima, K., Y. Fujita, K. Katsura, K. Maruyama, Y. Narusaka, M. Seki, K. Shinozaki and K. Yamaguchi-Shinozaki (2006). "Transcriptional regulation of ABI3- and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis." Plant Mol Biol 60(1): 51-68.

Nambara, E. and A. Marion-Poll (2005). "Abscisic acid biosynthesis and catabolism." Annu Rev Plant Biol 56: 165-85.

Neibaur, I., M. Gallo and F. Altpeter (2008). "The effect of auxin type and cytokinin concentration on callus induction and plant regeneration frequency from immature inflorescence segments of seashore paspalum (Paspalum vaginatum Swartz)." In Vitro Cellular & Developmental Biology - Plant 44(6): 480-486.

Neudecker, P., K. Schweimer, J. Nerkamp, S. Scheurer, S. Vieths, H. Sticht and P. Rosch (2001). "Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1." J Biol Chem 276(25): 22756-63.

Nishimura, N., N. Kitahata, M. Seki, Y. Narusaka, M. Narusaka, T. Kuromori, T. Asami, K. Shinozaki and T. Hirayama (2005). "Analysis of ABA hypersensitive germination2 revealed the pivotal functions of PARN in stress response in Arabidopsis." Plant J 44(6): 972-84.

Nishimura, N., K. Hitomi, A. S. Arvai, R. P. Rambo, C. Hitomi, S. R. Cutler, J. I. Schroeder and E. D. Getzoff (2009). "Structural mechanism of abscisic acid binding and signaling by dimeric PYR1." Science 326(5958): 1373-9.

Niu, X., T. Helentjaris and N. J. Bate (2002). "Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes." Plant Cell 14(10): 2565-75.

North, H. M., A. De Almeida, J. P. Boutin, A. Frey, A. To, L. Botran, B. Sotta and A. Marion-Poll (2007). "The Arabidopsis ABA-deficient mutant aba4 demonstrates that the major route for stress-induced ABA accumulation is via neoxanthin isomers." Plant J 50(5): 810-24.

OHare, T. J. and C. G. N. Turnbull (2004). "Root growth, cytokinin and shoot dormancy in lychee (Litchi chinensis Sonn.)." Scientia Horticulturae 102(2): 257-266.

Ohta, M., Y. Guo, U. Halfter and J. K. Zhu (2003). "A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2." <u>Proc Natl Acad Sci U S A</u> 100(20): 11771-6.

Pandey, G. K., J. J. Grant, Y. H. Cheong, B. G. Kim, G. Li le and S. Luan (2008). "Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination." <u>Mol Plant</u> 1(2): 238-48.

Pandey, S., D. C. Nelson and S. M. Assmann (2009). "Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis." <u>Cell</u> 136(1): 136-48.

Papp, I., L. A. Mur, A. Dalmadi, S. Dulai and C. Koncz (2004). "A mutation in the Cap Binding Protein 20 gene confers drought tolerance to Arabidopsis." <u>Plant Mol Biol</u> 55(5): 679-86.

Park, C. J., J. M. An, Y. C. Shin, K. J. Kim, B. J. Lee and K. H. Paek (2004a). "Molecular characterization of pepper germin-like protein as the novel PR-16 family of pathogenesis-related proteins isolated during the resistance response to viral and bacterial infection." <u>Planta</u> 219(5): 797-806.

Park, C. J., K. J. Kim, R. Shin, J. M. Park, Y. C. Shin and K. H. Paek (2004b). "Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway." <u>Plant J</u> 37(2): 186-98.

Park, S. Y., P. Fung, N. Nishimura, D. R. Jensen, H. Fujii, Y. Zhao, S. Lumba, J. Santiago, A. Rodrigues, T. F. Chow, S. E. Alfred, D. Bonetta, R. Finkelstein, N. J. Provart, D. Desveaux, P. L. Rodriguez, P. McCourt, J. K. Zhu, J. I. Schroeder, B. F. Volkman and S. R. Cutler (2009). "Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins." <u>Science</u> 324(5930): 1068-71.

Pascal Maeser, N. L. a. J. I. S. (2003). "The Clickable Guard Cell: Electronically linked Model of Guard Cell Signal Transduction Pathways." The Arabidopsis Book.

Pearson, W. R., T. Wood, Z. Zhang and W. Miller (1997). "Comparison of DNA sequences with protein sequences." Genomics 46(1): 24-36.

Pernisova, M., P. Klima, J. Horak, M. Valkova, J. Malbeck, P. Soucek, P. Reichman, K. Hoyerova, J. Dubova, J. Friml, E. Zazimalova and J. Hejatko (2009). "Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux." Proc Natl Acad Sci U S A 106(9): 3609-14.

Rayle, D. L., M. L. Evans and R. Hertel (1970). "Action of auxin on cell elongation." Proc Natl Acad Sci U S A 65(1): 184-91.

Razem, F. A., A. El-Kereamy, S. R. Abrams and R. D. Hill (2006). "The RNA-binding protein FCA is an abscisic acid receptor." Nature 439(7074): 290-4.

Razem, F. A., A. El-Kereamy, S. R. Abrams and R. D. Hill (2008). "Retraction. The RNA-binding protein FCA is an abscisic acid receptor." Nature 456(7223): 824.

Reyes, J. L. and N. H. Chua (2007). "ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination." Plant J 49(4): 592-606.

Riou-Khamlichi, C., R. Huntley, A. Jacqmard and J. A. Murray (1999). "Cytokinin activation of Arabidopsis cell division through a D-type cyclin." Science 283(5407): 1541-4.

Risk JM, M. R., Day CL. (2008). "FCA does not bind abscisic acid." Nature 456: E5-6.

Rock, C. D., T. G. Heath, D. A. Gage and J. A. Zeevaart (1991). "Abscisic Alcohol Is an Intermediate in Abscisic Acid Biosynthesis in a Shunt Pathway from Abscisic Aldehyde." Plant Physiol 97(2): 670-676.

Rock, C. D. and X. Sun (2005). "Crosstalk between ABA and auxin signaling pathways in roots of Arabidopsis thaliana (L.) Heynh." Planta 222(1): 98-106.

Ruzicka, K., K. Ljung, S. Vanneste, R. Podhorska, T. Beeckman, J. Friml and E. Benkova (2007). "Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution." Plant Cell 19(7): 2197-212.

Saavedra, X., A. Modrego, D. Rodriguez, M. P. Gonzalez-Garcia, L. Sanz, G. Nicolas and O. Lorenzo (2010). "The nuclear interactor PYL8/RCAR3 of Fagus sylvatica FsPP2C1 is a positive regulator of abscisic acid signaling in seeds and stress." Plant Physiol 152(1): 133-50.

Saez, A., N. Apostolova, M. Gonzalez-Guzman, M. P. Gonzalez-Garcia, C. Nicolas, O. Lorenzo and P. L. Rodriguez (2004). "Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling." Plant J 37(3): 354-69.

Sakurai, N. and Y. Masuda (1978). "Auxin-induced extension, cell wall loosening and changes in the wall polysaccharide content of barley coleoptile segments." Plant and Cell Physiology 19(7): 1225-1233.

Samanani, N., D. K. Liscombe and P. J. Facchini (2004). "Molecular cloning and characterization of norcoclaurine synthase, an enzyme catalyzing the first

committed step in benzylisoquinoline alkaloid biosynthesis." <u>Plant J</u> 40(2): 302-13.

Sambrook, J. and D. W. Russell (2001). "Molecular Cloning: a laboratory manual. 3rd edition." <u>Cold spring harbor laboratory press, Cold Spring Harbor, New York</u>.

Santiago, J., F. Dupeux, A. Round, R. Antoni, S. Y. Park, M. Jamin, S. R. Cutler, P. L. Rodriguez and J. A. Marquez (2009a). "The abscisic acid receptor PYR1 in complex with abscisic acid." <u>Nature</u> 462(7273): 665-8.

Santiago, J., A. Rodrigues, A. Saez, S. Rubio, R. Antoni, F. Dupeux, S. Y. Park, J. A. Marquez, S. R. Cutler and P. L. Rodriguez (2009b). "Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs." <u>Plant J</u> 60(4): 575-88.

Santner, A. and M. Estelle (2009). "Recent advances and emerging trends in plant hormone signalling." <u>Nature</u> 459(7250): 1071-8.

Sato, A., Y. Sato, Y. Fukao, M. Fujiwara, T. Umezawa, K. T. Hibi, M. Taniguchi, H. Miyake, D. B. Goto and Nobuyuki (2009). "Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase." <u>Biochem. J.</u> 424:439-448.

Scherer N.M., C. E. T., S. L. B. Loreta B. Freitas and F. M. Salzano (2006). "Evolutionary Analysis in Pathogenesis-Related Proteins." <u>NIC Series</u> 34: 193-196.

Schmidt, C., I. Schelle, Y. J. Liao and J. I. Schroeder (1995). "Strong regulation of slow anion channels and abscisic acid signaling in guard cells by phosphorylation and dephosphorylation events." <u>Proc Natl Acad Sci U S A</u> 92(21): 9535-9.

Schroeder, J. I., G. J. Allen, V. Hugouvieux, J. M. Kwak and D. Waner (2001). "Guard Cell Signal Transduction." <u>Annu Rev Plant Physiol Plant Mol Biol</u> 52: 627-658.

Schwartz, S. H., X. Qin and J. A. Zeevaart (2003). "Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes." <u>Plant Physiol</u> 131(4): 1591-601.

Schweighofer, A., H. Hirt and I. Meskiene (2004). "Plant PP2C phosphatases: emerging functions in stress signaling." <u>Trends Plant Sci</u> 9(5): 236-43.

Sekiya, J., T. Yasuda and Y. Yamada (1977). "Callus induction in tobacco, pea, rice and barley plants by auxins and their analogues." <u>Plant and Cell Physiology</u> 18(5): 1155-1157.

Selitrennikoff, C. P. (2001). "Antifungal proteins." <u>Appl Environ Microbiol</u> 67(7): 2883-94.

Seo, M., A. J. Peeters, H. Koiwai, T. Oritani, A. Marion-Poll, J. A. Zeevaart, M. Koornneef, Y. Kamiya and T. Koshiba (2000). "The Arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves." <u>Proc Natl Acad Sci U S A</u> 97(23): 12908-13.

Seo, M. and T. Koshiba (2002). "Complex regulation of ABA biosynthesis in plants." <u>Trends Plant Sci</u> 7(1): 41-8.

Shang, Y., L. Yan, Z. Q. Liu, Z. Cao, C. Mei, Q. Xin, F. Q. Wu, X. F. Wang, S. Y. Du, T. Jiang, X. F. Zhang, R. Zhao, H. L. Sun, R. Liu, Y. T. Yu and D. P. Zhang (2010). "The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition."

Plant Cell 22(6): 1909-35.

Shen, Y. Y., X. F. Wang, F. Q. Wu, S. Y. Du, Z. Cao, Y. Shang, X. L. Wang, C. C. Peng, X. C. Yu, S. Y. Zhu, R. C. Fan, Y. H. Xu and D. P. Zhang (2006). "The Mg-chelatase H subunit is an abscisic acid receptor." Nature 443(7113): 823-6.

Shinozaki, K. and K. Yamaguchi-Shinozaki (2000). "Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways." Curr Opin Plant Biol 3(3): 217-23.

Skoog, F. and C. O. Miller (1957). "Chemical regulation of growth and organ formation in plant tissues cultured in vitro." Symp Soc Exp Biol 54(11): 118-30.

Soderman, E., M. Hjellstrom, J. Fahleson and P. Engstrom (1999). "The HD-Zip gene ATHB6 in Arabidopsis is expressed in developing leaves, roots and carpels and up-regulated by water deficit conditions." Plant Mol Biol 40(6): 1073-83.

Srivastava, S., M. H. Rahman, S. Shah and N. N. Kav (2006 a). "Constitutive expression of the pea ABA-responsive 17 (ABR17) cDNA confers multiple stress tolerance in Arabidopsis thaliana." Plant Biotechnol J 4(5): 529-49.

Srivastava, S. and E. R. J. N. K. L. V. R. D. M. F. B. K. N. N. V (2006 b). "Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels." Plant growth regulation 49: 17-25.

Srivastava, S., R. J. N. Emery; and M. H. R. a. N. N. V. Kav (2007). "A Crucial Role for Cytokinins in Pea ABR17-mediated Enhanced Germination and Early Seedling Growth of Arabidopsis thaliana   under Saline and Low-temperature Stresses." Journal of Plant Growth Regulation 26(1): 26-37.

Staswick, P. E., W. Su and S. H. Howell (1992). "Methyl jasmonate inhibition of

root growth and induction of a leaf protein are decreased in an Arabidopsis thaliana mutant." Proc Natl Acad Sci U S A 89(15): 6837-40.

Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C. and Rathore, K.S. (2002). "Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP." Plant Mol Biol 50(3): 463-474.

Sunkar, R. and J. K. Zhu (2004). "Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis." Plant Cell 16(8): 2001-19.

Szostkiewicz, I., K. Richter, M. Kepka, S. Demmel, Y. Ma, A. Korte, F. F. Assaad, A. Christmann and E. Grill (2010). "Closely related receptor complexes differ in their ABA selectivity and sensitivity." Plant J 61(1): 25-35.

Tamimi, S. and R. D. Firn (1985). "The Basipetal Auxin Transport System and the Control of Cell Elongation in Hypocotyls." Journal of Experimental Botany 36(6): 955-962.

Tan, X., L. I. Calderon-Villalobos, M. Sharon, C. Zheng, C. V. Robinson, M. Estelle and N. Zheng (2007). "Mechanism of auxin perception by the TIR1 ubiquitin ligase." Nature 446(7136): 640-5.

Taylor, H. F. and R. S. Burden (1973). "Preparation and Metabolism of 2-(14C)-cis, trans-Xanthoxin." J. Exp. Bot. 24(5): 873-880.

Thompson, A. J., A. C. Jackson, R. A. Parker, D. R. Morpeth, A. Burbidge and I. B. Taylor (2000). "Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid." Plant Mol Biol 42(6): 833-45.

Torres, M. A. and J. L. Dangl (2005). "Functions of the respiratory burst oxidase

in biotic interactions, abiotic stress and development." <u>Curr Opin Plant Biol</u> 8(4): 397-403.

Tsuchisaka, A. and A. Theologis (2004). "Unique and overlapping expression patterns among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members." <u>Plant Physiol</u> 136(2): 2982-3000.

Umezawa, T., N. Sugiyama, M. Mizoguchi, S. Hayashi, F. Myouga, K. Yamaguchi-Shinozaki, Y. Ishihama, T. Hirayama and K. Shinozaki (2009). "Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis." <u>Proc Natl Acad Sci U S A</u> 106(41): 17588-93.

Uno, Y., T. Furihata, H. Abe, R. Yoshida, K. Shinozaki and K. Yamaguchi-Shinozaki (2000). "Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions." <u>Proc Natl Acad Sci U S A</u> 97(21): 11632-7.

Utriainen, M. and S. A. H. Kokko, O. Sarrazin, S. Kärenlampi, (1998). "PR-10 protein is induced by copper stress in roots and leaves of a Cu/Zn tolerant clone of birch, *Betula pendula*." <u>Plant, Cell & Environment</u> 21(8): 821-828.

Van Loon, L. C. a. V. S. E. A. (1999). "The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins." <u>Physiological and Molecular Plant Pathology</u> 55: 85-97.

Vernon, D. M., M. J. Hannon, M. Le and N. R. Forsthoefel (2001). "An expanded role for the TWN1 gene in embryogenesis: defects in cotyledon pattern and morphology in the twn1 mutant of Arabidopsis (Brassicaceae)." <u>Am J Bot</u> 88(4): 570-582.

Walter, M., C. Chaban, K. Schutze, O. Batistic, K. Weckermann, C. Nake, D.

Blazevic, C. Grefen, K. Schumacher, C. Oecking, K. Harter and J. Kudla (2004). "Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation." Plant J 40(3): 428-38.

Wang, C., J. Huang and H. J. (1999). "Characterization of two subclasses of PR-10 transcripts in lily anthers and induction of their genes through separate signal transduction pathways." Plant Molecular Biology 40: 807-14.

Weinl, S. and J. Kudla (2009). "The CBL-CIPK Ca(2+)-decoding signaling network: function and perspectives." New Phytol 184(3): 517-28.

Wilson, A. K., F. B. Pickett, J. C. Turner and M. Estelle (1990). "A dominant mutation in Arabidopsis confers resistance to auxin, ethylene and abscisic acid." Mol Gen Genet 222(2-3): 377-83.

Xigang Liu, Y. Y., Bin Li, Yanli Nie, Wei Li, Wei-Hua Wu, Ligeng Ma (2007). "A G Protein-Coupled Receptor Is a Plasma Membrane Receptor for the Plant Hormone Abscisic Acid." Science 315: 1712-1716.

Xiong, L., M. Ishitani, H. Lee and J. K. Zhu (2001). "The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression." Plant Cell 13(9): 2063-83.

Xiong, L., H. Lee, M. Ishitani and J. K. Zhu (2002). "Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in Arabidopsis." J Biol Chem 277(10): 8588-96.

Xiong, L. and J. K. Zhu (2003). "Regulation of abscisic acid biosynthesis." Plant Physiol 133(1): 29-36.

Yamamoto, Y. Y., P. Puente and X. W. Deng (2000). "An Arabidopsis

cotyledon-specific albino locus: a possible role in 16S rRNA maturation." <u>Plant Cell Physiol</u> 41(1): 68-76.

Yang, Y. (2003). "Signal transduction of abscisic acid in Arabidopsis thaliana:Identification and characterisation of protein interaction partners of ABI2." <u>Technical University, Munich</u>.

Yang, Y., R. Sulpice, A. Himmelbach, M. Meinhard, A. Christmann and E. Grill (2006). "Fibrillin expression is regulated by abscisic acid response regulators and is involved in abscisic acid-mediated photoprotection." <u>Proc Natl Acad Sci U S A</u> 103(15): 6061-6.

Yoshida, R., T. Hobo, K. Ichimura, T. Mizoguchi, F. Takahashi, J. Aronso, J. R. Ecker and K. Shinozaki (2002). "ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis." <u>Plant Cell Physiol</u> 43(12): 1473-83.

Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K. (2006 a). "The Regulatory Domain of SRK2E/OST1/SnRK2.6 Interacts with ABI1 and Integrates Abscisic Acid (ABA) and Osmotic Stress Signals Controlling Stomatal Closure in Arabidopsis." <u>J Biol Chem</u> 281(8): 5310-5318.

Yoshida, T., N. Nishimura, N. Kitahata, T. Kuromori, T. Ito, T. Asami, K. Shinozaki and T. Hirayama (2006 b). "ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs." <u>Plant Physiol</u> 140(1): 115-26.

Zalejski, C., Z. Zhang, A. L. Quettier, R. Maldiney, M. Bonnet, M. Brault, C. Demandre, E. Miginiac, J. P. Rona, B. Sotta and E. Jeannette (2005).

"Diacylglycerol pyrophosphate is a second messenger of abscisic acid signaling in Arabidopsis thaliana suspension cells." Plant J 42(2): 145-52.

Zhang, Z. (2004). "Research on ABP9/ABP7 in Arabidopsis thaliana." Technical University, Munich.

Zhu, S. Y., X. C. Yu, X. J. Wang, R. Zhao, Y. Li, R. C. Fan, Y. Shang, S. Y. Du, X. F. Wang, F. Q. Wu, Y. H. Xu, X. Y. Zhang and D. P. Zhang (2007). "Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis." Plant Cell 19(10): 3019-36.

Acknowledgments

During my Ph.D. study I have benefited greatly from the assistance of many people. First of all, I am indebted to Professor Dr. Erwin Grill. I sincerely appreciate his illuminating scientific ideas for this project, patient academic instructions, and valuable help during my pregnancy. I am more grateful than I can possibly say.

With true gratitude, I thank Dr. Alexander Christmann for his constant patience, academic discussions, helpful suggestions, and well-timed encouragements from the beginning to the end of my Ph.D. study.

I also extend significant thanks to Izabela Szostkiewicz and Dr. Arthur Körte for their discussions and huge contributions to this project.

I further would like to thank Dr. Michael Meinhard for his laboratorial instructions and patient help at the beginning. Thanks to Dr. Michael Helm for his important instructions on protein purification. Thanks to Dr. Kenneth Berendzen from the University of Tübingen for his help in conducting the FACS analysis.

Finally, I am thankful to Caroline Klaus, Lisa Held, Christoph Heidersberger, Christian Kornbauer, and Johanna Berger for their laboratorial support of this work. I would like to give special thanks to Beate Seeliger for her frequent help in my life in Freising. With no less gratitude, I thank all of my colleagues in Lehrstuhl für Botanik, Technische Universität München.

此外，我要感谢我远在中国的父母。没有你们的理解和支持，我不可能远赴德国、完成学业！我还要感谢所有曾经帮助过我的人们，你们的关怀我永记于心！

Curriculum Vitae

Personal Data:

Name:	Yue Ma-Lauer
Gender:	Female
Date of Birth:	2nd June, 1977
Place of Birth:	Beijing, P.R.China
Nationality:	Chinese

Education:

1984-1996:	Primary school and middle schools in Beijing
1996-2000:	Study of Environmental Biology and Ecology in the College of Life Sciences, Beijing University, P.R.China
July 2000:	Bachelor of Science
2000-2003:	Study of Ecology in the College of Life Sciences, Beijing University, P.R.China
July 2003:	Master of Science
December 2003-April 2008:	Ph.D. study in Lehrstuhl für Botanik, Technische Universität München, Germany Supervisor: Professor Dr. Erwin Grill

Publications:

Ma, Y., I. Szostkiewicz, A. Korte, D. Moes, Y. Yang, A. Christmann and E. Grill (2009). "Regulators of PP2C Phosphatase Activity Function as Abscisic Acid Sensors." Science **324**(5930): 1064-8.

Szostkiewicz, I., K. Richter, M. Kepka, S. Demmel, Y. Ma, A. Korte, F. F. Assaad, A. Christmann and E. Grill (2010). "Closely related receptor complexes differ in their ABA selectivity and sensitivity." Plant J **61**(1): 25-35.